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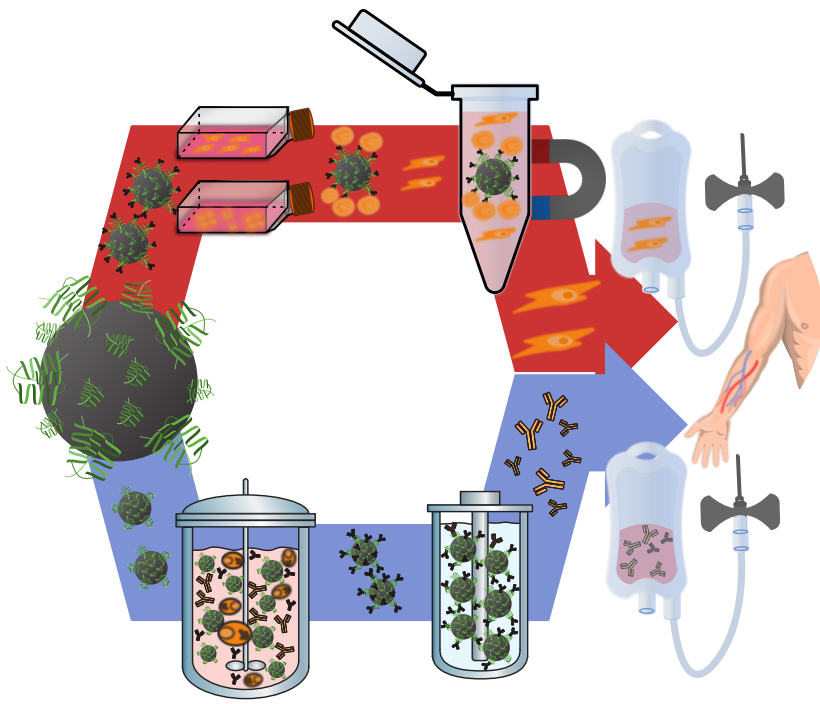
Doctoral Thesis in Biotechnology

Magnetic bead-based isolation of biological therapeutic modalities

NILS ARNOLD BRECHMANN



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NILS ARNOLD BRECHMANN

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'... he had gone eighty-four days now
without taking a fish'

-Ernest Hemingway (The Old Man and the Sea)

Abstract

Biopharmaceutical modalities, such as monoclonal antibodies or the less established cell therapies, are nowadays very important for the treatment of severe or incurable diseases. The manufacturing of such modalities is complex and costly, including the downstream processing, which is highly essential to ensure the safety and quality of the product.

Currently, monoclonal antibody downstream processes are heavily based on column chromatography, such as Protein A affinity capture, and highly depended on clarified liquid. This leads to a step intensive process, which is not only costly but also generates significant reduction of yield for every additional step. The cell clarification, in particular, for high cell density cultures can be insufficient and result in clogging of the following step due to remaining particles in the liquid. Alternatively, the clarification can lead to a higher contamination of product variants and process related impurities, such as antibody aggregations and Host Cell Proteins (HCPs). On the other hand, for large scale commercialization of allogenic cell therapy approaches based on human induced pluripotent stem cell (hiPSC) cell lines, efficient and reliable methods to ensure safety and quality of the cell product are needed. The presence of undifferentiated cells in a cell product derived from hiPSCs represent a risk of tumour and teratoma formation in the patient. The removal of undifferentiated cells in the cell therapy product is critical, and reliable and scalable methods are needed to support off-the-shelf production.

The work in this thesis aimed to develop an alternative downstream operational step based on magnetic beads linked with Protein A or Protein G and a magnetic separator system suitable for the purification of monoclonal antibodies or cell therapy products. Efforts were made to develop an efficient monoclonal antibody capture step, based on magnetic bead separation, directly applied on the harvest of monoclonal antibodies producing Chinese Hamster Ovary (CHO) cell cultures at different cell densities up to very high cell density (> 100 x 10^6 cells/mL) and scales ranging from small-scale to pilot-scale (up to 16 L). The system proved to be highly gentle towards the cell, minimizing aggregation and the release of HCPs (< 10 ppm) already complying with the regulatory

constraint after only one downstream operational step. Furthermore, the magnetic bead-based separation was applied for the negative isolation of cell subpopulations based on unique surface marker expression. Here a flexible isolation system was developed based on Protein A or based on Protein G magnetic beads providing high variability towards the surface receptor recognizing antibody. The magnetic beads were substantially larger compared to a cell resulting in a binding process where a bead is being covered by several cells. The system was evaluated towards different surface receptors, i.e. HER2, TRA2-49 and SSEA-4. The magnetic beads showed to be non-toxic towards the delicate human mesenchymal stem cells and iPSCs. The system also provided excellent negative selection of $HER2^+$ SKBR3 cells, taken as model, and $TRA2\text{-}49^+$/$SSEA\text{-}4^+$ iPSCs from different heterogenous model cell populations.

In conclusion, the present downstream strategies based on magnetic bead separation for the capture of monoclonal antibodies or for the negative selection of cell subpopulations showed great alternatives to resolve the challenges provided by intensified cultures in mAb manufacturing, and could provide a viable solution for cell therapy.

Keywords

Magnetic bead separation, Magnetic beads, Protein A, Protein G, Negative cell isolation, Monoclonal antibody, induced pluripotent stem cells, human mesenchymal stem cells, Allogenic cell therapy, Biomanufacturing, cell clarification

Sammanfattning

Bioläkemel, såsom etablerade monoklonala antikroppar eller ännu ej etablerade cellterapier, är mycket användbara för behandling av svåra eller obotliga sjukdomar. Tillverkningsprocessen av dessa är komplex och kostsam, särskilt reningsstegen. Icke desto mindre är detta arbete nedströms mycket viktig för att garantera produktens säkerhet och kvalitet. För närvarande är rening av monoklonala antikroppar (mAbs) starkt baserad på kolonnkromatografi, speciellt Protein A-affinitet, och är i hög grad beroende av klarifierad vätska. Detta leder till ett intensivt processsteg, som inte bara är kostsamt utan kan även leda till betydande reducering av utbytet i senare steg.

Klarifiering, särskilt på vätska med hög celltäthet, kan vara otillräcklig och leda till igensättning i följande steg på grund av kvarvarande partiklar i vätskan samt leda till högre kontaminering av produkt- och processrelaterade föroreningar, såsom värdcellsproteiner (HCP) och aggregering. Samtidigt behövs för storskalig kommersialisering av allogena cellterapimetoder, baserade på cellinjer av humaninducerade pluripotenta stamceller (hiPSC), effektiva och pålitliga metoder för att garantera säkerhet och kvalitet av cellprodukten. Odifferentierade celler i en cellprodukt, med hiPSCs som ursprung, ökar risken för tumör- och teratombildning hos patienten. Avlägsnandet av dessa odifferentierade celler i produkten är avgörande och tillförlitliga, skalbara metoder behövs för att cellterapiprodukter ska kunna massproduceras.

Arbetet i denna avhandling syftade till att utveckla ett alternativt reningssteg baserat på magnetiska pärlor kopplade med Protein A/G som ett magnetiskt separeringssystem lämpligt för rening av monoklonala antikroppar och produkter för cellterapi. Arbetet lades vid att utveckla ett effektivt infångningssteg baserat på separation med magnetiska pärlor, i syfte att implementeras direkt efter skörd av monoklonala antikroppar producerade av Chinese Hamster Ovary-celler (CHO) vid olika celltäthet, upp till mycket hög celltäthet (> 100 x 10^6 celler) /ml), samt i skalor som sträcker sig från liten till pilotskala (upp till 16 L). Systemet visade sig vara mycket skonsamt mot cellerna, samtidigt som det minimerade aggregering och frisläppning av HCP (< 10 ppm) så att gällande regelverk följs efter endast ett processteg nedströms.

Vidare applicerades separation med magnetiska pärlor på negativ isolering av cellsubpopulationer baserat på unika ytmarkörer. Här utvecklades ett flexibelt isoleringssystem baserat på magnetiska pärlor med Protein A och Protein G som ger hög variabilitet mot den ytreceptor som känner igen antikroppen. De magnetiska pärlorna var avsevärt större jämfört med en cell, vilket resulterade i en bindningsprocess där en pärla täcks av flera celler. Systemet utvärderades mot olika ytreceptorer, t.ex HER2, TRA2-49 och SSEA-4. De magnetiska pärlorna visade sig vara icke-toxiska mot de annars känsliga hMSCs och iPSCs. Systemet gav också utmärkt negativ selektion av HER2+ SKBR3-celler och TRA2-49+/SSEA-4+ iPSCs, tagna från olika heterogena modellpopulationer.

Sammanfattningsvis visar de presenterade strategierna för rening, baserade på separation med magnetiska pärlor, ett utmärkt alternativ för att lösa utmaningarna att rena mAbs från intensifierade cellodlingar, samt för att ge en praktisk lösning för allogen cellterapi.

Zusammenfassung

Monoklonale Antikörper (mAb) und Zelltherapien spielen eine ausgesprochen große Rolle für die Behandlung schwerer und unheilbarer Krankheiten. Jedoch ist die Herstellung dieser biopharmazeutischer Modalitäten aufwendig und kostenintensiv, vor allem in Bezug auf die Aufreinigung, welche eine wichtige Rolle für die Sicherheit und Qualität des Produktes spielt. Der Fokus des Aufreinigungsprozesses für monoklonale Antikörper basiert auf der Säulenchromatographie, im speziellen auf Protein-A-Affinitätschromatographie für welchen eine aufgereinigte Fermantaionsflüssigkeit benötig wird. Dies erhört die Prozesskosten deutlich und führt zu einer erheblichen Reduktion der Produktausbeute.

Einerseits kann die klassische Zentrifugation und Filtration der Fermentationsflüssigkeit meist unzureichend sein und zur Verstopfung durch die verbleibenden Partikeln führen. Andererseits kann die Zentrifugation und Filtration zu einer höheren Kontamination mit produkt- und prozessbedingten Verunreinigungen, wie zum Beispiel Proteine von der Produktionszelle (HCP) und Aggregationen führen. Für upscale Produktionen von allogenen Zelltherapieansätzen, welche auf Zelllinien menschlicher induzierter pluripotenter Stammzellen (hiPSC) basieren, werden effiziente und zuverlässige Methoden benötigt, um die Qualität des Zellprodukts zu gewährleisten. Undifferenzierte Zellen, die auf hiPSCs basieren, erhöhen das Risiko der Teratom-Bildung im Patienten. Die Eliminierung undifferenzierter Zellen in Zelltherapie ist entscheidend um die Sicherheit und Qualität der Therapie zu gewährleisten. Besonders in der upscale Produktionen werden zuverlässige und skalierbare Methoden zur Eliminierung dieser undifferenzierten Zellen benötigt.

Diese Dissertation beschäftigt sich mit der Entwicklung eines alternativen Downstreamschrittes, der für die Aufreinigung von monoklonalen Antikörpern und Zelltherapieprodukten geeignet ist. Der Fokus liegt auf magnetischen Partikeln und einem magnetischen Separatorsystem. Die Aufreinigung von monoklonalen Antikörpern basiert auf magnetischen Partikeln, die mit Protein A Liganden verbunden sind um diese monoklonalen

Antikörper zu binden. Diese magnetischen Partikel werden direkt nach der Beendigung der Kultivierung von unterschiedlichen Zelldichten bis hin zu sehr hoher Zelldichte (> 100 x 10^6 Zellen/mL) und Skalen vom kleinen Maßstab bis zum Pilotmaßstab (bis zu 16 L) der Eierstockzellen des chinesischen Hamsters (CHO) eingesetzt. Das System erwies sich als sehr schonend und minimierte die Aggregation sowie die Freisetzung von HCPs (< 10 ppm). Darüber hinaus wurde die magnetische Trennung für die negative Isolierung von Zellsubpopulationen basierend auf einzigartigen Oberflächenproteinen angewendet. Hierzu wurde ein flexibles Isolationssystem basierend auf Protein A - und Protein G - magnetischen Partikeln entwickelt, welches eine hohe Variabilität gegenüber dem Oberflächenrezeptor-erkennenden Antikörper bietet. Das System wurde auf verschiedene Oberflächenrezeptoren, d. h. HER2, TRA2-49 und SSEA-4, untersucht und die magnetischen Partikel erwiesen sich als nicht toxisch gegenüber den empfindlichen humanen mesenchymalen Stammzellen (hMSCs) und iPSCs. Darüber hinaus lieferte das Isolationsystem eine effektive negative Selektion gegenüber $HER2^+$ SKBR3-Zellen und $TRA2\text{-}49^+/SSEA\text{-}4^+$ iPSCs aus verschiedenen heterogenen Modellzellpopulationen.

Zusammenfassend lässt sich sagen, dass die vorgestellten Downstream Strategien auf der Grundlage der magnetischen Partikeltrennung für die Isolierung monoklonaler Antikörper und die negative Selektion von Zellsubpopulationen eine vielversprechende Alternative darstellen.

Populärwissenschaftliche Zusammenfassung

In der heutigen Medizin spielen Antikörper eine große Rolle, besonders im Kampf gegen Tumor - oder Autoimmun-Erkrankungen. Außerdem, werden auch immer weiter neue Therapieformen und Medikamente erforscht, zum Beispiel bestehend aus menschlichen Zellen.

Antikörper Therapien können genutzt werden um Tumorzellen gezielt zu erkennen, damit das menschliche Immunsystem diese findet und vernichten kann. Diese Antikörper werden künstlich designt und markieren gezielt Tumor- und Krebs-Zellen. Ebenfalls können diese auch zusammen mit toxischen Substanzen verabreicht werden um eine noch effektivere Therapie zu ermöglichen. Im Vergleich zu den traditionellen Chemotherapien, haben Antikörper basierte Tumor- und Krebs Therapien den Vorteil, dass sie selektiv nur befallene Zellen eliminieren anstatt ganze Zellgruppen.

Die zweite Therapieform, die so genannte Zelltherapie, die auf menschlichen Zellen basiert, hat erst in den letzten Jahren an Bedeutung gewonnen. Diese Therapieform existiert in zwei Formen. Erstens die sogenannte autologe Therapie und zweitens die allogene Therapie. In der autologen Therapie werden dem kranken Patienten selbst Zellen entnommen, aufgearbeitet und als Medikament wieder zu geführt. In der allogene Therapie allerdings werden die Zellen von einem gesunden Spender entnommen, aufarbeitet und an mehrere Pateienten verteilt. Besonders im Blickpunkt als allogene Therapie sind sogenannte induzierte pluripotente Stammzellen. Diese weisen mehrere Vorteile auf: 1) Diese Zellen können sich in alle Zellen differenzieren, außer Reproduktionszellen wie zum Beispiel Eizellen, 2) die ethischen Bedenken sind geringer als bei anderen pluripotenten Zellen, 3) die Zellen können aus spezialisierten Zellen, zum Beispiel Hautzellen, gewonnen werden und 4) die spezialisierten Zellen können relativ einfach vom Spender entnommen werden. Diese induzierten pluripotenten Stammzellen können dann als universale Basis genutzt werden um unterschiedliche Zelltypen zu produzieren und zum Beispiel totes Gewebe im Patienten zu ersetzen.

Allerdings ist für beide Medikamente (Antikörper und Zelltherapie) der Produktionsprozess sehr aufwendig. Bei

Medikamenten, welche direkt in Zellen produziert werden oder die Zelle selbst sind, kommt der Reinigung eine besondere Aufgabe zu. Besonders wichtig ist es die Qualität des Medikaments sicherzustellen und Verunreinigungen, welche durch den Produktionsprozess oder des Produktionsorganismus verursacht werden, zu entfernen.

Diese Arbeit beschäftigt sich mit der Entwicklung eines alternativen Prozesses basierend auf kleinsten magnetischen Partikeln, die zur Reinigung für Antikörper und ganze Zellen verwendet werden können. Die magnetischen Partikel binden selektive Antikörper oder Zellen und werden in einem magnetischen Feld gesammelt und die Verunreinigungen können somit einfach abgereichert werden. Zudem erwiess sich der Schritt als sehr schonend gegenüber Zellen und hoch selektiv. Dieser alternative Prozess kann helfen Herausforderungen in der Produktion dieser Medikamente zu lösen und diese dadurch einer breiten Masse einfacher zukommen zu lassen.

Public defense

This thesis will be defended on December 9th, 2022 at 10:00, in room M1, Brinellvägen 64A, Building M, KTH campus, Stockholm for the degree of Doctor of Philosophy (PhD)in Biotechnology.

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List of publications and manuscripts

The complete publications and manuscript are appended at the end of the thesis and are re-printed with permission of the copyright holders. The thesis is based on the following 3 published articles in peer-reviewed journals and one manuscript.

Paper I

Brechmann, N.A.; Eriksson, P.-O.; Eriksson, K.; Oscarsson, S.; Buijs, J.; Shokri, A.; Hjälm, G. and Chotteau, V. Pilot-scale process for magnetic bead purification of antibodies directly from non-clarified CHO cell culture. *Biotechnology Progress* **2019**, *35*, e2775.
Respondent's contribution: Designed the study, performed most of the experiments (cultivations and purification), process and data analysis, and wrote and revised the manuscript.

Paper II

Brechmann, N.A.; Schwarz, H.; Eriksson, P.-O.; Eriksson, K.; Shokri, A. and Chotteau, V. Antibody capture process based on magnetic beads from very high cell density suspension. *Biotechnology and Bioengineering* **2021**, *118*, 3499-3510.
Respondent's contribution: Designed the study, performed the experiments (cultivations and purification), process and data analysis, and wrote and revised the manuscript.

Paper III

Brechmann, N.A.; Jansson, M.; Hägg, A.; Hicks, R.; Hyllner, J.; Eriksson, K.; Chotteau, V. Proof-of-Concept of a Novel Cell Separation Technology Using Magnetic Agarose-Based Beads. *Magnetochemistry* **2022**, *8*, 34.
Respondent's contribution: Conceptualized and methodized the study, performed the investigation, formal analysis, visualization and writing (original draft, review and editing).

Paper IV

Brechmann, N.A.; Hägg, A.; Andersson, C.; Hicks, R.; Hyllner, J.; Eriksson, K.; Chotteau, V. Negative selection of human pluripotent stem cells (hiPSC). (Manuscript)
Respondent's contribution: Conceptualized and methodized the study, performed the investigation, formal analysis, visualization and writing (original draft, review and editing).

Related work not included in the thesis

Chotteau, V.; Eriksson, K.; Oscarsson, S. and **Brechmann, N.A.** Purification process based on magnetic beads. WO2021064244A1. 2021. (patent application)

Other scientific contribution

Magnetic bead purification of antibodies from cell broth at pilot-scale, Bioprocess international (BPI) European summit, Amsterdam, Netherlands, April 2018, *Poster presentation.*

Magnetic bead purification of mAb from CHO cell broth at pilot-scale, European Society of Animal Cell Technology (ESACT) 26th meeting, Copenhagen, Denmark, May 2019, *Oral presentation.*

Continuous antibody capture step based on magnetic beads, Integrated continuous Biomanufacturing IV, ECI conference series, Brewster (Cape Cod), MA, USA, October 2019, *Poster presentation.*

Purification process including cell separation using Protein A magnetic beads, American Chemical Society (ACS) Virtual Meeting & Expo Fall, San Francisco, CA, USA, August 2020, *Oral presentation.*

Antibody capture based on magnetic beads from culture at density > 100 x 10^6 cells/mL, American Chemical Society (ACS) Hybrid Meeting & Expo Fall, Atlanta, GA, USA, August 2021, *Oral presentation.*

Proof-of-concept novel cell separation technology using magnetic agarose-based beads, Advancing Manufacturing of Cell and Gene Therapies VII, ECI conference series, Coronado, CA, USA, February 2022, *Poster presentation.*

One-step clarification, capture and recovery process of antibodies from very-high cell densities using magnetic beads, Bioprocess international (BPI) European summit, Vienna, Austria, Mai 2022, *Oral presentation.*

Proof-of-concept novel cell separation technology using magnetic agarose-based beads, **Antibody capture process based on magnetic beads from very high cell density**, European Society of Animal Cell Technology (ESACT) 27th meeting, Lisbon, Portugal, June 2022, *Poster presentation.*

Abbreviations

ACT	Adoptive cell therapy
ADCC	Antibody dependent cell cytotoxicity
ADCP	Antibody dependent cell phagocytosis
AEX	Anion exchange chromatography
ASC	Adult stem cell
ASN	Asparagine
ATMP	Advanced therapy medicinal products
ATPS	Aqueous two-phase systems
B/E	Bind and elute
CAPEX	Capital expenditure
CAR-NK	Chimeric antigen receptor natural killer cell
CAR-T	Chimeric antigen receptor T-cell
CDC	Complement-dependent cytotoxicity
CDR	Complementary determining region
CEX	Cation exchange chromatography
CHO	Chinese hamster ovary
CIP	Clean in place
COG	Cost of gods
DO	Dissolved oxygen
DSP	Downstream process
EDTA	Ethylenediaminetetraacetic acid
EMA	European medicines agency
FACS	Fluorescence Activated Cell Sorting
FDA	Food and drug administration
FT	Flow through
HCP	Host cell protein
HEK293	Human embryonic kidney cells
HER2	Human epidermal growth factor receptor 2
HIC	Hydrophobic interaction chromatography
hMSC	Human mesenchymal stem cells
HMW	High molecular weight
HUVEC	Human umbilical vain endothelia cell
IEX	Ion exchange chromatography
Ig(A, D, G, E, M)	Immunoglobulin
IMS	Immunomagnetic separation
iPSC	induced pluripotent cell
iPSC	Induced pluripotent stem cells

LMW	Low molecular weight
LRV	Logarithmic reduction value
mAb	Monoclonal antibody
MACS	Magnetic Activated Cell Sorting
mDia	mammalian homolog of diaphanous
MW	Molecular weight
NK-cell	Natural killer cell
pAB	Polyclonal antibody
PCC	Periodic counter current chromatography
PrA / SpA	Staphylococcal Protein A
PrG / SpG	Streptococcal Protein G
PSC	Pluripotent stem cell
PTM	Posttranslational modification
R&D	Research and development
ROCKi	Rho-associated kinase inhibitor
SEC	Size exclusion chromatography
SSEA-4	Stage-specific embryonic antigen-4
TRA2-49	Tumour related antigen2-49
UF HF	Ultra-filter hollow fibre
USP	Upstream process
WCB	Working cell bank

Notations

c^*	Bulk concentration at equilibrium
C_H	Heavy chain's constant region
C_L	Light chain's constant region
F_d	Drag force
F_g	Gravitational force
F_m	Magnetic force
H	Magnetic field
k_a / k_{on}	Association constant
K_D	Equilibrium dissociation constant
k_d / k_{off}	Dissociation constant
L	Ligand
q	Adsorbed solute
q_{max}	Maximum adsorption of the solute
Q_{max}	Static binding capcity
T	Target
V	Volume
V_H	Heavy chain's variable region
V_L	Light chain's variable region
χ_V	Magnetic susceptibility

Contents

Chapter 1

Biopharmaceutical Industry

The field of pharmaceuticals has been undergoing a drastic change since the end of the last century, from small molecular weight chemical compounds to biopharmaceuticals emerging in the 1980s [1], leading to a division into pharmaceuticals (small molecules) and biopharmaceuticals.

Pharmaceuticals are represented by small molecule drugs consisting of organic compounds produced most often by chemical synthesis and include drugs such as Aspirin® and antibiotics [2, 3]. On the contrary, biopharmaceuticals or biologics are much more sophisticated than small molecule drugs [3]. Biopharmaceuticals are drugs that are produced in biological systems, converting a low-value substrate into a high-value product [3]. In comparison with small molecule drugs consisting of only a single amino acid or a few molecules, biopharmaceuticals are much larger compounds of several hundred amino acids, and contain much more complex structures, such as proteins, hormones, enzymes or even viruses and whole cells [2, 4], making them harder to not only produce but also to ensure consistency and safety.

Biopharmaceuticals have gained more and more influence especially for the effective treatment of severe diseases, for instance various types of cancer. Small molecules still present the largest fraction on the pharmaceutical market [5]. However, biopharmaceuticals have dramatically increased their market value over the last two decades and not only catching up in economic importance but also with a improved time to market than early biopharmaceuticals [6].

This thesis will further focus on the development of alternative purification strategies for biopharmaceuticals, specifically recombinantly produced proteins in mammalian cells and whole cells, therefore in the following only biopharmaceuticals are further discussed.

Today, the biopharmaceutical industry can be divided into established modalities and new emerging modalities. Established modalities are herein referred to as mostly proteins, such as: enzymes and/or monoclonal antibodies (mAbs), while new modalities are seen as products such as viruses for gene therapy or whole cells for cell therapy approaches.

These modalities are not only highly complex in their structure and behaviour but the production strategies have to also be highly sophisticated, representing a large challenge for the biotech industry in terms of know-how and investment costs (R&D, production sites) [4]. For a better insight into the manufacturing process a typical production process is described below.

Typically, all the manufacturing processes are divided in two major parts and then further subdivided into multiple unit operations (see Figure 1.1). Independently of the product, the process consists of the upstream processing and the downstream processing [7, 8].

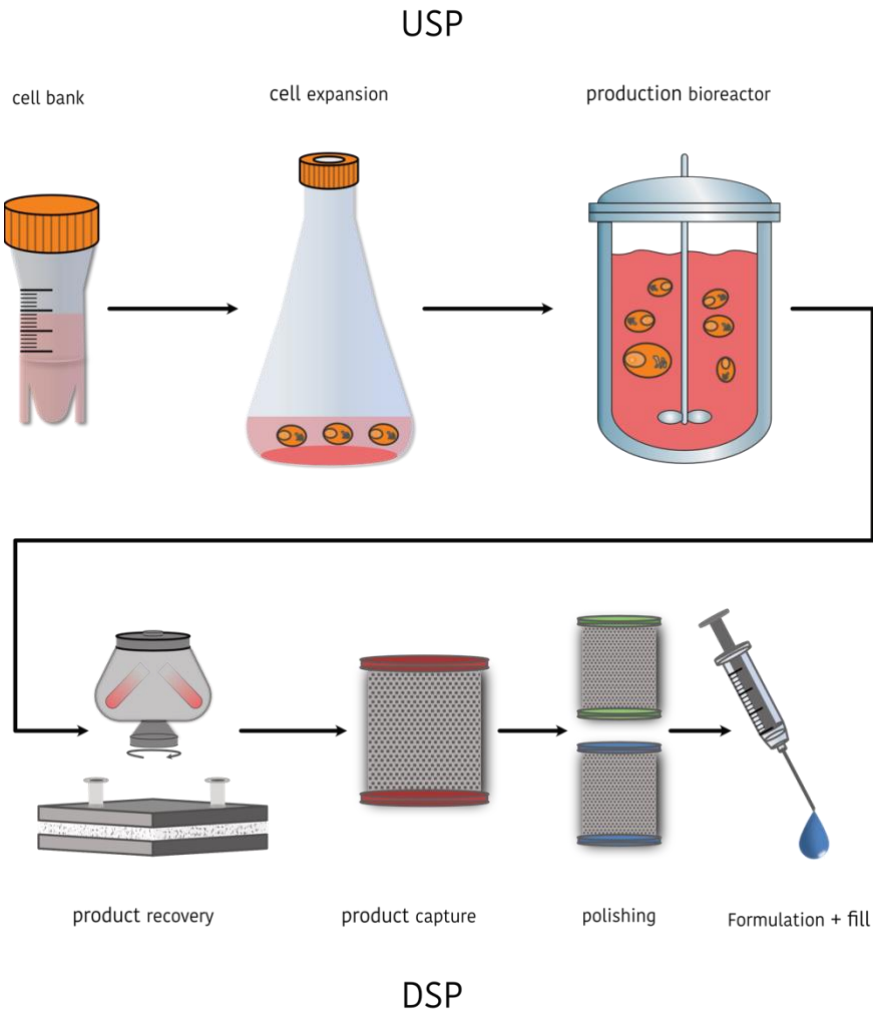

Figure 1.1: Schematic representation of manufacturing process for biopharmaceutical modalities

The schematic process shows a common categorization of USP (upstream process) and DSP (downstream process) with a schematic representation of the different unit operation

1.1 Upstream process

The upstream process (USP) describes the production of the desired target e.g. mAbs, meaning the cultivation of the biological system, in which the protein is produced. Prior to the start of the cultivation the right cell line needs to be selected, screened and developed. Commonly, the first step is to decide what cell system is used, prokaryotic cells or eukaryotic cells. Both provide advantages and drawbacks and need to be carefully considered. Prokaryotic cells, e.g. *Escherichia coli*, are for example well studied, easily to grow and maintain, while on the downside they are unable to produce complex glycoproteins with human like glycosylation, such as mAbs. Eukaryotic cells, e.g. Chinese hamster ovary (CHO) cells, on the other hand are much more complex and require a much higher level of maintenance to facilitate growth. However, they are capable of producing human like glycosylation, which makes them the organism of choice to produce biopharmaceuticals. As these cells do not naturally produce these biopharmaceuticals, the protein information is delivered in form of plasmids that carry the DNA information for production and assembly of the desired protein. The cell system of choice is then transfected with the plasmid(s) which is taken up by the cell. The information can be integrated in the genome, which result in a stable cell line, or a so-called transient integration in which the information remains in the plasmid and is not integrated resulting in a transient cell line. Transient cell lines are easier to develop but require constant selective pressure for the production, otherwise the cell line might lose the plasmid and with it the ability to produce a recombinant product. Stable cell lines are more favourable for production cell lines, as the quality of the product remains the same over the course of production with the information integrated in the genome. Therefore, most commonly stable mammalian cell lines are used for the production of biopharmaceuticals with human like glycosylation. Once the right cell line is selected the aim of the USP is to produce the product of interest and to reach a high productivity and an overall high product concentration. The cell culture is divided into 2D- and 3D-cell culture [9, 10]. In 2D-culture the cells adhere to the surface of the culture vessel. This technique is routinely used in laboratories, however they are considered suboptimal [10], as they are restricted to the growth area of the container they are in. In contrast, 3D-cell

culture alleviates some of the limitations, and are more easily amenable to more sophisticated operational modes, for instance growing cells on a scaffold, carriers or fully in suspension. In this case, lager area in all dimensions can be utilized for the cell growth, and better supply and distribution of nutrients, as well as advanced control of pH and gassing can be achieved. 3D-cell culture is preferred and often routinely used in the production of biopharmaceuticals, due to the advantages described above. The duration of the culture depends on the mode of cultivation and can range from a few days to several weeks [7]. Commonly, four different cultivation modes are applied for the production of recombinant proteins: (I) batch cultivation, (II) fed-batch cultivation, (III) chemostat cultivation and (IV) perfusion cultivation [11], with increasing complexity in their operation.

Batch-mode cultivation is seen as the simplest form, in which the cells are inoculated in the culture medium, containing all nutrients required for proliferation. The culture has a fixed volume during the production and is harvested once, typically when all nutrients are depleted and toxic metabolites such as ammonia and lactate have accumulated and cell death has occurred [11].

A more advanced set up of the batch-cultivation is an operational mode called fed-batch. In this set up the cells are inoculated with a certain volume of culture medium, similar to the batch mode, however, unlike batch mode fresh medium or a nutrient-rich supplement is added during the culture [11-13]. Through this addition of fresh medium, the duration of the cultivation can be significantly prolonged [13], because new nutrients are supplemented and toxic metabolites, inhibiting the growth, are diluted. This culture mode can result in a higher cell density, higher productivity and higher product concentration [13] compared to a batch operation. Therefore, fed-batch processes are often used in large scale production, as they are well established and relatively easy to operate [14, 15]. However, besides its advantages the fed-batch mode suffers from the drawback of a constantly changing cell environment, potentially effecting the product quality.

The chemostat mode is operated with a constant cell environment, keeping the volume at a fixed level as well as cell density (growth rate), nutrients and metabolites [11]. The reactor is operated with a constant inflow of medium and a constant outflow of cell broth containing cells, nutrients, metabolites and product.

However, the greatest limitation of this mode is that the cell's growth rate needs to be larger than the dilution rate of the process, otherwise the cells are washed out of the reactor [11]. For this reason, chemostat processes are rarely used for mammalian cell culture as their growth rate is often too low and would lead to an insufficient nutrient supply.

The last mode is the so-called perfusion mode. This set up is a development of the chemostat mode, in which a constant cell environment is maintained. In a perfusion process a constant inflow of fresh nutrients is supplied to the cells in order to maintain cell growth, while a constant outflow is removing metabolites and product. The difference compared to the chemostat process is that cells are retained in the reactor, leading to an accumulation and hence high cell density [11]. The cell retention is based on different physical properties mainly size and density. For the retention based on density, sedimentation or continuous centrifugation is used. In order to retain the cells based on size, a filtration device is used, often based on hollow-fibre filter [16-18]. Filter-based separation devices can be operated in two different versions depending on whether or not the product can pass through the filter. A microfilter that enables the removal of the product from the bioreactor was originally developed for the production of unstable proteins but has increasingly gained interest for the production of stable proteins [17]. The resulting constant product stream can be utilized in a continuous set up. The second variation which does not allow the product to pass through the filter is obtained through the use of an ultrafilter, with a molecular weight cut-off smaller than the product of interest [19-22], leading to product accumulation in the bioreactor. This perfusion variant, also referred to as intensified fed-batch, has the advantage that a single harvest at the end of the culture can be applied, providing a higher concentrated and lower total harvest volume than regular perfusion cultivations. The downside of this process is that it is not suitable for unstable proteins and provides a challenge for the downstream process, which was part of the investigations in this work.

1.2 Downstream process

The downstream process (DSP), accounting for the majority of the production costs [23-26], is initiated after the cell culture and includes a large range of operational steps aiming to decrease volume, increase product concentration and to ensure suitable product purity and quality [27]. Depending on expression system used in the upstream process the product can be obtained through different means. The target is either found within the intracellular space, or it is secreted and transported to the extracellular space, which is often the case for mammalian cells. The secretion of proteins in mammalian cells is a complex pathway that includes folding and post translational modification (PTMs) in different cell organelles. Commonly, the polypeptide sequence of the protein is transported to the *Endoplasmic Reticulum* (ER) where the sequenced enters the ER-lumen. Here the sequence is cleaved, matured and folded. The matured protein is then transported in ER-vesicles to the *Golgi-Apparatus* where the protein is further modified, e.g. glycan maturation. Once the maturation process of the protein is finalized, proteins with a secretion signalling are transported from the *Golgi* in a vesicle to the cell membrane. The vesicle fuses with the membrane and releases the protein into the extra cellular space [28]. The product can be found in a soluble or insoluble form. Therefore, the operational steps are highly product specific but can be divided into four different categories.

(I) The recovery step (Figure 1.1), which is ultimately the separation of solids and liquid. Frequently used methods for this step are centrifugation and filtration. Depending on the location and form of the product either the liquid or the solid faction is further processed. To give an example: in case of a secreted product the liquid fraction contains the product of interest and vice versa if the product is not secreted and remains within the intracellular space the solid fraction contains the product and is therefore further processed. If the product is retained in the intracellular space additional operations to the clarification step to disrupt the cells and obtain the product are required.

(II) The capture step (Figure 1.1) is typically seen as the main step to isolate the product in a rather high purity. The bulk purification is performed at this step with the removal of the majority of impurities such as Host cell proteins (HCP), DNA and

other culture components [7, 29, 30]. Typically, this step is performed through precipitation, extraction or adsorption [23] with different levels of selectivity for the product. However, adsorption is especially relevant for biopharmaceutical proteins and is very frequently used in the form of packed-bed chromatography [31, 32]. The adsorption principle is based on a reversible interaction between the product and the surface of the packed-bed and is influenced by the chemical structure and biological function [33]. This results in interactions that can be based on electrostatic forces, hydrophobic forces, Van-der-Waals forces, hydrogen bounds or a combination of these [34]. These interactions are exploited in form of affinity, ion exchange and hydrophobic interaction chromatography (often governed by electrostatic, hydrophobic forces, and hydrogen bonds), while size exclusion only relies on the hydrodynamic radius for separation.

(III) The polishing step (Figure 1.1) is performed to remove the manufacturing related impurities for instance low and high molecular fractions of the product (e.g. aggregates), charge variants, etc. [35, 36].

The last step (IV) is the product formulation (Figure 1.1). In this step the product is conditioned for long term storage and prepared to obtain the drug substance [35].

1.3 Antibody manufacturing

Antibodies play an existential role in the human immune system primarily in the recognition and defence against pathogens [37]. They specifically detect and bind to pathogen specific antigens and activate a cascade of immune effector functions to annihilate the pathogen [37]. Antibodies as commercial products can be found in two variations. Once in form of polyclonal antibodies (pAb) and once as monoclonal antibodies. These two variations have essential distinct characteristics although they can bind to the same antigen. Polyclonal antibodies are essential produced by different immune cells (B-cells), therefore polyclonal antibodies are always a heterogenous mix of antibodies binding to the same antigen but different epitopes of that antigen [37]. On the other hand, monoclonal antibodies are produced by a single B-cell. Hence, mAb populations are homogenous and bind to a single epitope [38]. Both

are used as therapeutic application but monoclonal antibodies are nowadays much more relevant as a therapeutic, because of their high specificity and low variability. Monoclonal antibodies contribute significantly to this thesis. Therefore, their structure and specific production process is presented in detail in this chapter.

Monoclonal antibodies play a major role in the treatment of severe and difficult to cure diseases such as cancer, e.g. carcinomas, myelomas and melanomas, autoimmune diseases, e.g. arthritis, multiple sclerosis and asthma [39]. The emergence of hybridoma technology in 1975 by Köhler et al. [38] recognised by their Nobel prize, was quickly followed by the first approval of an antibody based drug muromonab-CD3 (Orthoclone OKT3, Johnson and Johnson) [39, 40] targeting CD3 [41] based on a monoclonal murine antibody. Since then, the developed and approval by FDA and EMA of therapeutic monoclonal antibodies has skyrocketed with 97 on August 2020 [41] and the 100th mAb was approved in 2021 [42] and currently 141 mAbs that have been approved or are under revision in the EU and US [43]. Their high versatility makes them attractive for the treatment of various diseases and has led to the antibody market to be a multibillion-dollar business [1, 44].

1.3.1 mAbs

Immunoglobulins such as mAbs or naturally occurring antibodies i.e. in serum, can usually be divided in 5 isoforms/types IgA, IgD, IgG, IgE and IgM, with further subdivision, depending on the C_H variation [45]. The IgG isoform is the most abundant isoform not only for naturally occurring but also as a therapeutic, specifically the IgG1 subtype [44, 46]. As IgG1 (Trastuzumab) has been the model mAb used in this thesis its structure will be discussed in more depth.

The IgG1 mAb is a large Y-shaped protein (Figure 1.2) with a size of approximately 150 kDa [47, 48] consisting of two identical heavy chains (≈ 50 kDa each), two identical light chains (≈ 25 kDa each), with N-Glycosylation and disulfide bonds providing a high degree of heterogeneity [48]. The light chain can appear in two forms, as lambda (λ) and kappa (κ), which are encoded by different genes on different chromosomes. The lambda light chain is encoded on chromosome 22 [49, 50] while the kappa light chain is encoded on chromosome 2 [51, 52]. The four chains are further divided into two regions referred to as the constant region (C_H and C_L) and the

variable region (V_H and V_L) [48]. The IgG1 structure for the heavy chain contains three constant domains (C_{H1-3}) and one variable domain (V_H) and for the light chain one constant domain (C_L) and one variable domain (V_L) [53] (see Figure 1.2). The constant domains C_{H2} and C_{H3} form the so-called Fc part (fragment crystallizable) of the mAb (Figure 1.2) which is the effector and binding site for molecules or cells. The variable domains V_L and V_H and constant domains C_L and C_{H1} form the fragment antigen binding site, the Fab fragment [48, 53]. The variable domain of both chains contain three hypervariable regions [48, 53], which are referred to as the CDR loops (CDR1-3), the complementary determining region. With the sequence and size of these regions forming a complementary surface to the epitope of the antigen which gives the IgG1 its specificity [54].

Beside the structure of the monoclonal antibody, posttranslational modifications are crucial for their function and can give the mAb different characteristics. Disulfide bonds are important for the integrity of the mAb, specifically interconnecting the different chains. Commonly, IgG1 contains four interconnecting disulfide bonds and 12 disulfide bonds connecting the different domains resulting in a total of 16 disulfide bonds [55]. Two of them link both heavy chains together at the hinge region (Figure 1.2), while the other two disulfide bonds link the light chain and the heavy chain via the C_{H1} and C_L region [48]. Furthermore, N-Glycosylation is another crucial posttranslational modification. The N-Glycosylation resides at the amino acid ASN-297, which is conserved in both heavy chains and situated in the C_{H2} domain [56-58] (Figure 1.2). The correct glycosylation is critical for the mAb, meaning that not only the presence but especially the microheterogeneity and combination of sugars of the glycosylation effects the half-life, solubility and can determine the right function [58] and mAb's activities [59-63].

A fully functional mAb has, as indicated above, two main functions. (I) The antigen binding via the CDR region, resulting in two independent binding sites [48, 64] that target extracellular receptors or soluble proteins [65] or other structural components of cells and pathogens. This leads to a cascade of different mechanisms of action, such as complement-dependent cytotoxicity (CDC) and apoptosis. Secondly, (II) the Fc-effector provides additional mechanisms in combination with the antigen binding. The Fc-

effector function can introduce cytotoxicity or phagocytosis induced by the mAb via the Fcγ receptor (FcγR) [66], so-called antibody dependent cell cytotoxicity (ADCC) and antibody dependent cell phagocytosis (ADCP), in which the Fc part is used by immune cells e.g., NK-cells for the recognition of a malignant cell [48, 65]. On the other hand, the Fc-region also interacts with the FcRn (neonatal Fc receptor) which governs the half-life of the mAb in the blood plasma [66].

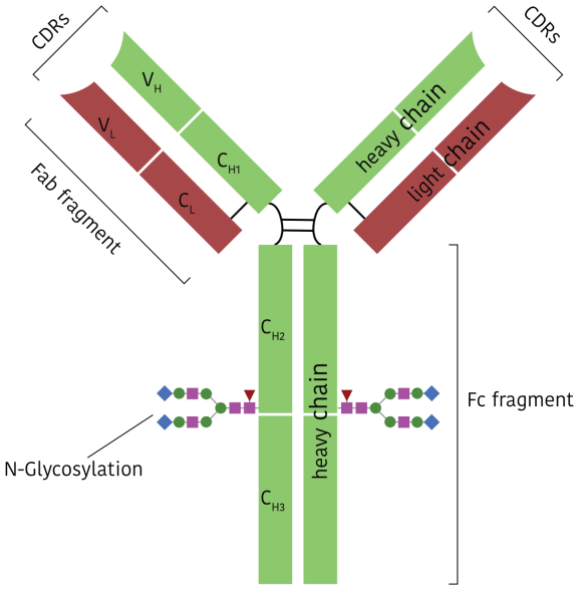

Figure 1.2: Schematic mAb structure

Y shape IgG1 molecule consisting of two identical light and heavy chains containing each one side for N-Glycosylation.

1.3.2 Manufacturing process

In this chapter the current state of the art mAb production process is explained. The process for the specific production of monoclonal antibodies is built as described in the chapter 1, consisting of an upstream part (USP) and a downstream part (DSP).

The cultivation of the expression system is mostly performed in large-scale stainless steel stirred tank bioreactors or disposable plastic bioreactor. For the expression system, several different cell lines are in use. It is important that the expression system is able to produce complex and large proteins with the correct posttranslational modifications (PTMs) [67] for instance N-glycosylation. As the therapeutic mAbs are glycoproteins the right glycosylation plays an important role for their function, but equally important incorrect glycosylation can provoke a severe immune response. The expression system needs to be able to assemble a glycan structure close to or identical to those of a human to avoid a negative immune response. Hence, only mammalian cell lines are currently used as expression systems for the production of full-length therapeutic mAbs.

Commonly, these cell lines are Chinese hamster ovary (CHO), which is by far the most used cell line [67, 68] and NS0 and Sp2/0 [7, 69], which are both murine myeloma cells. As these cell lines are derived from animals their PTMs are close to human PTMs but not identical, which has led to the study of human derived cell lines such as HEK293 (human embryonic kidney cells) for the production of glycoproteins. However, CHO cells are still primarily used as they present attractive process attributes such as rapid growth, high expression, robustness and the ability to grow in chemically defined suspension media [7]. The CHO cells used for the production of mAbs were originally grown adherently, meaning attached to a surface. This however is quite inefficient as the expansion space is limited. Therefore, CHO cell lines were adapted to grow in suspension and over the years different variations from the CHO origin have been derived and used. For example, the CHO-M cell line, used in the present investigation, which has been derived from the CHO-K1 cell line.

Today a consensus process (Figure 1.1) has emerged for the production of mAbs throughout different manufactures [7]. At the beginning of the process is the thaw of the cell line from a vial of the working cell bank (WCB) (Figure 1.1). This is followed by several steps of expansion (seed-train) in order to generate a cell mass suitable for the inoculation (inoculum train) of the bioreactor. The number of seed train steps depends on the concentration of cells in the inoculum and the size of the final bioreactor. The seed bioreactor is the stage before the production bioreactor, also referred to as the

N-1 stage. This stage is used to generate the final cell mass in order to inoculate the production bioreactor with the correct target cell density and a large culture dilution. The productions are typically operated in fed-batch mode, which is still the preferred mode of operation, in bioreactors of sizes 5000 to 25000 L [7]. Typically, the duration of such a production stage bioreactor is between 7 and 21 days. This system accumulates titers between 1 and 5 g/L, which can in some cases even be higher with levels of 10 - 13 g/L [7]. Once the termination criteria are reached (e.g., low viability, titer, time), the cultivation is terminated. To draw an exact line on when the USP stops and DSP start is somewhat controversial, as sometimes the clarification steps is accounted into the USP and sometimes into the DSP. However, from this authors point of view the harvest marks the end of the USP and the DSP is initiated with the cell clarification steps.

The current DSP trains have been subject to intensive standardisation and optimization [70], as seen in Figure 1.3 in which the same platform process has been adapted by different manufactures. The DSP train starts with the cell clarification step (Figure 1.1) of the cell broth, which is a critical step for all following DSP steps, as they depend on non-particulate clarified liquid. In order to achieve this, solid-liquid separation methods are used. Firstly, large solid particles are removed via a continuous disk stack centrifuge [7], followed by one or more filtration steps, e.g. depth filtration, to remove the lower density particles [29]. Depending on the cell mass and volume, centrifuges and depth filters can reach limitation regarding the solid removal. Additionally, these steps are quite harsh and can lead to cell damage and the release of host cell proteins (HCPs) [71]. Alternatively, a higher degree of cell debris and colloids in the cell culture broth, which places an increased burden on the clarification train, can be pre-treated by flocculation/precipitation. This can reduce the required depth filter surface [29]. However, it should be carefully considered as additional contaminants are added to the liquid that can impact the further downstream steps [29]. Once a clarified supernatant is obtained the capture step of the mAb follows. Affinity separation has emerged as the preferred choice for the capture of mAbs. This is mainly caused by the availability of Protein A as a universal affinity ligand. Protein A is a bacterial surface protein that has been found to specifically bind to many IgG species. Protein A chromatography

has a great advantage as it reduces the volume significantly, is highly selective towards the target mAb and removes the bulk impurities. Resulting in a highly concentrated and purified product, which is unique for this step as there is no universal platform technology with these characteristics available for any other target molecule. The Protein A binds mainly to the Fc-region of the mAb but has also shown interaction with the Fab-region [72-75]. Protein A is explained in chapter 2.1.5. In order to reverse the selective binding and elute the mAb from the affinity ligand a low pH buffer is used. This step also serves as a crucial viral inactivation step with the product being held in the low pH environment for 30-60 min to inactivate enveloped viruses [76]. Commonly, two to three viral inactivation/removal steps with orthogonal mode of actions [77] are used in DSP of products derived from mammalian cell culture due to the risk of virus contamination leading to an immune response by the patient as mammalian cells are closely related to humans. Commonly, low pH treatment, viral filtration and a chromatography step are used to ensure a sufficient virus inactivation/removal. The logarithmic reduction of the virus particles is essential rather than the number of steps. Commonly, a logarithmic reduction value (LRV) of ≥ 4 per step is required [77] so that an overall LRV greater than 6 is achieved, although ICH Q5A guidelines do not define a concrete criterion [78]. After the low pH treatment two polishing steps for the further removal of impurities follow. Mostly ion exchange (IEX) chromatography is used in form of cation (CEX) and anion exchange (AEX). However, hydrophobic interaction chromatography is sometimes used instead of one of the IEX (Figure 1.3). The different adsorbers (IEX and/or HIC) are usually also used in different modes of action to not only target different chemical and physical properties but to select for specific impurities. Cation exchange is mostly performed in bind and elute mode (B/E) to target the mAb and wash away the impurities. This step usually leads to an even higher degree of product concentration and removal of positively charged impurities. On the other hand, AEX and HIC are usually performed in flow through (FT) mode in this platform process. Specifically targeting impurities such as host cell DNA (HDNA), HCPs, endotoxins, leached Protein A and product related impurities [29, 30, 79]. This is then followed by the formulation and filling of the drug substance to end the whole process.

Even with an almost uniform platform process for the purification of mAbs (Figure 1.3) [80], the DSP is responsible for the majority of the consumables with up to 80 % of the total costs, with Protein A chromatography having the highest share [30]. With the high costs and the multiple operational steps, the DSP is perceived as a crucial bottleneck in this platform process. The multiple operational steps not only add costs to the overall process but also reduce the product yield significantly. Therefore, optimization of the DSP productivity needs to be addressed to make this platform process efficient and transform it into the 21st century.

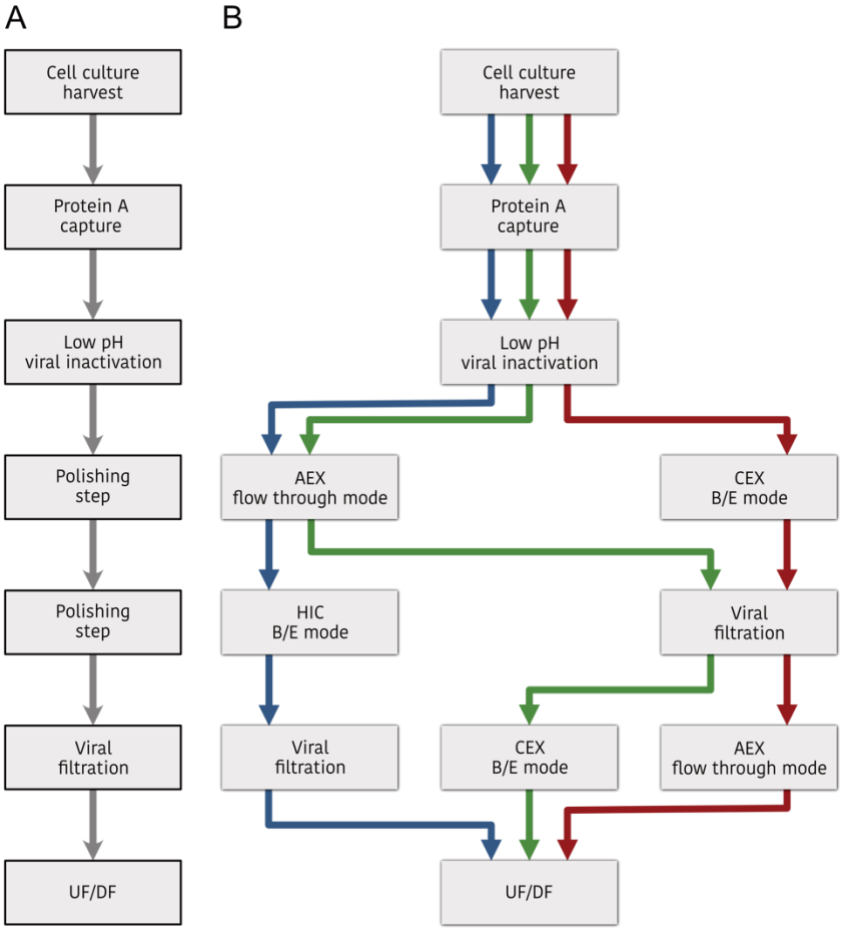

Figure 1.3: mAb DSP platform process

Platform DSP adapted by different manufactures. (A) The grey process (grey arrows) represents the standardized platform process with the different operational steps. The operational steps include the culture harvest, mAb capture, various steps of viral reduction operations, several polishing steps and formulation steps. (B) The processes blue, green and red (blue, green and red arrows) represent adaptation by different manufactures. Green and red process are adaptation by Genetech and the blue process is an adaptation by Biogen. Figure adapted from Shukla, 2017.

1.4 Cell therapy manufacturing

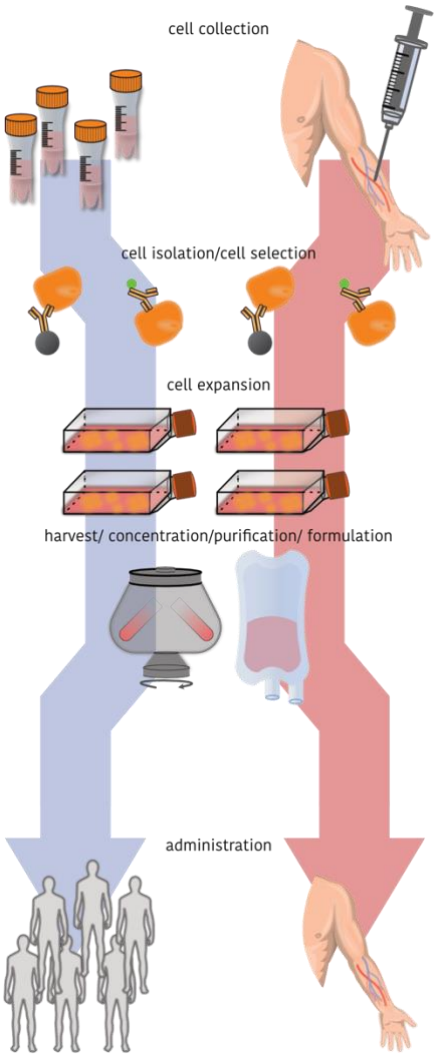

Figure 1.4: Allogenic and autologous cell therapy workflows

Schematic representation of an allogenic (the donor-to-patient(s)) workflow (blue arrow) and an autologous (patient-to-patient) workflow (red arrow). The workflow presents the different process steps with cell collection, cell isolation/cell selection, cell expansion, harvest/concentration/purification/formulation and administration.

The second biotherapeutic target in this thesis is cell product, more precisely differentiated cells from induced pluripotent stem cells (iPSCs), used for cell therapy. iPSCs are a special class of cells. These cell, as indicated by their name, are pluripotent. Whereby the term describes cells that are capable of differentiating into any cell type except reproductive cells. However, iPSCs are not naturally occurring cells and are generated by reprogramming of adult cells such as fibroblasts [81]. In comparison embryonic stem cells, which are naturally occurring pluripotent cells, raise ethical concerns when used as therapeutics due to their nature and the donor material (embryonic cells) is difficult to obtain. This is not the case for iPSCs with easily accessible donor material and low ethical concerns. Cell therapy usually refers to the transplantation of human cellular material into a patient to treat damaged or malfunctioning tissue or cells [82, 83]. This type of therapy started to emerge in the early years of 2000 but suffered from limited technology and knowledge to operate such complex therapies [84]. It has taken until nowadays for this area to re-emerge as a therapeutic modality. Cell therapy belongs to the large emerging field of advanced therapy medicinal products (ATMP), which includes gene therapy, somatic cell therapy and tissue-engineered products [85, 86]

ATMPs are seen as a very promising area in biopharma for the treatment of complex diseases, such as Alzheimer's, malfunctioning tissue, and genetically inherited diseases. In 2021 the ATMP market was estimated to a value of ≈ US$ 10 billion [87]. The area is predicted to grow up to ≈ US$ 60 billion by 2030 with an annual growth rate of ≈ 21 % [87]. North America provides currently the largest market share, which can also be seen by the amount of authorised therapies by the FDA, but Asia is the largest growing market. Cell therapies have shown an astonishing efficacy for a broad range of diseases previously untreatable, which ultimately leads to this fast market growth. This does not only show that a large financial power is behind these new modalities but also that there is a market for these therapies due to their high efficacy. However, current costs for treatments are not sustainable and can be in the tens-to-hundreds of thousands of US$ per treatment leading to the necessity of improved manufacturing strategies and

off-the-shelf solutions [88] in order make the therapies more accessible for patients.

1.4.1 Cells

Commonly, ATMPs are divided into autologous and allogenic therapies (Figure 1.4), which describes the origin of the therapeutic cells [83].

Autologous therapy referrers to an approach in which the cells are taken from a patient and are given back to the same patient (patient-to-patient), as seen in Figure 1.4. In this approach no other donor, than the patient itself, is involved for the donation of therapeutic cells. Autologous cells have the advantage that they are non-immunogenic [89] and usually only a 'single' dose needs to be produced, but these cells have the limitation of their unpredictable variability and function caused by the characteristics of the host (age, comorbidity, genetic structure) [90]. Also, the sampling requires invasive techniques to obtain the material. The manufacturing process is complex and suffers from a very high fluctuation of the starting material, the patient cells [90]. Autologous therapy approach is not an off-the-shelf treatment.

On the other hand, the allogenic therapy refers to the use of therapeutic cells from healthy donors, that are then transplanted into many patients (Figure 1.4). The allogenic approach became even more important with the creation of iPSCs by Yamanaka et al. [81], which was awarded with the 2012 Nobel Prize in Physiology or Medicine, together with a better understanding of developmental biology. This made it possible to have defined cell lines for any differentiations, opening for many therapeutic targets. The pluripotent cells have the advantage that they do not have the same unpredictable variability as patient cells since they are issued from a defined master cell bank (Figure 1.4) and derived from healthy tissue [90]. This makes them very uniform and allows for a defined, well qualified raw material [90]. Furthermore, the creation of these cell banks is simple as the donor material can easily be obtained from high available adult tissue (fibroblasts) and following be reprogrammed and differentiated into the desired cell type. Therefore, is the allogenic approach very well suited as an off-the-shelf drug to respond immediately to a disease [90]. This requires

the production quantities to be several times higher as many patients are treated with the same batch, which leads to the challenge of large-scale manufacturing including both culture and the purification of large cell quantities. The purification is crucial as treatment safety is one of the highest concerns for the production of allogenic cell therapies. Impurities can trigger an immune response in the patient, as the cells originate from another human. But with iPSCs as the basis for off-the-shelf allogenic therapy the pluripotency raises an even more important safety concern with the aspect of pluripotent cells' potential to from tumours. As explained, pluripotent cells have the ability for unlimited self-renewal and to differentiate into all three germ layers and any cell type except reproductive cells. Especially, the unlimited self-renewal of pluripotent cells can lead to abnormal and disruptive growth, resulting in tumour formation after the cell transplantation [91, 92]. But also the high differentiation ability of pluripotent cells can promote and can give raise to tumour formations of randomly differentiated cells called a teratoma [93, 94]. Therefore, it is imperative the cell product has to be free of undifferentiated cells prior to the administration to reduce the risk of teratoma formation [95-101]. Providing the high safety of a cell transplant and ensuring the complete removal of pluripotent cells is one of the greatest challenges for the production of allogenic therapies.

Overall, there have been very few direct comparisons of autologous and allogenic approaches regarding safety and efficacy. Until now it is hard to draw a conclusion on which cell therapy approach will be permanently established and most likely both will co-exist. Currently, as of July 2022 there have been 23 approved and valid authorisation of ATMPs by the U.S. Food and Drug Administration (FDA) [102] with the majority of it being autologous approaches, including somatic and tissue repair therapies as well as gene therapy with CAR-T cells [86, 103]. But with the significant advantages of allogenic cell therapy it is expected that the field might be moving into that direction [90] with the chance of developing off-the-shelf drugs.

To further push the field in the direction of effective treatments and cost-effective manufacturing, more emphasis needs to be placed on the development of allogenic approaches in order to manufacture an off-the-shelf product and to utilize a platform

process to lower the cost of such therapies to make them available to a large group of patients.

Cell types that have a potential to be used in cell therapy can generally be categorised into non-specialized cells (e.g. iPSCs) and specialized cell (e.g. immune cells) [82]. This also includes genetic engineering of cell, as seen in some immune cell therapies. To give an overview, different cell types are presented in the following.

Non-specialized/unspecialized cells for cell therapies

Unspecialized cell can be found not only in embryonic cells, but also in adult tissue. These cells can differentiate in different cell types and provided the potential of unlimited renewal [82, 104]. Depending on the stem cell type the differentiation potential can vary from highest potential (totipotentcy) to the lowest potential (unipotency), whereby, for example, embryonic stem cells are pluripotent meaning they can differentiate into the three germ layers and any cell type excluding reproductive cells [82, 105].

Therapies based on unspecialized cells can be further divided into pluripotent stem cells (PSCs) and adult stem cells (ASCs) [82].

PSCs give rise to cells of the three germ layers and any cell type excluding reproductive cells [82]. Embryonic stem cells and iPSCs fall into this category. The group of pluripotent stem cells are the most promising types for cell therapy. But with the ethical concern over embryonic stem cells and the advantage of well-defined iPSC from a master cell bank, the main focus for clinical applications is shifted to iPSCs [82]. iPSCs are promised to be the basis for an off-the-shelf cell therapy as they can be differentiated into different cells.

The second category are adult stem cells (ASCs) belonging to the somatic cells, and are undifferentiated cells that have however, committed to a cell type with limited self-renewal and differentiation potential. Their main purpose is to replace lost cells for the healing of damaged tissue by giving rise to progenitor cells that will then form the differentiated cell types. Adult stem cells are for example: mesenchymal stem cells, neural stem cells and hematopoietic stem cells [106], and can be found for instance in the bone marrow of adult humans. For therapy applications all kinds of ASCs are of interest but commonly hematopoietic and mesenchymal stem cells have been used [82].

Specialized cells for cell therapies

In contrast, specialized cell-based cell therapies are being also developed and are mostly based on somatic cells that are not stem cells, which usually are isolated from a donor and processed, meaning cell isolation, selection and expansion, and following administrated to a patient [82]. Specialized cell-based therapies include a variety of different somatic cells and the most present are probably immune cells, for instance NK-cells, T-cells and macrophages. These somatic cells are already committed and highly specialised

Immune cell-based cell therapy belongs to the group of adoptive cell therapy (ACT) [107]. These therapies can be performed with a variety of different immune cells, such as: infiltrating lymphocytes, modified T-cells (modified T-cell receptor and antigen receptor) [108, 109] and different killer cells (NK-cells, lymphokine-activated killer cells and cytokine-induced killer cells) [109]. These immune cells are already highly specialized and can be genetically engineered for example by grafting a chimeric antigen receptor (CAR) onto a T-cell to form a CAR-T cell [82, 110].Most frequently the modified immune cells are used as a strategy to target cancer, but are also suitable for the treatment of chronic infections and autoimmune diseases [82, 109]. In these treatments the modified immune cells are usually part of a complex cascade initiating tumour reactivity or antitumour activity of the human immune system.

Besides immune cells other somatic cells are under investigation/use for cell therapies. For example: fibroblasts, chondrocytes, hepatocytes, pancreatic islets and keratinocytes. As a key difference, compared to immune cells, these cell types are mostly used as cell grafts [82]. This application however progressed slowly since the transplantation of cells is limited due to technology and data supporting durability and efficacy [82]. Besides the common cell transplantation, whole skin and bone marrow transplantation, which to be perfectly candid are not part of this group. The cell availability, engraftment success rate and non-specific inflammatory as well as thrombotic mechanisms post transplantation [82] are one of the major hurdles prior to application.

1.4.2 Manufacturing process

As seen in the previous section cellular-based therapies have a huge potential and can be based on a large variety on different cell types. The manufacturing process for autologous or allogenic therapies and the different cells have similarities, see Figure 1.4. In the following text the basic manufacturing of cell therapy products is described.

Overall, the process consists of 5 steps: (I) cell collection, (II) cell isolation/selection, (III) cell expansion, (IV) cell harvest/concentration/purification/formulation and (V) administration (Figure 1.4). Depending on the type of therapy (autologous or allogenic, see Figure 1.4) the starting material is different but the overall production process remains similar. Starting with the (I) cell collection from a donor which can be performed in a variety of ways. But is usually defined by the desired cell type. For example, if mesenchymal stem cells are required then a biopsy or arthroscopy from different tissue such as: bone marrow, adipose tissue or synovial fluid can be performed [111]. On the other hand, if cells are collected from human blood leukapheresis can be performed [112]. These collection methods have to be performed for each patient in autologous therapies. In comparison to allogenic approaches, the cell collection needs to be performed fewer times on only a few healthy donors to obtain the desired cell type. This can be a reprogrammable cell type to ultimately obtain iPSCs or ASC, from which cell lines are developed. The collection of cells is usually followed by a variation of (II) cell isolation/selection (Figure 1.4), which can be different for the different cell types but are commonly sorted regarding their specific surface maker expression. Typically, for this step the expressions of different cell surface markers are used, as they are easily accessible and specific. Two different methods (see Figure 1.4) can be used for that with Fluorescence Activated Cell sorting (FACS) or immunomagnetic separation (IMS), based on magnetic beads as seen in Magnetic Activated Cell Sorting (MACS). Both methods are frequently used for this step. FACS is based on fluorescence conjugated antibodies. These antibodies usually target receptors which are unique for the desired subpopulation, e.g. a pluripotent maker such as SSEA-4. Once the subpopulation is labelled with fluorescence antibodies, the whole population is processes. The fluorophores that are conjugated to

cells are excited by a laser and the emission is detected. Based on the detection the different cell subpopulations are packed in differently charged droplets. The trajectory of the droplets is then manipulated with an electromagnet and the different subpopulation are separately collected. On the other hand, IMS utilizes antibodies conjugated to magnetic beads for the isolation/selection. The mechanism of action is quite similar to the fluorescence labelling. The magnetic beads also target specific surface maker, depending on the conjugated antibody, leading to a magnetic labelling of a subpopulation. The movement of the magnetically labelled subpopulation is then manipulated in a magnetic field and retained, while the unlabelled population is collected. Once the magnetic field is removed the labelled cells are collected. The third step, cell expansion (Figure 1.4), is performed in order to achieve the cell quantity needed for the administration. This step is crucial for the manufacturing of cell therapy products. The cell expansion can be performed in different ways. Due to the mostly adherent nature of cells used in cell therapy most of the expansion is commercially performed in 2D. T-flasks systems can be scaled up into layered flask system or larger flask, providing a larger surface area to support higher numbers of cells. However, cell expansion can also be accomplished in 3D bioreactors, with a large variety of bioreactors that can be used, among them, are the stir-tank, packed-bed or rocker-based bioreactors, which can support the growth of cells in suspension as single cells, cell aggregates or adherent cells on growth supports (so called micro carriers). After the cell expansion is done, step IV (Figure 1.4), culture harvest/concentration/purification/formulation of the therapeutic cells is followed [113]. This is a critical step to ensure the safety of the cell product and to adjust the desired cell number and media composition [114] for the administration. Cells and culture medium are separated, commonly by centrifugation and/or filtration steps [113]. Lastly, this step includes the formulation of the cell product. Commonly, the therapeutic cells are cryopreserved for longer storage, which requires specific cryopreservation media in the formulation. The adjustment of the formulation buffer or cryopreservation media is usually also achieved by cell wash via centrifugation or filtration. The therapeutic cells are then transferred into a suitable container for storage or administration

[114]. The last step (V) is the final administration to the patient (Figure 1.4).

Chapter 2

Purification of biomolecules and new modalities

The DSP of a biopharmaceutical production process or more specifically the purification of the active drug substance, contributes significantly to the safety and efficacy of the drug to ensure purity and quality of the drug substance. The purification is the crucial step in the manufacturing process to remove process and product related impurities that otherwise could provoke immune response, impair with the drug's efficacy and lower the half-life.

As presented in the previous chapter, biopharmaceuticals, including mAbs, are produced recombinantly leading to the challenge of removing unwanted impurities related to the host organism and to the process. Specifically, in the case of monoclonal antibodies theses are HCPs, metabolites and product variation (aggregates, charge variants and glycosylation profile).

In the case of cells for cell therapies the impurities are of different nature. Typically, these are undesired cell types, serum, metabolites and other medium components [115]. Specially, for allogenic therapies based on differentiations from iPSCs, the removal of undifferentiated cells is crucial.

The purification of recombinant proteins and especially mAbs relies mainly on packed-bed chromatography techniques, on the other hand purification for cell therapies mainly utilizes techniques such as centrifugation and filtration for cell wash and concentration.

In the following chapter, techniques for the purification of both products will be presented and the theoretical background of chromatography is explained.

2.1 Packed-bed chromatographic separation

This chapter will focus on solid-phase separation commonly associated with packed-bed chromatography. The presented work is similar in principle to packed-bed chromatography, which is therefore an excellent example to discuss the theory.

The general principle can be discussed independent from the ligand or type of chromatography used and is based on chemical and physical attributes. Commonly, it is distinguished between the mobile phase and the stationary phase. The mobile phase describes the liquid containing the product of interest, while the stationary phase describes the solid support (resin) that contains the active group. The separation in this technique is based on the fact that different components in the liquid phase travel at a different rate through the stationary phase. This phenomenon is the case for all types of chromatography, i.e. affinity and non-affinity.

2.1.1 Support material

The solid support that contains the active group(s), used to interact with the product of interest, is essential for packed-bed chromatography. Commonly, the solid support is also referred to as chromatographic resin. This solid support consists of small uniform beads ranging from a few μm for analytical purposes to tens of μm for preparative or production purposes. These beads can be comprised of different materials and can have different structures. The materials can be distinguished into organic or inorganic and can have characteristics of xerogels or aerogels. Xerogels are resin materials that can swell in water resulting in enlarged bead size. On the contrary, aerogels are unaffected by water and keep their bead

size. The material for the resin should provide characteristics such as neglectable affinity for proteins, hydrophilicity, withstand extreme environments such as high flow, possibility to add active groups, and different sizes and porosities. Inorganic bead material for example are silica beads or glass beads. These beads are usually solid and provided a lower capacity due to the lower surface area. However, they withstand extreme environment such as high flow or high pressure. On the other hand, commonly used organic materials are polysaccharides such as agarose, cellulose or dextran or synthetic organic materials such as polyacrylamide or polystyrene. These organic materials are usually porous and provide a larger surface area resulting in high capacity. The beads can be micro porous, macro porous or a combination. In order to reach high porosity different material are cross-connected. Nowadays, agarose beads cross-linked with a polymer (e.g. dextran) are frequently used.

2.1.2 Chromatographic process

The chromatography process can be operated either as *bind-and-elute* mode or *flow-through* mode. The *bind-and-elute* mode is a process in which the product of interest is actively retained on the stationary phase (*adsorption*) and unwanted impurities travel through the stationary phase without interacting with it. This step is usually referred to as the *wash step(s)*, as all unbound material is washed away by excess liquid. Once the product is isolated the dissociation of the product (*elution*) occurs by modification or change of the composition of the mobile phase. Vice versa, in the *flow-through* mode no interaction occurs between the product of interest and the stationary phase, instead the impurities interact.

2.1.3 Mass transfer

Mass transfer within the chromatography material is defined by three different dynamic behaviours known as convection, diffusion and dispersion. Convection in relation to chromatography describes the intraparticle fluid movement (void), which in return with a high fluidic movement transports molecules to free ligands promoting faster adsorption of the molecule. In packed-bed chromatography the convection depends on the linear velocity and particle size.

Therefore, higher fluidic movement can result in better mass transfer on the surface of the chromatographic beads. Identically important is the phenomenon of diffusion for the mass transport into the chromatographic resin. The diffusion only becomes apparent in porous resin and is highly affected by pore size, molecule size (diffusion coefficient), concentration gradient and linear flow rate (mixing). Diffusion is defined as the process of random molecular movement from a region of high concentration to low concentration. Diffusion is the dominant force for local adsorption behaviours into the pores, as the mixing inside the pores can be close to zero at higher flow rates. Diffusion can be a limiting factor with slower diffusion coefficient, depending of the molecular size, necessitating a lower flow rate in packed-bed chromatography. On the contrary batch adsorption is almost completely independent of the flow rate. Here the diffusion is mostly influenced during the batch adsorption phase. Hence the diffusion can be less dominant and equilibrium can be reach faster. Lastly, dispersion has an impact on the mass transfer. Dispersion is a common phenomenon in laminar flow scenarios in which the flow velocity is zero at the surface of a channel due to the friction of the liquid on the wall. This can compromise the diffusion efficiency in column chromatography. Although dispersion can be a critical phenomenon, it is only severe in plug-flow operated packed-bed chromatography, while the batch adsorption phase in magnetic bead separation is unaffected by dispersion as the mixing is operated in well mixed system, generating turbulent flow mode.

2.1.4 Non-Affinity Chromatography

Non-affinity chromatography includes all the methods that do not contain a specific affinity ligand that binds to a specific surface. A large variety of methods are available for chromatography separation, which can generally be categorised into size exclusion chromatography (SEC), ion exchange chromatography (IEX) and hydrophobic interaction chromatography (HIC).

Size exclusion chromatography

SEC is the separation of molecules based on their size differences more specifically on the difference of the hydrodynamic radius of the molecules [116, 117]. Generally, the separation is accomplished through a filtering effect by the stationary phase [116]. The

stationary phase consists of porous particles with defined pore size allowing molecules to diffuse through the stationary phase according to their molecular size [116]. This means that large molecules have a shorter flow path as they cannot penetrate the pores as deep as small molecules. This principle can only be found in packed-bed chromatography. This technique is mostly used for analytics but does have some application in preparative and production chromatography, where it is applied as a polishing step for the removal of aggregation for instance.

Ion exchange chromatography

The separation in this technique is based on the charge difference of molecules and is distinct by the competitive binding (attraction) due to ionic or electrostatic interactions of charged molecules and opposite charged stationary phase [118]. IEX plays one of the, if not the biggest role in the purification process for molecules and proteins, due to the versatile applicability and the varying charge properties of molecules [118]. Additionally, IEX resins provide very high capacity, can be used at all purification steps for capture and polishing, and are cost effective compared to special affinity ligands. In ion exchange chromatography the stationary phase is linked with a charged grouped resulting in a charged stationary phase, which is either positively charged, so called anion exchanger (AEX), or negatively charged, so called cation exchanger (CEX) [118]. In order to attract the protein to the stationary phase it needs to have the opposite charge, as stated above. This can be achieved by adjusting the pH of the mobile phase in relation to the isoelectric point (pI) of the protein. The isoelectric point is defined as the pH at which a protein has a net charge of zero. For example, if the pH of the mobile phase is below the protein's pI, the overall net charge of the protein will be positive, ergo it will bind to a CEX resin. Vice versa when the pH of the mobile phase is above the protein's pI, the negatively charged protein will bind to an AEX resin. The bond between the protein and stationary phase can be reversed by either changing the pH of the mobile phase to reach a neutral net charge of the protein or increasing the ionic strength of the mobile phase with the addition of salts.

Hydrophobic interaction chromatography

The third category of non-affinity chromatography is hydrophobic interaction chromatography. As the name implies hydrophobic interaction between the protein and stationary phase is the basis for

this separation technique [119]. The stationary phase is typically linked with hydrophobic ligands leading to a interaction with exposed hydrophobic patches of the proteins in an aqueous solutions [119]. An essential feature of the mechanism of action is a high salt concentration in the mobile phase promoting water structure, therefore HIC is also referred to as 'salting out chromatography' [119]. The salt ions interact with the water molecules and anti-chaotropic salts lead to a more structured water. This results in exposing non-polar region of the protein, as the water molecules surrounding the protein are displaced into the bulk water, leading to lower solubility of the protein [119]. Hence, the hydrophobic patches interact with the hydrophobic ligand of the stationary phase, as this leads to a more favourable low free energy. Elution in HIC is accomplished by lowering the conductivity or changing to more chaotropic salts [119]. HIC can be used in different steps during the purification process of proteins especially when the salt concentration is already high in the 'sample'. Commonly, in mAb purification, HIC is used as a polishing step after high salt elution from IEX chromatography to remove product and process related impurities. However, the high salt concentration used for the binding needs careful consideration as it might lead process related impurities such as aggregation.

2.1.5 Affinity Chromatography

Affinity chromatography is the second large group of methods for purification purposes. Affinity chromatography techniques are the most powerful and specific tools for the purification of specific proteins from complex supernatant [120]. For certain targets, affinity chromatography might be the only efficient way of purifying the target. Affinity chromatography is very powerful due to the high specificity. It enables not only the recovery of the bulk protein and reduction of the liquid volume but also a high initial purity of the target molecule. This makes the affinity step highly efficient as it combines two purification steps in one; the capture step and the polishing step. The interaction in affinity chromatography is based on biological interactions, for instance between antigen and antibody, receptor and ligand, or enzyme and substrate [120]. These interactions are not only highly selective but also reversible, which

make them a perfect fit for affinity chromatography. The disruption of the affinity complex alters the binding affinity between the affinity ligand and the target reversing the binding [120, 121]. This is achieved through a combination of lowering the pH, or changing the ionic strength or by addition of competing molecules [121]. For immunoglobulin binding affinity ligands, low pH is the preferred elution method.

Affinity chromatography has been through intensive improvements in the recent years [120] with different support materials, higher capacity and improved sanitation resistance being developed. This is on one side, caused by the antibody industry, as Protein A is their main purification tool, and the pursuit of improved Protein A resins increases the process economics drastically leading to larger quantities of mAbs to be purified. On the other side affinity chromatography is improved by the development of new ligands enabling new targets to be purified with highly selective resins. The first affinity chromatography was already exploited in the early part of the 20th century with the binding properties of amylase to starch [120]. Since then, affinity chromatography exploited many different ligands for a large variety of targets, such as sugars, enzymes and co-enzymes, proteins and many more [120].

Importantly, for the resin is the solid support of the stationary phase, commonly microporous uniform particles are used as they provided a sufficiently large surface. Especially for Protein A, cross linked agarose has emerged as the dominant solid support [121]. The selection of a suitable affinity ligand is essential for a working resin, besides the general biological function the ligand should have a sufficiently high affinity towards the target to enable a rapid adsorption [120]. The affinity ligand is immobilised on the solid support via covalent coupling. In order to avoid steric hindrances most affinity ligands are coupled via a spacer [122]. Commonly, the affinity ligand is coupled N-terminally or C-terminally via a amine group or a carboxyl group, but thiol groups are frequently used as well [120]. Protein A however, has a very dominant role in affinity chromatography and will be discussed in more detail in the following section together with the other related immunoglobulin binding proteins.

Immunoglobulin binding proteins

Proteins binding to immunoglobulins occur naturally and can be found as bacterial surface proteins [123] and are part of a bacterial

defence system to avoid recognition by the immune system [124]. Three different immunoglobulin binding proteins are commercially relevant, namely: Protein A (SpA), Protein G (SpG) and Protein L. They bind to various species of immunoglobulins with different affinities.

Staphylococcal Protein A

Staphylococcal Protein A is naturally occurring and uniquely found on the surface of *Staphylococcus aureus*. As off today it is the most prominent immunoglobulin binding protein [123]. With the increasing demand of mAbs for therapeutic purposes Protein A affinity chromatography, with the described advantages, has taken an important place in the DSP for these modalities [123]. SpA consists of 509 amino acids resulting in a size of ≈ 58 kDa [125] and provides a high affinity towards various immunoglobulin G (IgG) species. Three different domains are found in the naturally occurring SpA; the signalling sequence (S), the homologous binding domains (E,D,A,B and C) and the anchoring domain to the cell wall (X and M) (Figure 2.1) [123]. Interesting for the use in affinity chromatography are only the five binding domains with all of them independently capable of binding to the Fc region of IgG [123]. A single domain consists of three α-helices anti-parallel arranged with a stabilizing hydrophobic core (Figure 2.1) [123]. Out of the three helices only helix 1 and helix 2 are involved in the specific binding to the region between C_{H2} and C_{H3} of the IgG's Fc region [72, 123]. Additionally, the domain also provides binding toward the Fab fragment, specifically the heavy chain (V_H) through helix 2 and 3 [75, 123] (Table 2.1). Most interesting for industrial applications is the B domain, which is the most explored and engineered domain. Commonly, known now as the Z-domain (Figure 2.1) [123] with beneficial features such as lower binding to the Fab-region of IgGs and tolerance to harsh conditions used in CIP applications [123, 126], while preserving the high affinity. This engineered Z domain is now the base for many Protein A affinity resins.

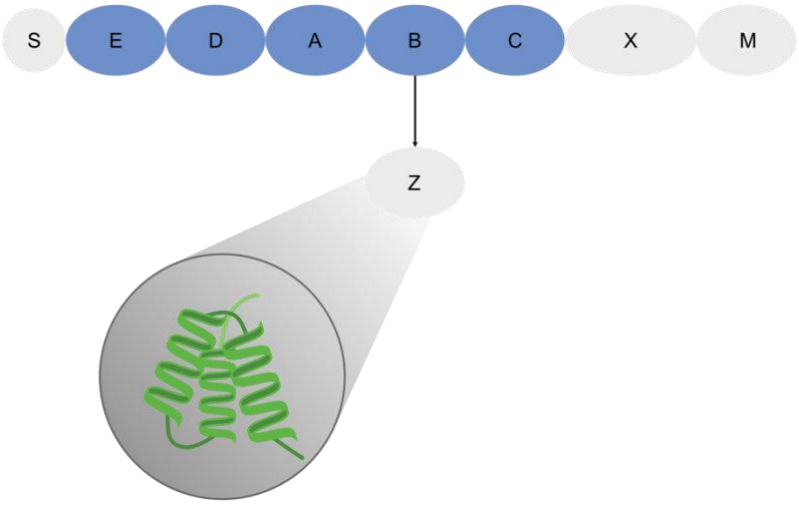

Figure 2.1: Structural regions of Staphylococcal Protein A

Different regions of the Staphylococcal Protein A including the signalling region (S), the achoring regions (X,M) and the IgG binding domains (E,D,A,B,C). Additionally displayed the commonly engineered Z domain originating from the B domain and a structural representation of the Z domain consisting of 3 α helices. Adapted from Hober et al. 2007.

Streptococcal Protein G

Streptococcal Protein G (Table 2.1), in comparison to Protein A, is found in various species of the Streptococcal family mainly from group C and G [127, 128]. Similar to SpA, Protein G also interacts with the Fc and Fab region. Protein G interacts with roughly same part of the Fc region (C_{H2} and C_{H3}) [127, 129], but the binding domain provides no sequence homology to the one from SpA [130]. The interaction in the Fab region, however, takes place at the C_{H1} domain. The secondary structure of Protein G consists of four-stranded β-sheets linked by one α-helix [127]. Beside the binding domains for IgGs, Protein G also provides binding domains towards Albumin [127]. However, the most industrial relevant difference of SpG is the affinity to other IgG subclasses. Mainly, human IgG3 can be of interest as SpA does not provide interaction to that subclass [123].

Finegoldia magna Protein L

Unlike SpA and SpG, Protein L (Table 2.1) is expressed in *Finegoldia magna* [131], a gram-positive bacterium belonging to the species of *Peptococcus magnus* [132]. Protein L has, until now, not been prominently used for affinity purification, due to the difference in the binding interaction as Protein L binds only to the Fab-fragment. Specifically, Protein L binds to the κ-light chain of human immunoglobulins [131, 133, 134] but does not discriminate between the immunoglobulin subtypes, giving it a broader range of interaction. However, with the differences to SpA and SpG, the binding characteristics might play a larger role in the future with the new modalities entering the pharmaceutical market. Reversibly targeting the Fab-fragment becomes particularly valuable for purification purposes. For example, for various derivates of antibodies only containing the Fab-fragment or potentially CAR-T cell purification utilizing the binding towards the CAR fragment.

	Origin	Binding site	Human IgG preferences
Protein A	*Staphylococcus aureus*	Fc region (C_{H2} and C_{H3} domain) and Fab region (V_H)	IgG1, IgG2 and IgG4
Protein G	Streptococcal	Fc region (C_{H2} and C_{H3} domain) and Fab (C_{H1})	IgG1, IgG2, IgG3 and IgG4
Protein L	*Finegoldia magna*	Fab (V_L but only κ-light chains)	IgG1, IgG2, IgG3 and IgG4

Table 2.1: Immunoglobulin-binding proteins

2.2 Non-packed-bed and non-chromatographic separation

This chapter will focus on alternative approaches compared to the traditional packed-bed chromatography. The above-described

mechanisms in packed-bed chromatography and derivatives of it are also used for these alternative approaches. Some of the techniques are similarly based on solid-phase separation while others use different mechanisms. Traditionally, packed-bed chromatography is the dominant choice for the purification of therapeutic proteins, especially in preparative scale up to manufacturing scale. Due to the fact that the alternative strategies have been limited by costs, effort of changing a current platform and lack of large-scale devices [121]. Limitations like low purity and yield should not necessary be associated to these alternative approaches, as affinity forms of these approaches can resolve these limitations. Alternative separation methods do have the ability to overcome the limitation of diffusion [121] that restricts the mass transport and flow rate in packed-bed chromatography. Lately, these alternative methods have shown advances and improvements to resolve their limitations and might become a real alternative in the near future for the DSP of therapeutics. Alternative separation methods can be categorised in four different methods, with varying degrees of practicality.

2.2.1 Membrane-based separation

One category are membrane-based separation methods, more specifically membrane based adsorbers. These adsorbers are based on the same physiochemical properties as solid supports in packed-bed chromatography and can be equipped with affinity ligands, charged groups or hydrophobic chains [79]. They potentially allow for fast binding behaviour at high flow rates due to the reduced mass transfer resistance resulting in fast processing of liquids [135]. Additionally, membrane adsorbers provide potentially a smaller footprint, are less expensive [135] and have a simpler preparation procedure to that of standard packed-bed methods [136]. On the contrary, membrane absorbers fight limitations such as: low binding capacity, moderate resolution, flow-through operation and limited availability [135, 137].

2.2.2 Precipitation

The second category is precipitation, which can be used in a variety of forms for the purification of therapeutic modalities. Commonly used is for example the ammonium sulphate precipitation. However, it is mainly interesting for proteins, as cells used in cell therapy might be compromised by this process. Precipitation possess a great potential for large-scale [121] and continuous manufacturing [138], in order to cope with the current process intensification. The mechanism of action behind the protein precipitation is the 'salting out' effect, similar to HIC chromatography, in which salts are added to the supernatant to increase the water structure. Leading to exposed hydrophobic patches and lower protein solubility, until proteins precipitate [139]. Precipitation is attractive due to the combination of simplicity, robustness and high concentration factor [121], which makes it an interesting tool at the early stage of the DSP as contaminates are tolerated and a high product concentration is achieved. However, common disadvantages can be observed with precipitation as well such as low specificity and the additives leading to further contamination in the sample. Additionally, protein aggregation can be a critical parameter [138], especially for therapeutic mAb, as it can impact the protein's efficacy. But precipitation is a present subject of research as it very feasible in a continuous set up.

2.2.3 Aqueous two-phase systems

The next category is very similar to the precipitation approach, but is potentially more suitable for mAb processing. Aqueous two-phase systems (ATPS) have been used for several decades already, but mainly for the purification of enzymes [140, 141]. The core of ATPS are two immiscible aqueous solutions, each based on a different component. Typically, a salt-based solution (phosphate, citrate or sulphate [142]) and a polymer-based solution (polyethylenglycol or dextran) are used. The formation of the two phases appears spontaneously, but can be accelerated by external forces such as centrifugation. Depending on the design (polymer MW, polymer concentration, salt concentration, temperature, etc) of the system the target protein can be obtained in either one of the two solutions.

For improvement affinity ligands can be coupled to the polymer to facilitate the selectivity towards the target [143]. However, due to the complexity of the system a large range of factors can influence the partitioning, leading to a labour-intensive evaluation and process development prior to the implementation. Nevertheless, the ATPS provides advantages with regards to scalability, high capacity, continuous operation, low cost and high throughput [79, 143]. Furthermore, ATPS have the potential to gently separate cell for a feedstock, which makes this technique potentially useful for the manufacturing of cell therapy products [143, 144].

2.2.4 Expanded bed-chromatography

The last category describes alternative approaches that are very similar to the packed-bed chromatographic techniques. These approaches are similarly also based on solid-phase separation but not within the constraints of a packed-bed column and included techniques such as expanded bed chromatography/adsorption (EBA), monolith chromatography and magnetic separation. In expanded bed chromatography the particles are not packed in a bed but rather loosely contained within a column. During the adsorption, the mobile phase is used to extend the particles along the whole column in order to free space between the particles and avoid clogging, while simultaneously capturing the target molecule. For the elution the flow of the mobile phase is reversed and the particles are packed for an efficient elution. Expanded beds provide therefore a substantial advantage over packed-bed-based chromatography as it would allow adsorption from particulate cell suspension, reducing the number of steps [79]. However, until now EBA has not been widely implemented nor used, as the particle density and size are highly critical for the mechanism of action and give a fairly narrow range of operation [79, 145]. Furthermore, this system has limitations regarding sanitation and high cell densities and product titers [79], which makes it not usable to solve current manufacturing bottlenecks. Similar, monolithic chromatography can be used to process particulate cell suspension. In monolithic chromatography, the bed consist of a continuous material [146] rather than single beads. This has the advantage of a lower backpressure [146]. However, monoliths usually provide low

capacity and are therefore not used in larger set ups, their use has been restricted to analytical purposes. Lastly, magnetic separation is an alternative approach based on solid phase separation. As it has been the research objective of this thesis more detail will be given in the following chapter.

Chapter 3

Magnetic separation

The focus of the thesis was to develop a magnetic separation-based tool that can be used as an alternative in the DSP for the manufacturing of biopharmaceuticals. The DSP based on packed-bed chromatography in mAb production represents not only a bottleneck in terms of productivity but also in terms of process costs. The DSP is the major cost source in the whole manufacturing process. Aside from the material costs another factor is highly important, the economic value of the DSP, which is the number of operational steps. Multiple steps in the DSP lead to loss of product resulting in overall low process yield and low throughput. Commonly, a column chromatography-based DSP for mAbs requires between 7 – 9 operational steps [80, 147], depending on the design of the process. A single operational step naturally provides a yield that is below 100 %, whereby excellent yields are expected around 90 %. These operational step yields multiply to an overall yield that is easily below 50 %. This summarizes the DSP dilemma quite well, by implementing multiple steps to meet regulatory requirements the yield becomes increasingly low, leading to the need of higher production. Besides implementing intensified culture systems to enhance the mAb titer, step reduction in the DSP can be an effective method to improve productivity and resolve

some of the bottlenecks. This is the core of the majority of the presented work, providing magnetic separation to eliminate several operational steps for suitable large-scale manufacturing of biopharmaceuticals.

3.1 Magnetic separation history overview

The phenomenon of magnetism has been known for centuries being described in ancient Greece in the year 550 BC [148]. But magnetic separation took until the middle of the 19 century before it was described [148] and following has since been translated into countless commercial applications, for instance in the mining industry. Since the early 1940's magnetic separation has also been used in wastewater treatment applications [149]. However, no biotechnologically relevant applications have been introduced. The relevant use for the biotechnological area is firstly mentioned in the 1970's by Dunnill and Lilly [150] with investigation regarding the immobilization of an enzyme on a magnetic support material. Dunnill and Lilly further developed the technique for the immobilization of affinity ligands to capture enzymes from cell homogenates [151]. Following the developments by Dunnill and Lilly, the use of agarose magnetic beads for magnetic separation has been firstly described in 1977 [152] by Guesdon and Avrameas. Overall, the magnetic separation gained interest in the late 1970's in the work of other groups about different materials, application and deeper understanding of the phenomenon [149, 153-156]. In 1979, Ugelstad set the ground for more effective magnetic separation by the manufacturing of uniform polymer particles [157, 158] and developed it further with the introduction of magnetic iron oxide into the monosized polymer particles [159, 160]. This led to the development of the Dynabeads™, which are still commercially available and widely used. Early on, these beads were used for the targeted separation of cells, for example from blood [159] or the removal of tumor cells [161]. Magnetic separation since then has become commercially very successful with a large variation of applications such as: cell fractionation, enzyme immobilization, magnetic affinity chromatography, immunoassays, and magnetic based extraction [149, 154]. Importantly these applications are primarily restricted to bench-scale application, with most of them related to sample preparation. Although, magnetic separation was

originally developed for large-scale bioseparation, it has never been implemented in such processes. Mainly, the lack of suitable large-scale equipment, suitable beads and optimized operation seem to be responsible for this [149].

Since the early 2000's, a wave of new developments has highlighted the re-emerging importance of magnetic separation for biotech applications, with focus on scale-up. As shown by pilot-scale studies for the purification of biomolecules [162-164] and development of suitable equipment [165]. Following the next 10-20 years magnetic separation for biomolecules has been extensively researched with large increase in the numbers of publications [166]. A large focus has been put on industrial applications [167-170] and in particular as an alternative in mAb purification using Protein A coated beads [22, 171, 172]. Considerably improvements have been made to magnetic particles for instance on the support material and variety of ligands, as well as improved equipment for large-scale, understanding of processing time and cleaning requirements. Overall, this has led to a broader understanding of the process. However, magnetic separation has not been transferred into manufacturing operation for biomolecules for instance mAb manufacturing until now. Today, packed-bed chromatography plays the most important and significant role in the DSP of mAb manufacturing processes.

3.2 Magnetic separation workflow

For a comprehensive understanding of the advantages and drawbacks magnetic separation provides, a general description of the simplified workflow is valuable. In the following, the workflow is presented and shortly described. A simplified scheme is shown in Figure 3.1. Magnetic separation consists of typically three phases: (I) target adsorption on the magnetic beads, (II) Separation of magnetic bead-target complex in a temporary applied magnetic field, (III) bead wash and target recovery. Similar to column chromatography the magnetic beads need to be equilibrated, which is part of phase (I). Since the magnetic beads are dispersed freely in suspension, the buffer replacement can be, depending on the volume, rapid and the equilibration step is finalized within a matter of seconds or minutes. Once the adsorption conditions are adjusted the sample is applied to the magnetic beads. The liquid that contains

the sample needs to comply with the condition adjusted during the equilibration. In the batch adsorption phase the sample and magnetic beads are incubated together with constant motion (mixing or shaking). Motion of the suspension is crucial as the magnetic beads' high mass leads to rapid sedimentation and secondly to insufficient interaction between the ligand (bead) and the target. The duration of the batch binding is highly dependent on the mixing and kinetics between ligand and target. In the case of mAbs and Protein A the kinetics is fast, leading to relatively short incubation times. The batch binding process will be explained in depth in the following chapter. (II) After the free target is adsorbed onto the magnetic beads, an external magnetic field collects the magnetic beads in the magnetic pole region. Application of a magnetic field with a high intensity retains the agglomerated magnetic beads in the pole region to ensure the withdrawal of the sample liquid containing the unbound impurities and contaminants, for instance: HCPs or cells. The theoretical understanding of the magnetic separation is explained in depth in the following chapter. (III) Several wash steps are followed in order to remove unspecifically bound impurities or loosely bound proteins. The wash steps are performed identically with resuspension of magnetic beads in wash buffer, application of the external magnetic field and withdrawal of the liquid, often repeated several times. Once impurities are removed the *elution* step follows, which is the desorption of the target from the ligand. In fact, the desorption of the mAb is performed identically to Protein A based affinity chromatography with a low pH buffer displacement. The purified and concentrated mAb is found in the elution buffer, which is withdrawn from the retained magnetic beads. After the target, specifically mAb, is obtained in the elution the pH of the buffer should be adjusted to physiological pH to avoid aggregation. After the separation has been performed, cleaning in place (CIP) procedure with NaOH is used to clean the magnetic beads to avoid cross contamination in the next cycle and minimize fouling effects. Once the magnetic beads are cleaned, they can be reused in a new cycle of target capture.

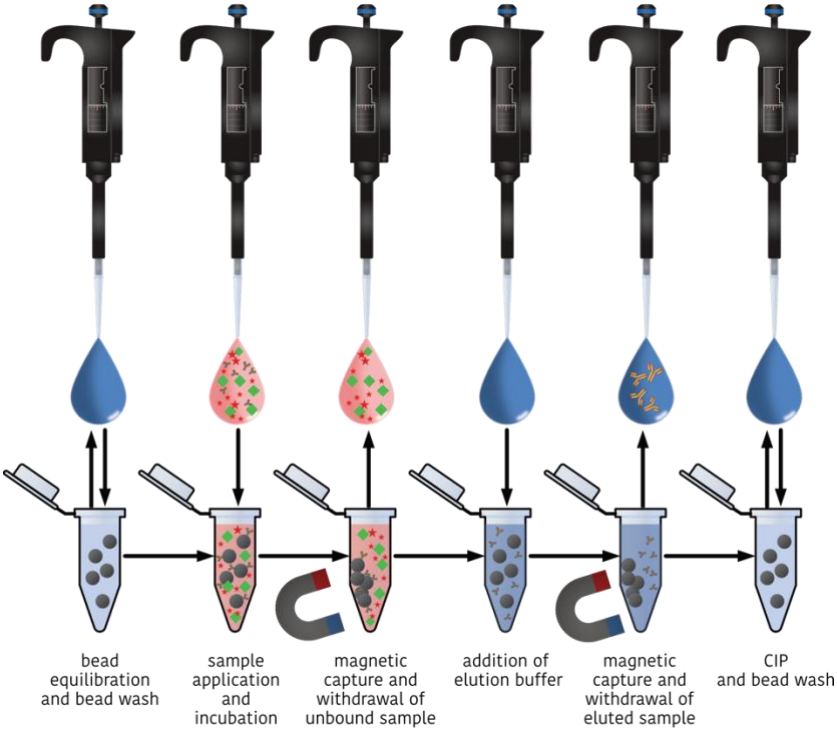

Figure 3.1: Magnetic separation workflow

Typically, magnetic workflow in small-scale separations of targets of interest. The process is typically divided into three steps (I) target adsorption which includes bead equilibration, sample application and incubation. (II) Isolation of magnetic beads in a temporary magnetic field, described as magnetic capture and withdrawal of unbound sample and (III) bead wash and target recovery including elution buffer addition and withdrawal of eluted sample.

3.2.1 Principle of batch adsorption

The adsorption of the target molecule, as mentioned, is based on the principle of batch adsorption. This process herein is explained on the basis of Protein A and monoclonal antibodies. However, the described process can easily be extrapolated to other affinity-based batch adsorptions.

Generally, the process depends on the reversible interaction between two proteins as all affinity-based systems do [173]. In this

case a ligand, i.e. Protein A (L) and target, i.e. mAb (T) as described in equation 1:

$$T + L \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} TL \tag{1}$$

Herein in reaction 1, with k_{on} and k_{off} being equal and the strength of the interaction between target and ligand, the affinity is described by the concentration ratio at equilibrium of free target [T] and ligand [L] to target and ligand complexes [TL]. The affinity is determined when the system is at equilibrium, meaning adsorption and desorption of target to the ligand occurs at the same rate. The affinity (K_D) is expressed in equation 2.

$$K_D = \frac{[T][L]}{[TL]} = \frac{k_d}{k_a} \tag{2}$$

The equilibrium dissociation constant K_D, can also be expressed as the ratio of the dissociation constant (k_d/k_{off}) and the association constant (k_a/k_{on}) [173].

Systems that provide a fast and robust interaction, a high affinity interaction, are commonly characterized by fast binding and slow release of the free target. This means that the target binds relatively rapidly to the ligand and the formed complex exists over a longer period, but the time to reach equilibrium will take longer, compared to low affinity interaction (high K_D).

However, the affinity between ligand and target only describes one aspect of the batch adsorption. The overall adsorption is another parameter for its characterization. The batch adsorption process is the determining factor and has crucial influence on the time and efficiency of the magnetic separation. As described in the workflow during the batch adsorption the magnetic beads and the feed (sample) are mixed, whereat the feed can be different biological liquids [22, 162, 164, 170-172, 174] clarified or non-clarified. The solution of magnetic beads and target is kept in suspension over the whole course of the batch adsorption process through constant mixing. The mixing is an important factor in the batch adsorption as the dynamic of the fluid has an important impact on the uptake-time of the target. Compared to packed-bed chromatography the

adsorption processes (target application and uptake) are, by essence, two systems with entirely different dynamics. Whereas the traditional packed-bed chromatography it is seen as a plug flow bioreactor and the residence time of the target is the defining factor. On the contrary, batch adsorption in magnetic separation can be described as a batch stirred tank reactor, in which the motion of the fluid together with the uptake of the target is significantly influenced by the mixing speed rather than by the residence time only [22]. However, on a local level the adsorption for both methods is identical as shown in equations 1 and 2. The batch adsorption process is performed close to the state of equilibrium of the system, leading to a variation in the residence time in batch depending on the desired target uptake. A classical model to describe the adsorption process, especially for saturation systems, is the Langmuir adsorption model [175], although most of the conditions required in this model are not coherent with the reality in adsorption [176]. The Langmuir model can be expressed as the isotherm equation 3 [149, 176]:

$$q = \frac{c^* q_{max}}{K_D + c^*} \tag{3}$$

Where q is the adsorbed solute (target), q_{max} the maximum adsorption of the solute, c^* the bulk concentration of the target in suspension at equilibrium and K_D the equilibrium dissociation constant. The binding process and the duration on the local level is highly depended on this kinetics, which in turn, as seen in equation 3, is depended on the ligand and target (K_D) and diffusion. On the global scale, the mixing, as mentioned above, plays also a vital role. The mixing can manipulate c the bulk concentration at the local level and therefore influences the diffusion and can shorten the duration of the process. Additionally, the equilibrium, and therefore the yield and duration of the process, can be manipulated by the amount of free binding sites (q); specifically in two ways: (i) q > c (more binding sites than target concentration) or (ii) q < c. In scenario (i) with an excess of binding sites the yield is potentially higher and the duration is shorter, but the magnetic beads are then inefficiently utilized as many binding sites are not occupied. In scenario (ii) the effect is the opposite with better utilization but potentially lower yield and longer duration.

Finally, batch adsorption has two other factors that makes it distinctively different from the adsorption in packed-bed chromatography. As previously mentioned, the batch adsorption can easily cope with non-clarified supernatant, which would clog the packed-bed. Secondly, batch adsorption is independent from the flow rate, which means that the total amount of target is presented immediately to all the theoretical binding sites or within the mixing time. Therefore, batch adsorption, if the parameters are kept equal, is theoretically the same at 1 mL and at 1000 L, making it a powerful tool for large-scale operation. This is not the case for column chromatography in which only a fraction of the target is presented to a fraction of the binding sites (plates), which makes the processing of large quantities of fluid difficult.

3.2.2 Principle of magnetic separation

Generally speaking magnetic separation is as any other separation technique, the sorting of two different spaces [155]. This means that the magnetic beads are separated from a space of low magnetic strength (liquid) to a space of high magnetic strength (pole of a permanent magnet), resulting in the attraction of the beads to the pole of the permanent magnet. Magnetic separation is restricted to separation of particles from liquid and is not applicable directly to molecules [155]. This can however be circumvented with the magnetic labelling of molecules. The magnetic materials used in magnetic separation determine the success of the separation. Thus, the material of the magnet and the particles are highly critical for a successful separation. In the presented work, the magnetic field was solely generated with one or more permanent magnets. Commonly, the magnetic material, which accounts for the permanent magnet and the magnetic particles, is distinguished into four categories, depending on the physical characteristics of the material: ferromagnetic, ferrimagnetic, paramagnetic and diamagnetic. The underlying physical phenomenon is based on the orientation of the electrons of the material's atoms, specifically the amount of unpaired or spin-paired electrons [155]. This can be described in terms of positive or negative magnetic susceptibility. Unpaired electron spins are attracted to a high magnetic field and provided therefore a positive magnetic susceptibly. On the other hand,

materials with paired electron spins are repelled from a magnetic field [155] and provided a negative magnetic susceptibility. The magnetic force of all magnetic material is determined by this phenomenon, as it is the sum of unpaired electron spins. Ferromagnetic materials, for instance iron, have a high degree of atoms with unpaired electrons, hence it has a high degree of magnetization (magnetic moment / magnetic dipole per volume) [155]. The second class, the ferrimagnetic material, also provides a high degree of magnetic dipoles per volume. The third category are paramagnetic materials which also provide a high degree of unpaired electrons, but are much less attracted to a magnetic field compared to the two first categories. The last category, diamagnetic materials, has no unpaired electron spins. This material is regarded as magnetically unresponsive, although they are weakly repelled by a magnetic field [154, 155].

Permanent magnets, as used in the present work to apply the magnetic field, usually belong to the group of ferromagnetic materials. However, the number of unpaired electron spins in a material does not make a material a permanent magnet. The unpaired electrons or atomic dipoles need to be aligned by an external force in order to create a permanent/temporary magnet [155]. This can be done by an external magnetic field. This phenomenon is used to produce permanent magnets in industry and is the same principle that is used for the magnetize the magnetic beads and attract them to the pole of the permanent magnet.

The material of the magnetic beads is of equal importance as the material of the permanent magnet. Similarly, the magnetic beads contain ferromagnetic material, mostly iron oxide, which provides a high magnetic susceptibility and can lead to a high magnetization saturation of the beads, if a strong magnetic field is applied [154, 155]. Other magnetic materials are usually not used for magnetic beads, as a rapid separation is desired. Commonly, extremely small ferromagnetic particles are used for magnetic beads ranging from the lower nm to below 100 Å [154, 177-179]. Due to their small size these ferromagnetic particles become superparamagnetic, which gives them highly interesting characteristics, as they provide a high magnetic susceptibility and a high magnetization saturation but lose the effect of permanent magnetization or the retention of magnetic memory, so called magnetic hysteresis [154].

The separation of magnetized beads in a magnetic field is, in general, dependent on three forces: the magnetic force (F_m) acting on the bead, the gravitational force (F_g) and the drag force (F_d) of the fluid. If the ratio, R, of these three forces is greater than 1, magnetic separation is expected to occur, as described in equation 4 by Hirschbein et al., 1982.

$$R = \frac{F_m}{F_g + F_d} \tag{4}$$

As described in equation 4 the magnetic force is the dominant force that counters the gravitational force and the drag force obtained by a liquid movement (Figure 3.2). If there is no constant flow of liquid through the system then equation 4 can be modified:

$$R = \frac{F_m}{F_g} \tag{5}$$

This, for instance, is the case for steady systems with no liquid exchange during the magnetic separation (small-scale separation). The magnetic force (F_m) in relation to the distance is dependent on the magnetic susceptibility (χ_V) of the magnetic material used in the bead, the volume of the magnetic beads (V), the magnetic field (H) applied by the permanent magnet and the magnetic field gradient ($\frac{\partial H}{\partial x}$) as described by Whitesides et al. 1983 and Hirschbein et al., 1982.

$$F_m = \chi_V V H \frac{\partial H}{\partial x} \tag{6}$$

In case the surrounding medium has a magnetic susceptibility, it needs to be considered in the formula as well. However, in the current case the medium susceptibility can be neglected. Therefore, the success of a magnetic separation depends on the described factors above. The susceptibility and volume are dependent on the used magnetic particle, while the magnetic field and field gradient depend on the used magnet. Whereby the magnetic field gradient plays a highly important role, it defines the change of the magnetic field in relation of the distance to the bead.

The magnetic field gradient is a major practical limitation for magnetic separation. With restricted field strength and declining gradient, the separation is limited to small volume otherwise, the distance between magnetic bead and magnetic field becomes too large. In order to circumvent this effect local gradients can be applied, for example multiple magnets, to cover a larger volume.

As presented above, magnetic separation is an interplay of different forces and depends on the separation conditions. With a magnetic force high enough to counter gravitational force and drag of the liquid, magnetic separation can be achieved. In scenarios without liquid flow, no drag force needs to be considered. In this case, the gravitational force seems trivial for such small beads ranging in the µm range, but the bead size together with its settling velocity can have a large impact on the success of the separation [154]. Overall, if all the factors are considered, magnetic separation can potentially be the most rapid and convenient method for separation of particles.

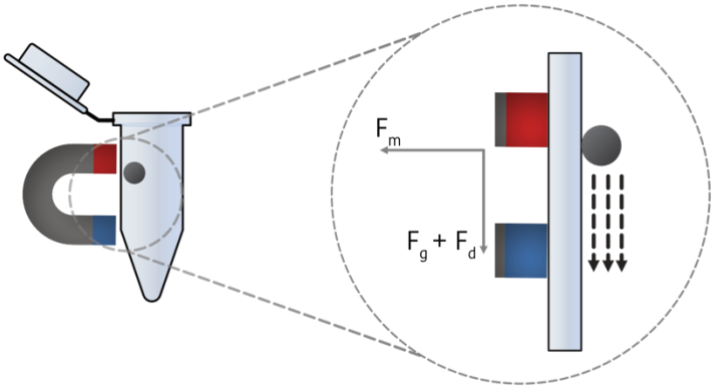

Figure 3.2: Schematic description of the interplay of forces during the magnetic separation

3.2.3 Magnetic beads

A large variety of different magnetic beads are currently available also accompanied by many custom-made beads [180]. These beads are used for an even larger range of products, many of which are molecules of interest for the biopharmaceutical or biotechnological industry, such as enzymes, saccharides, antibodies, DNA/RNA, peptides, globulins and other proteins, and cells [174, 181]. In particular, for the aim of a large-scale magnetic separation or the overall aim of implementing magnetic separation into commercial processing, the magnetic beads have a crucial role. Therefore, the properties of the beads are pivotal for the success. According to Franzreb et al. 2006, the properties can roughly be characterized into four categories: (I) magnetic characteristics, (II) morphology, (III) surface attachments and (IV) availability. To contextualize these categories, they are described shortly in the following paragraph. The magnetic characteristics describe the level of magnetization and the type of magnetism. It is desirable to use magnetic beads with a high magnetization (> 35 emu/g) and superparamagnetic characteristics, to ensure that the beads will not retain a magnetic memory and are easily resuspended [180]. The morphology describes the shape, size, density uniformity and stability [180]. The size can vary quite significantly from small beads (≈ 50 nm) to large beads (≈ 100 µm). The right size of magnetic beads is still a subject of discussion as different approaches are pursued. In case solid beads are used, small sizes are preferred as this helps to provide a large surface area to volume ratio. However, with the use of porous beads, the larger size will not affect this ratio negatively. The beads should also be monodispersed and spherically shaped to ensure identical behaviour in the magnetic field and efficient packing in the magnetic filter [180]. Furthermore, the density and the robustness of the bead's construction are other important factors of the morphology [180]. In category three the surface, the attachments and the roughness of the magnetic beads are listed. High roughness of the surface is desirable as it increases the surface area. Also, the surface should be easy to derivatized and should provide easy access to the ligands [180]. The last category comprises the availability of beads. The magnetic beads should preferably be easy to access, e.g. purchase, and low in cost as this will substantially determine the economic viability [180].

The beads, used in the presented thesis comply with the categories given, but diverge from the view of Franzreb et al. 2006, regarding size and cheapness of the ligand. All studies used the commercial MAGicBeads from MAGic Bioprocessing (Uppsala, Sweden), see Table 3.1. The MAGicBeads provide high magnetization of 40 emu/g and superparamagnetic behaviour. The MAGicBeads consist of 4 % agarose resin with an average diameter between 90 and 100 µm [22, 95, 172] and can be coupled with various ligands, such as Protein A, Protein G, quaternary amines and sulfur trioxide. The highly porous beads provide a high surface area resulting in a capacity that can compete with traditional packed-bed chromatography resin. The downside of the high porosity is the longer time needed for the target to diffuse into the cavities. Additionally, the probability of fouling effects is higher in porous beads, as proteins and such can remain in the beads' cavities after the separation, but this can be circumvented with sufficient wash and CIP protocols as it denatures residual proteins.

Magnetic Bead	Composition	Size [µm]	Reference
MAGicBeads	Agarose	90 - 100	[22, 95, 172]
Dynabeads	Polystyrene	1 -2.8	[182, 183]
MagnaBind	Silane	1 – 4	[184]
NEB magnetic prA beads	N.A.	N.A.	[185]
SureBeads	N.A.	2.4 – 3.4	[186]
Abcam PrA Magnetic Beads	cross-linked agarose	75 – 150	[187]

Table 3.1: Short overview of commercial beads for mAb purification

The table shows a small fraction of commercially available beads linked with Protein A or Protein G for the purification of antibodies.

3.3 Target oriented magnetic separation process

This subchapter will discuss the specific differences in the separation for the targets used in this work, starting with the mAb purification for which many notions have already been introduced and followed by the isolation of cells and its specific characteristics.

3.3.1 Magnetic separation of antibodies

The separation of antibodies has already been used as a model throughout this chapter, so it is presented here as a concise summary. The magnetic separation of monoclonal mAbs depends highly on the affinity of the Protein A ligand and the subtype of the targeted antibody. For different origins and IgG subtypes the affinity can significantly change [123]. Therefore, it is highly important to identify the IgG subtype prior to the purification. When, the chosen system is suitable for the IgG, the process is identical and has a workflow, as previously described in Figure 3.1 and is shown below in Figure 3.3. The purification process for antibodies based on magnetic beads starts with the termination of the cell culture.

In order to determine the amount of magnetic beads used for the purification the fraction of the maximum capacity at equilibrium can be used. After the conditioning of the magnetic beads (*equilibration*), the beads can be directly added either to the culture supernatant or the non-clarified cell broth. Once a sufficient mAb adsorption or the equilibrium has been reached, the magnetic beads are retained in a magnetic field and then the cell culture liquid (i.e. cells and conditioned medium) is removed. The beads are washed several times, depending on the amount of cells in the cell broth. The elution is then performed and followed by neutralization of the eluted fraction to avoid aggregation. Finally, sanitation of the magnetic beads with 0.1 M NaOH takes place.

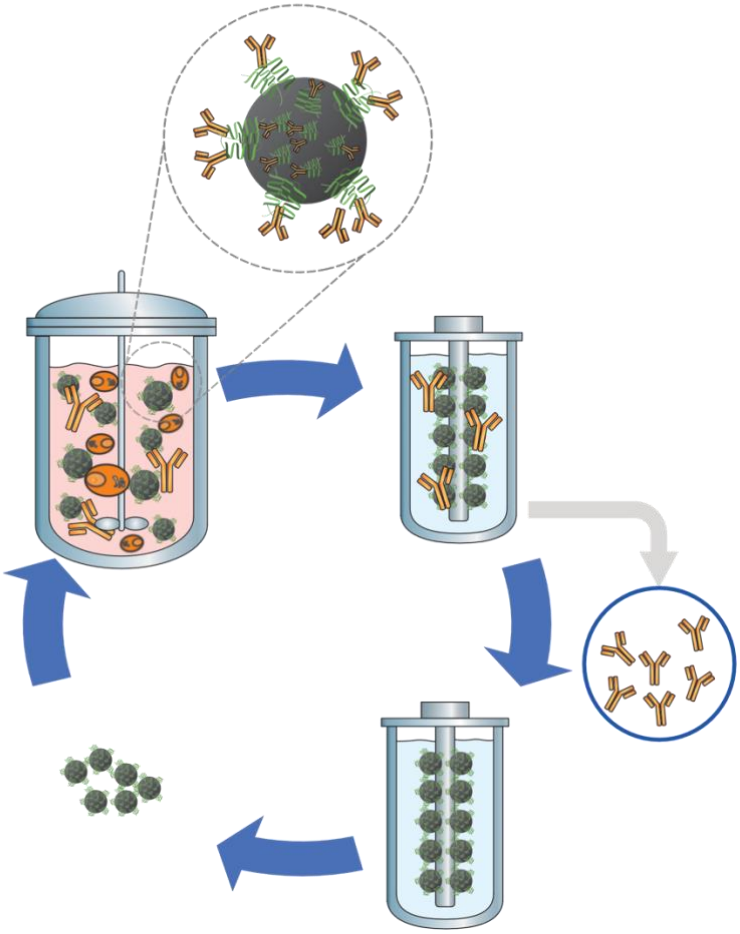

Figure 3.3: Schematic magnetic separation in large-scale

Schematic representation of a magnetic separation of mAbs including the three phases of magnetic separation: (I) target adsorption which includes bead equilibration, sample application and incubation with the cell broth, (II) isolation of magnetic beads in a temporary magnetic field and (III) bead wash and target recovery including elution and withdrawal of eluted sample. After the magnetic separation the beads are sanitized and re-sued in a circular process.

3.3.2 Magnetic separation of cells

The isolation of cells is to some extent different from the separation of antibodies. The basic principle described earlier still applies but with an important modification. Firstly, cell isolation is categorized in either *negative selection* or *positive selection*, these are opposite methods to isolate a desired subpopulation. In *negative selection* the unwanted subpopulation is targeted and removed, while the desired subpopulation is retained in the liquid, sometimes also referred to as cell depletion. Opposite to it, *positive selection* targets the desired subpopulation and the unwanted cells are washed away [188]. *Positive selection* is seen as the more selective method as it targets the desired subpopulation directly and is therefore highly useful for unprocessed samples, for instance whole blood [188]. However, it requires a release system for the isolated cell population. *Negative selection* is a simpler method, which has a lower specificity as it targets the unwanted cell, but does therefore not interfere negatively with the desired cell type. Therefore, it is commonly applied if the desired subpopulation needs to remain untouched [188] as antibodies interacting with the surface receptor this can lead to unwanted cell change. Both methods can be used in combination in order to enable highly specific sorting.

In short, magnetic cell isolation can be divided into the steps of (I) antibody/surface receptor selection (conjugated beads), (II) magnetic labelling and (III) magnetic capture (Figure 3.4). As *negative* and *positive selection* are highly identical and merely differ if the isolated subpopulation or untouched subpopulation is desired. In this setup, a cell and not a molecule is targeted, which provides a target significantly larger compared to antibodies. The first step is the selection of the desired surface receptor for the specific subpopulation. This receptor should preferably be unique for this subpopulation to selectively target it. The second step that is followed is the magnetic labelling. Here the antibody conjugated to the magnetic beads interacts with the cell's surface receptor, which is commonly achieved by incubating and mixing the beads and cells. The labelling of the subpopulation is a rapid process facilitated by the constant mixing and highly selective antibody. The last step of the process is the magnetic capture. Here the magnetically labelled cells are retained in a magnetic field while the populations not expressing the surface maker are removed. Depending on the type

of cell isolation either the magnetically labelled fraction or the unlabelled fraction is further processed.

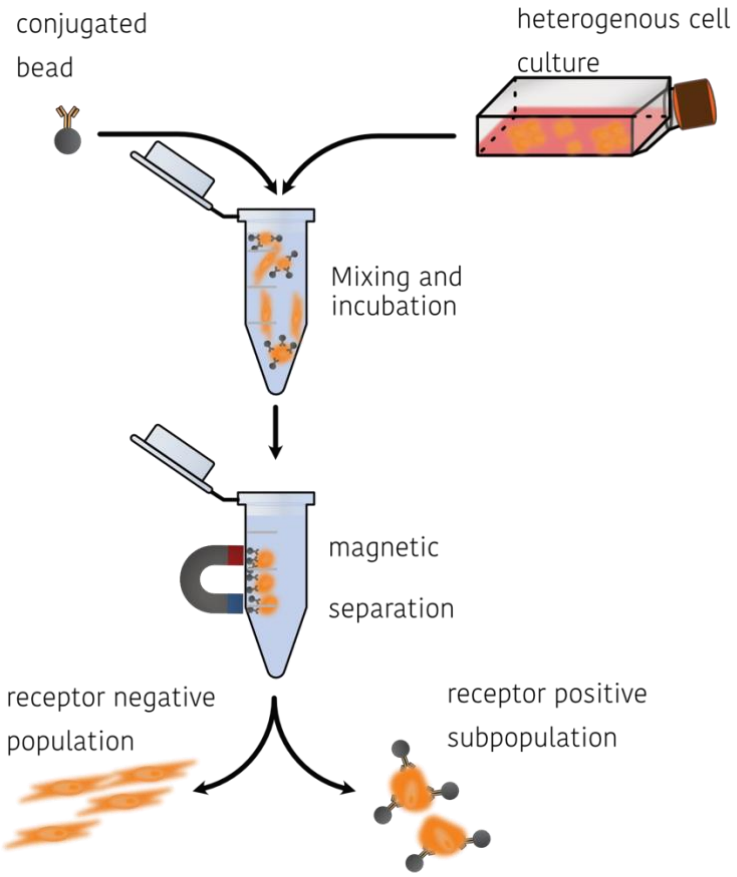

Figure 3.4: Magnetic isolation of cell subpopulations from heterogenous populations

Representation of a magnetic isolation of cell subpopulations as either *positive* or *negative selection* of receptor positive cells divided in the typical workflow of (I) antibody/surface receptor selection, (II) magnetic labelling including mixing and incubation, and (III) magnetic isolation

Chapter 4

Challenges in intensified mAb manufacturing

This chapter will give a brief overview on the perspective of mAb manufacturing with regards to process intensification and continuous manufacturing. Today, a large majority of manufacturing process for mAbs rely on batch operation such as: fed-batch cultivation or batch DSP.

In the recent years, continuous manufacturing [189-193] and process intensification [194-201] have been a burgeoning field of research in both academia and industry [202]. Culture intensifications based on medium renewal in which the volumetric productivity is notably increased due to much larger cell concentration [22, 203] can be seen as the spearhead of this movement. Intensification and continuous operations for mAb production can provide large economic benefits [202] with higher throughput, higher product titer and higher flexibility coupled with lower capital expenditure (CAPEX), lower cost of goods (COG) and smaller footprint due to smaller production plants [203].

4.1 Limitations

Although the described advances have pushed mAb manufacturing into new spheres not only from an economic point of view but also from a product quality standpoint with a much more sophisticated and controlled cell environment, this intensification also creates bottlenecks and challenges for the downstream operations. Especially, the DSP has to cope with the increased volumetric productivity in the form of higher cell mass and mAb titer.

In general, the challenges can be divided in two categories: (i) clarified liquid (ii) particle containing liquid. The first category refers to purified harvest streams, coming from cell clarification steps or perfusion operation that sieves the product out. In process intensification, this is commonly the case with a cell separation device that retains the cells and removes product and metabolites via e.g. a hollow fibre or other cell retention devices. For category (i) innovative methods have been put in place to cope with large quantities of clarified liquid. For example the capture step is operated in a so called “periodic counter-current” (PCC) mode [204]. In this setup multiple columns are connected together to facilitate the capture process. Commonly, this is operated as a three-column system in which two columns are interconnected during the loading while the third column is washed and eluted [205]. On the contrary, if the capture steps downwards of an intensified culture are operated in batch mode, the large amount of liquid can present a major challenge for the DSP. In batchwise operated packed-bed-based DSP, the sample loading and washing phase, depend on the volume, and take the majority of the processing time [162]. The loading and washing steps are limited to a maximum flow rate to avoid a high pressure drop, depending on the design and dimensions of the bed. The loading of several hundred litres of feed can take up to hours or even days. This forces the splitting of the capture step in several cycles as columns cannot be scaled-up indefinitely. Also, single loading would result in inefficient resin utilization leading to economical drawbacks and loss of process intensification. In comparison the batch adsorption process, used in magnetic separation, is a very rapid process. The loading phase is theoretically finalized immediately, however practically restricted by the maximum flow rate of the used pumps. This is similar for the following wash steps, which are performed immediately as well

providing another speed advantage. Overall, due to the independence from the flow rate in magnetic separation, liquid processing can be achieved faster compared to the flow dependent packed-bed-based separation.

On the other hand, category (ii) provides different kind of challenges for the DSP. The large number of particles, i.e. cells, in cell culture broth, commonly obtained by large fed-batch processes or by intensified operation such as intensified fed-batch cultures challenge mainly the cell clarification steps [206]. From an economic standpoint these steps are time intensive and costly due to consumables and/or equipment [206]. From a process point of view this step can negatively interfere with the feed stream and can lead to cell lysis causing an increase in levels of host cell proteins (HCP) and DNA [71, 207]. Furthermore, not only the release of HCP can be critical but very high cell density feed streams provides another challenge for the cell clarification, as centrifugation and filtration methods can reach their limitation leading to an insufficient removal of solids and a yield reduction of the steps [208, 209]. Additionally, centrifuges can still cause cell disruption which leads to product degradation and decrease of clarification efficiency [22, 208]. Furthermore, the depth filtration step presents another limiting factor with solid still present after the centrifugation. This leads to potential filter clogging or poor utilization of the filter [206, 208]. Therefore, an alternative approach has been introduced, in which the high cell density feed stream is pre-treated to improve the clarification step for instance with flocculation agent or low pH. This can improve the clarification as a large fraction of solids are removed prior to the clarification step. But the supplementation of the feed stream with new components, which need to be removed during the DSP, or unsuitable low pH can be cumbersome for the following steps [29, 206, 210]. Thus, category (ii) still remains a bottleneck especially for intensified cultures. In comparison, magnetic separation provides desirable properties, such as being gentle, rapid and compatible with complex crude cell broth [22, 162, 164, 171, 172, 174]. Beside the fact that magnetic separation can cope with non-clarified cell broth, the gentleness of the process is another interesting feature. Due to the mechanically gentle process, cell lysis is decreased leading to lower levels of HCP and DNA. Magnetic separation can potentially cope with issues related to very high cell

densities and can therefore be an alternative for the discussed operational steps.

Chapter 5

Challenges in cell therapy manufacturing

This chapter will discuss the challenges and limitations for cell therapy manufacturing with the focus on cell sorting. The status quo of current manufacturing strategies has been described already in Chapter 1.3

Cell therapy has gained vibrant interest in the recent years with an increasing number of clinical trials and usage of cell types [103, 211]. Using living cells as a therapeutic has the potential to dynamically perform many complex biological functions simultaneously that cannot be achieved with the use of conventional drugs [103]. With the maturation of this therapy approach, the manufacturing has to develop likewise [211]. The success of cell therapies relies on the future development and solutions for critical challenges, such as; biological challenges, manufacturing challenges and regulatory challenges [103]. In the following text, the manufacturing challenges are discussed.

5.1 Limitation

The manufacturing of cell therapy products, independent from the type of therapy, lacks common methods. The processes are mostly not standardized, hardly automated, not performed in closed systems and have limited scalability [211]. This all leads to incredible costs for single therapy doses which can hardly be sustained. Especially, the large number of open and manually performed steps are making the process costly and inefficient [211]. The field is still emerging and developing and it was shown that manufactures have problems meeting the product specification [212]. This means for autologous therapies that the patient might be unable to get a treatment if the batch has to be discarded, similarly, in allogenic therapies if a batch is out of specification the resulting financial impact can increase the cost to the patient. As cell therapy products are highly complex and specific and depend on complex raw material (donor cells), reduction of complexity and variability of the manufacturing process needs further development [212]. Additionally, the dosage per patient can vary from millions to tens of millions of cells per patient depending on the application [213]. Manufactures strive today to orientate towards the implementation of bioprocess principles already established for biomolecule production [211]. Cell manufacturing can be daunting as it is more complex compared to biomolecules. These cellular products are also more vulnerable and process variations can lead to ineffective or low quality products [115]. It is critical for the manufacturing to have a deep understanding of cell biology, mode of action as a therapeutic, and the manufacturing process [115] to achieve robust, efficient and cost-effective processes as well as high-quality and safe cell products.

As described in Chapter 1.3 the manufacturing process of cell therapy products consist commonly of 5 steps, with certain variations depending on the product. These 5 steps in turn can also be divided in UPS and DSP of the process. While the steps of cell collection, cell isolation/selection and cell expansion belong to the USP, cell harvest/concentration/purification/formulation are accounted in the DSP. The administration is seen as a separated step and not further discussed in the section. Multiple challenges exist in the manufacturing workflow for both autologous and allogenic therapies.

Aside from the collection of raw cell material, which can be highly complex itself and is not discussed in here, the isolation and cell selection is already a critical parameter. The step of cell isolation/selection also represent a step that can be present in both USP and DSP. FACS and IMS, as introduced in Chapter 1.3, are commonly used here. However, they have different advantages and drawbacks. FACS enables processing of multiple high purity subpopulations but suffers from costly equipment, long processing times and substantial cell loss [95, 214, 215]. IMS provides processing of larger quantities, higher throughput, lower cell loss, but usually provides lower purity [95, 214, 216, 217]. But most importantly both methods are only able to process relatively small volumes. For example, IMS uses low magnetized magnetic beads and a scaffold to enhance the magnetic gradient for the separation of beads and liquid. This can be problematic as the scale-up can be limited by the low magnetization of the beads. Therefore, these methods are commonly used before the cell expansion.

The following cell expansion is highly limited, due to the adherent nature of a lot of the cell therapy products. The restricted surface area in adherent cultures (2D-cultres) limits the production of sufficient amounts of cells for treatment or clinical studies [115]. Although there have been advances, overall the scale up is logistically impractical and fundamentally inefficient [115]. But with the dosing and the number of cells needed per dose being determining factor for the expansion. The cell expansion needs to overcome the limitation of adherend cultures (2D-cultres) and need to move to suspension cultures (3D-cultures). The exact dosing still can vary with the application, but is estimated to be in the range of 10^9 cell/patient [218]. This would place a significant burden on the expansion, especially in allogenic approaches where one batch is used to treat several patients. Depending on the size of the therapy application it can be expected that commercial scale manufacturing needs to accommodate a high number of cells per year leading to potential batch sizes between 200 to 2000 L for allogenic therapies [218].

The DSP is a more critical factor for the success of cell therapies as it ensures safe and high quality cell products. DSP for cell therapy products include the step of cell harvest/concentration/purification/formulation [115]. Firstly, the time window during the DSP should be kept to a minimum,

commonly 6 – 8 h, as the cells can be affected by prolonged periods in suboptimal conditions [115]. With growing culture volumes and number of cells that need to be process the time factor becomes highly critical.

The cell harvest together with washing and concentration presents a particular challenge, as each step depends on different factors from the cell expansion (culture system, cell type, etc.). Filtration and centrifugation can be used to efficiently separate cells from the culture medium and further wash and concentrate them. These methods can be limited by throughput (centrifugation) or introduction of harsh mechanical conditions and clogging (filtration). Due to the fragile nature of human cells this step can easily compromise the cell's integrity. Therefore this step needs to be mild and gentle enough to maintain the cell's integrity [113]. But even more importantly the currently used methods for cell harvest/concentration/purification/formulation are non-specific and therefore unable to isolate or remove cells based on surface maker expression. This becomes an even more significant problem in allogenic therapy approaches where higher cell quantities, derived from iPSCs, are produced in commercial scale-ups. Here it is necessary to remove undifferentiated cells prior to patient administration to eliminate the risk of tumour or teratoma formation [96-101]. Therefore, reliable cell isolation methods are needed that are efficient, robust, simple and generic to ensure the cell's functionality and safety [95, 219].

Chapter 6

Present investigation

The work presented in the following studies aimed to contribute to a solution for legacy process limitations and development of an alternative capture method. A novel magnetic separation technique based on highly magnetized superparamagnetic beads and permanent magnet-separator was introduced for the isolation of biological therapeutic modalities. The work was divided into the development of an alternative capture process in monoclonal antibody manufacturing and the development of a novel scalable isolation system for the purification of cell subpopulations.

A magnetic separation process enables the integration of several operational steps into a single step. This was demonstrated in different systems such as small-scale and pilot-scale studies with variation on the cultivation mode, low and very high cell density and different cell lines i.e., CHO cells, hiPSCs, hMSCs, HUVECs and SKBR3 cells. The application of the alternative of magnetic separation can help to efficiently target current bottlenecks in mAb manufacturing processes, as this process shows great improvement within one step that includes the clarification and capture compared to state-of-the-art processes. When it comes to cell therapy products, such as pluripotent cells, the alternative of magnetic

separation might help to target future bottlenecks of scale-up operation that emerge with these new modalities.

The contribution of the present alternative magnetic separation is divided in two categories, outlined below.

1. Alternative capture for monoclonal antibody manufacturing
    - *Paper I*: Pilot-scale process for magnetic bead purification of antibodies directly from non-clarified CHO cell culture
    - *Paper II*: Antibody capture process based on magnetic beads from very high cell density suspension
2. Large scale tool for cell isolation in allogenic therapy approaches
    - *Paper III*: Proof-of-concept of a Novel Cell Separation Technology Using Magnetic Agarose-Based Beads
    - *Paper IV*: Negative selection of human induced pluripotent stem cells (hiPSC)

In papers I – II, an alternative capture step based on magnetic agarose beads and magnetic separation was introduced, which replaces the operational steps of centrifugation, filtration and mAb capture by a single step process integrating cell clarification and mAb capture. The process was developed, characterised and optimized with regards to pilot-scale and very high cell density operation. The magnetic system was further evaluated as a tool for the isolation of cell subpopulations in papers III-IV. Herein, the magnetic beads were used as a highly flexible tool combining the advantages of current cell isolation techniques.

6.1 Alternative capture for monoclonal antibody manufacturing

Monoclonal antibodies are the biggest blockbuster in biopharmaceuticals and are expected to grow further. This ever-growing demand places a significant burden on the manufacturing, especially on the capture and downstream operations. As already described in Chapter 4, the DSP for monoclonal antibodies highly depends on clarified cell culture supernatant. The requirement for multiple cell clarification steps prior to the capture can result in a significant loss of product that counteracts the improvements from the USP. Therefore, reduction of operational steps at large-scale can improve the yield and throughput. However, the improvements from the USP by highly intensified culture processes create another issue. Due to the high cell mass generated in these systems the following clarification steps can become difficult to operate. These problems are addressed in papers I and II where an alternative solution is shown for the current manufacturing processes for mAbs.

Paper I – Pilot-scale process for magnetic bead purification of antibodies directly from non-clarified CHO cell culture

Paper I develops and then implements the magnetic separation based on Protein A magnetic resin suitable for pilot-scale processes in order to circumvent the limitations of product loss in current fed-batch processes for the production of monoclonal antibodies. Essentially, the magnetic separation based on Protein A magnetic resin operated as an integrated step replacing centrifugation, filtration and product capture, improving upon the traditional column-based Protein A capture.

Firstly, the new Protein A magnetic resin was evaluated with regards to its binding kinetics, specifically the Langmuir adsorption kinetics and affinity. This is important for the characterisation of the magnetic resin, as it determines the maximum binding capacity (Q_{max}) for antibodies. The maximum binding capacity is not only used to rank the resin in relation to traditional Protein A resins, but also for the desired process operation. The maximum binding

capacity is a key indicator used in process scaling and gives the maximum amount of antibody that can be adsorbed per mL of resin. Figure 6.1 shows the Langmuir kinetics obtained at equilibrium with two different antibody samples: polyclonal antibodies and monoclonal antibody. The experimental data from all saturation conditions were fitted according to the Langmuir isotherm. At adsorption saturation or reaction equilibrium, the static binding capacity of the resin is obtained. The polyclonal antibodies had a maximum binding capacity of 56 mg/mL and the more relevant monoclonal antibody IgG1 65 mg/mL (Figure 6.1). In relation to commercial non-magnetic Protein A resins with capacities of 64 mg/mL for rProtein A Fast Flow, 66 mg/mL for Mabselect SuRe and 63 mg/mL for Captiv A PriMAB and Amsphere Protein A JWT203 [172, 220] the presented magnetic resin provided a superb capacity. However, for process development the static capacity is of secondary interest as it only displays the binding at equilibrium. The capacity that is dependent on process parameters is more useful, which is why, in packed-bed chromatography, the dynamic binding capacity (DBC) is commonly used. This puts the capacity in relation to the flow rate and ultimately, the retention time. Magnetic separation does not provide such an index number due the nature of the process used. Therefore, the fractional saturation of the magnetic beads was used as the scaling parameter for the pilot-scale operations.

Once the key process parameters were identified in small-scale, the pilot-scale magnetic separation was developed. The used magnetic separator is a chamber containing 8 retractable permanent magnets in which the magnetic beads are attracted to the magnetic field and isolated from the liquid. Firstly, the capabilities to separate and magnetically hold the resin along with buffer flows were evaluated. This was followed by a development run using clarified supernatant (CF). The purification was performed with 26 L of supernatant at a concentration of 440 mg mAb/L. The magnetic separator proved to be capable of handling the supernatant and 1 L of beads at a selected target flow of 100 L/h and resulted in a final elution of 87 % in 2.5 L of buffer.

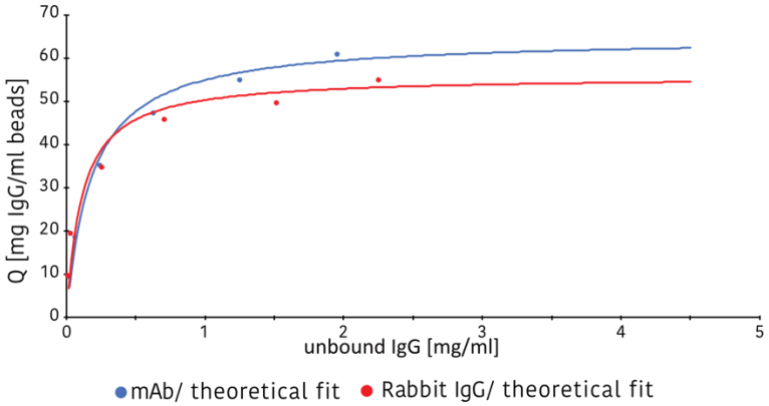

Figure 6.1: Langmuir isotherm

Capacity assay for a monoclonal IgG1 and a polyclonal rabbit IgG determined at the reaction's equilibrium. Blue dots represent the measurement points for the mAb, while the blue line shows the theoretical fit. The rabbit IgG is shown as red dots for the measurement points and red line as the extrapolated fit.

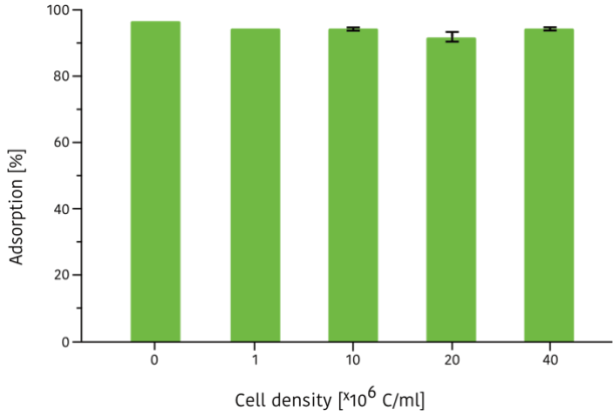

Figure 6.2: mAb adsorption in cell broth at various densities

Adsorption of free mAb from cell broth containing cell densities up to 40 x 10^6 cells/mL with cell free adsorption as a reference. All Adsorption are performed at 20 mL scale containing a set amount of mAb (20 mg).

Additionally, for evaluation of the adsorption behaviour in cell containing liquid, small-scale studies were performed with a

range of cell densities (Figure 6.2). Cell-free supernatant, i.e. supernatant in the absence of cells, was used as a reference. All the adsorption runs were treated identically with the same amount of mAb and beads, but with varying cell densities (0, 1, 10, 20 and 40 x 10^6 cells/mL). The adsorption proved to be identical in all the conditions with mAb captures larger than 90 % (Figure 6.2). The identical adsorption of the different conditions showed that mAb capture was feasible from non-clarified supernatant and that the cell density had only a very small influence.

The efficient capture of mAb and the demonstrated functionality of the magnetic protype separator in the presence of cells built the premises to perform pilot-scale purifications on non-clarified cell broth. Two pilot-scale separation runs were performed, run B1 and run B2 (Figure 6.3), based on two identical fed-batch cultivation with CHO cells resulting in cell broth of 15.57 (1.31 g/L mAbs) and 16.25 L (1.51 g/L mAbs), respectively. Run B1 and run B2 used the same magnetic separator, beads and similar setting as the CF run. The amount of magnetic resin was determined based on the culture volume and mAb titer one day prior to the separation. Based on a conservative fractional saturation of the beads, the separations were performed with 0.8 L and 1L of beads for runs B1 and B2, respectively. Both separations were operated with slightly different cell densities, viabilities and volumes (Table 6.1). Based on the experience gained from run CF the total adsorption time was reduced from 4 h to 2 h. Although both cell broths were slightly different, the adsorption behaviour obtained was highly identical for both pilot-scale separations, showing an efficient mAb uptake within 1 h of 99 % and 95 % for runs B1 and B2, respectively (Figure 6.3 A). The following elution was also adjusted based on the experience from run CF. The magnetic resin was reassembled in a semi-packed bed for a more efficient elution profile (Figure 6.3 B). This generated a column-like elution with highly concentrated product. However, run B1 showed an insufficient elution of 52 %, which was found to be caused by erroneous buffer pH that was above 3.5 instead of 3.0, highlighting the importance of correct elution buffer pH. The elution in run B2 was adjusted and performed at a pH of 2.8. This resulted not only in a high yield of 86 % but also in an elution volume of 1 L (Figure 6.3 B). The elution volume of 1 L was equal to the bead volume generating an elution volume similar to packed-bed chromatography and providing a

concentration factor of 16.25. The collected elution fractions from runs B1 and B2 were analysed for HCP levels. The level of HCP contamination in the final mAb product is one of the most important critical quality attributes [36, 46, 221-223] and an important regulatory requirement. Runs B1 and B2 showed a logarithmic reduction of 2.95 and 2.9, respectively. This reduction was in a similar range as traditional column chromatography [46, 224]. However, the magnetic separation provided superior HCP levels in the eluted products with 4.2 and 7.5 ppm/mg mAb. This represents a significant decrease compared to the legacy technique based on column Protein A capture including cell clarification (300 ppm/mg)[36, 222, 225].

	run CF	run B1	run B2
Feed volume [L]	26	15.57	16.25
mAb titer [g/L]	0.44	1.31	1.51
Viability [%]	-	89.9	75.9
Amount of magnetic beads [mL]	1000	800	1000
Total process time (including adsorption)	N/A	≈7.5 h	≈ 5.5 h

Table 6.1: Process parameters

Parameters for the used supernatant and cell broth obtained from two fed-batch cultures used for the development and evaluation of the pilot-scale magnetic separator.

Both pilot-scale runs based on non-clarified cell broth showed that a purification of mAb in pilot-scale was not only feasible but also eliminated the need for a separated cell clarification step. Magnetic separation provided the advantageous feature of batch adsorption that enabled the capture throughout the whole volume of magnetic resin. Furthermore, the presented approach provided an elution technique that circumvented the cumbersome elution commonly seen for larger scale magnetic separation. Finally,

the assessment of HCP levels in the eluted product revealed excellent purification qualities with levels at the lower end of the regulatory requirements for biopharmaceuticals (< 1 - 100 ppm) [70, 221, 226-228]. This showed that the magnetic separation provided a very gentle and efficient system. With the adsorption directly applied to the cell broth, the integrity of the cells remained intact, and therefore were less likely to release large amounts of HCPs compared to processes that include clarification steps.

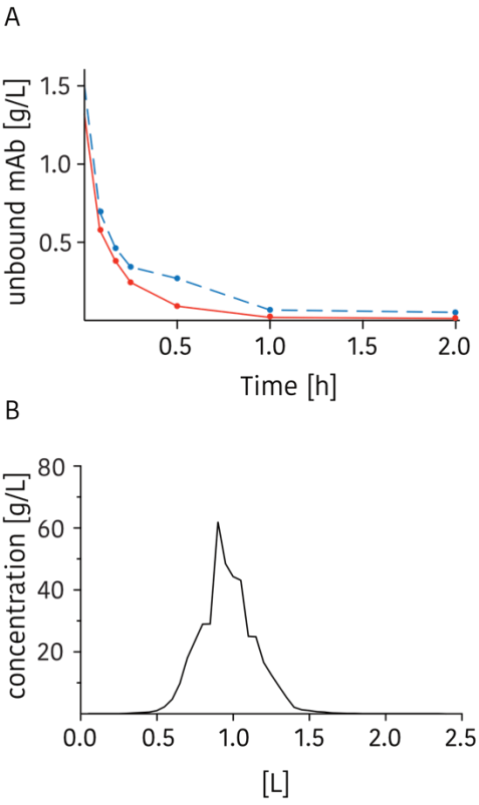

Figure 6.3: Pilot-scale purification on non-clarified cell broth from fed-batch cultivations

(A) Adsorption efficiency from run B1, 15.73 L (red/solid), and run B2, 16.25 L (blue/dashed) at mAb concentrations of 1.31 and 1.51 g/L. (B) reassembled profile from run B2 elution taken as spot samples and measured at A_{280}.

Paper II – Antibody capture process based on magnetic beads from very high cell density suspension

In paper II the magnetic separation technology for the capture of monoclonal antibodies was further investigated with the aspect of implementation for process intensification. The growing interest in process intensification, especially cell culture based on the continuous exchange of medium, in order to increase the productivity has generated new challenges for the following operational step: centrifugation, filtration and mAb capture. Cell culture intensification through medium exchange can be accomplished through different approaches but ultimately leads to an increase in cell density and with it, increase in productivity. One of the concepts used for process intensification is referred to as intensified fed-batch or concentrated fed-batch. Similar to perfusion process, this technique uses a hollow fiber filter as cell separation device to renew cell culture medium. In contrast to perfusion, intensified fed-batch uses an ultra-filter which has a much smaller cut-off (≤ 50 kDa). This enables not only very high cell density but also accumulation of the mAb, combining the advantages of perfusion and the traditional fed-batch process. As intensified fed-batches are typically harvested only once, at the end of the culture duration, the very high cell mass represents a severe challenge for the clarification step [206].

The magnetic bead-based separation was operated for the first time to capture mAbs from non-clarified high cell density cell broth obtained in intensified fed-batch cultures, shown as a schematic in Figure 6.4.

Three independent intensified fed-batch cultures were operated in small-scale glass stir tank bioreactors with a working volume of 200 mL with ultra-filter (UF) hollow fiber (HF) cartridges. All three cultures were performed identically, except for the cell density and the hollow fiber filters of identical cut-off resulting in the cultures named cult_01, cult_02 and cult_03. The evolution of the culture's density, viability and mAb concentration as a function of time can be seen in Figure 6.5. The cultures covered a large range of cell densities, commonly found in industry, from low (≈ 20 x 10^6 c/mL), medium (≈ 50 x 10^6 c/mL) and very high cell density (≈100 x 10^6 c/mL) (Figure 6.5 A). These different conditions were used for the subsequent magnetic separations, named run_01,

run_02 and run_03. Cult_01 was used as the initial evaluation of the system, the relatively low cell density (≈ 20 x 10^6 c/mL) was a result of early termination due to filter clogging. Cult_02 showed a constant cell growth until the medium cell density of ≈ 50 x 10^6 c/mL was reached and maintained for several days through the cell bleed.

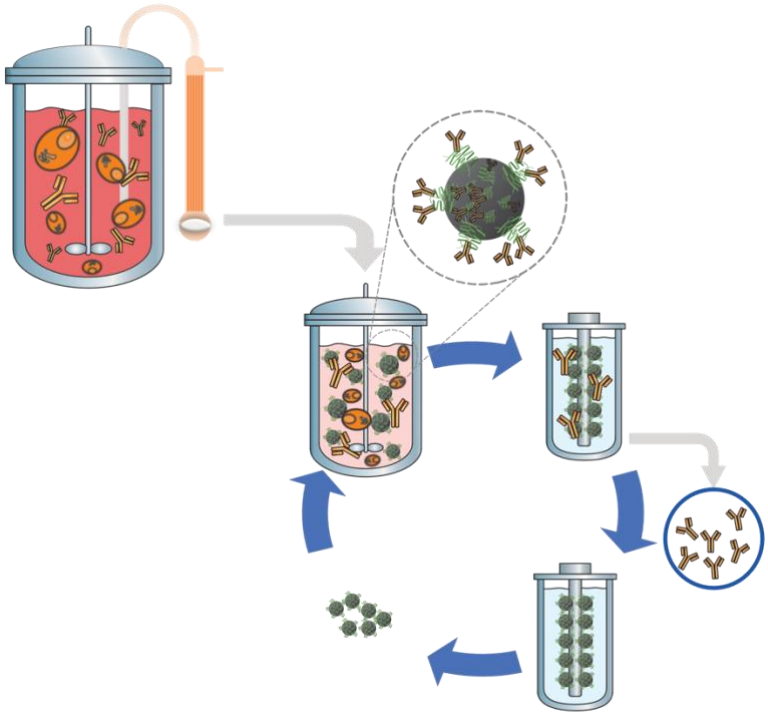

Figure 6.4: Magnetic separation downwards of an intensified fed-batch process

Schematic representation of a magnetic separation of mAbs from an intensified fed-batch culture including the three phases of magnetic sepration: (I) target adsorption which includes bead equilibration, sample application and incubation with the cell broth, (II) isolation of magnetic beads in a temporary magnetic field and (III) bead wash and target recovery including elution and withdrawal of eluted sample. After the magnetic separation the beads are sanitized and re-used.

Finally, the last culture cult_03 provided a similar growth to cult_02 but was not controlled through cell bleed and was pushed until collapse followed by the culture harvest. At the end of all three

cultures the harvest provided a large range of product concentration ranging from 0.8 mg/mL to 10.8 mg/mL (Figure 6.5 B and Table 6.2).

The magnetic separations (run_01, run_02 and run_03 of cult_01, cult_02 and cult_03, respectively) were immediately performed on the cell broth after the harvest of the culture. The single magnetic separation integrated the operational steps of centrifugation, filtration and product capture. The removal of the free mAb was monitored for all separation runs with samples taken at regular intervals (Figure 6.6). The adsorption of all the separation runs could be described by an asymptotic decline of the free mAb and rapid capture within the first 15 min. In all three separations, complete adsorption of 98 - 99 % was reached (Figure 6.6). Subsequently, the elution was performed in a semi-packed column in order to minizine the washing and elution volumes. For all the runs high yields, with 80 %, 90 %, and 94 % (Figure 6.6) and high concentration factors were obtained 3.9, 3.3, and 1.8 for run_01, run_02 and run_03, respectively.

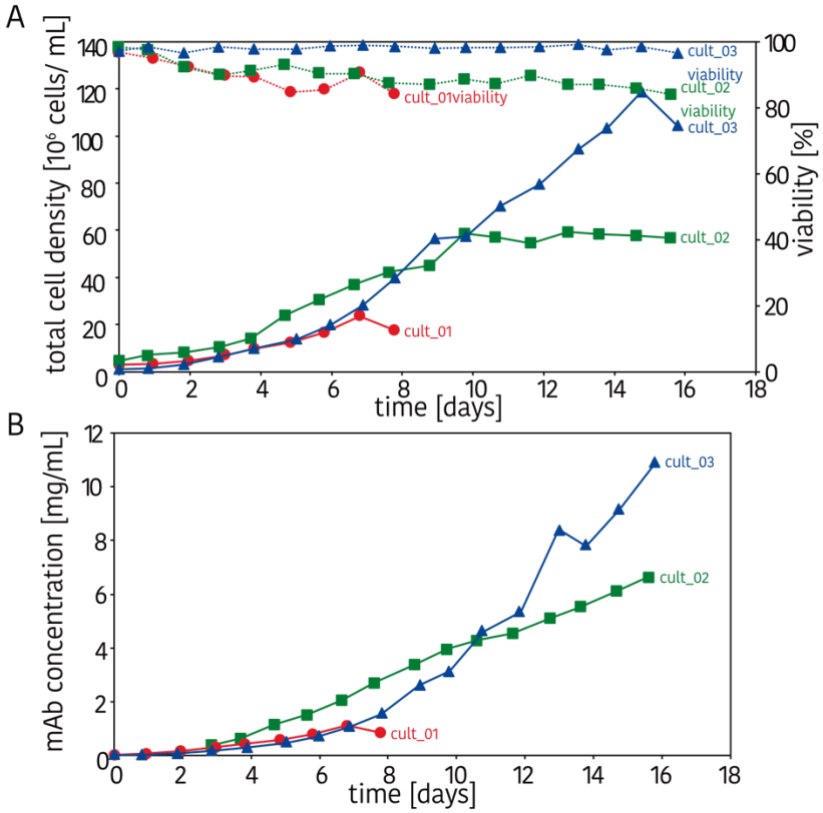

Figure 6.5: Intensified fed-batch cultivation data for all three cultures

A) Cell viability and total cell density as a function of cultivation time. (B) production titer in the bioreactor shown as mAb concentration as a function of cultivation time.

The quality of the purified mAb obtained after the magnetic separation was analysed with regards to important quality attributes, mainly residual HCP levels, size distribution, variation of charge variants and N-glycosylation isoforms.

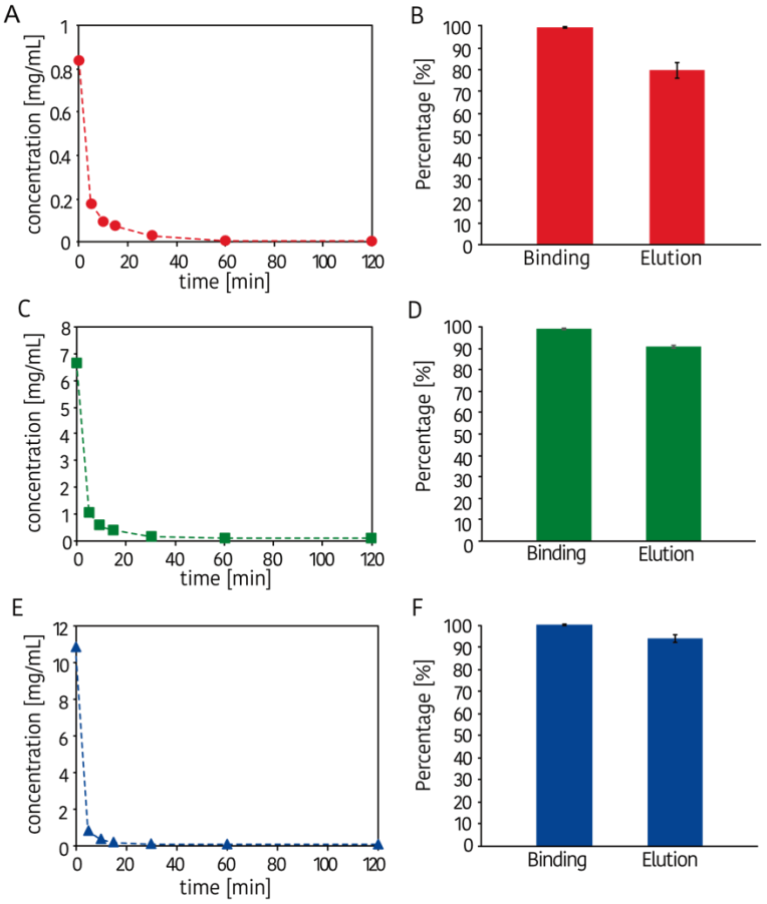

Figure 6.6: Magnetic purification directly applied to the harvest of intensified fed-batch cell suspension

(A, C, E, left panels) Adsorption efficiency of three independent magnetic purifications shown as decreasing free mAb concentration in the cell suspension over time. (B, D, F, right panels) represent the total binding after 2 h of incubation and the total elution yield. (A, B) Magnetic purification run_01 performed with cult_01 with a mAb concentration of 0.8 g/L and a total cell density at harvest of 17.5 x 10^6 cells/mL. (C, D) Magnetic purification run_02 performed with cult_02 with a mAb concentration of 6.6 g/L and a total cell density at harvest of 56.8 x 10^6 cells/mL. (E, F) Magnetic purification run_03 performed with cult_03 with a mAb concentration of 10.8 g/L and a total cell density at harvest of 103.9 x 10^6 cells/mL.

The remaining HCP levels and HCP reduction are for the assessment of a mAb purification method probably the most important attributes. The HCP level was measured for all three magnetic separations by an ELISA assay. The obtained logarithmic reduction was similar for all the runs with 3.0 and 2.3 (Table 6.2), which was in agreement with the reduction in traditional Protein A chromatography. However, the total HCP level in the purified material was exceptionally low with concentrations of ≈ 0.6 ppm for run_01, ≈ 3.5 ppm for run_02 and ≈ 5 ppm for run_03 (Table 6.2). The low HCP levels indicates that the magnetic separation is a gentle process preserving the integrity of the cells and minimizing the release of HCPs. The following analysis of other quality attributes showed a high monomer content of the purified mAb, charge variant distribution with a main peak of ≈ 69 % and a similar N-Glycosylation pattern for all three runs. The size distribution revealed that the high molecular weight (HMW) and low molecular weight (LMW) species were below 4 %, showing very low protein aggregation. As protein aggregation is likely impairing the efficacy of the drug and can potentially provoke immune response [229], it is essential to avoid its formation or to remove it prior to administration. The monomer content did not differ greatly among the runs, which advocates that the cell density had no impact on the aggregate formation. Additionally, the mild conditions provided by the magnetic separation, seen in the low HCP levels, were also favourable to minimize the formation of new aggregates. The charge variants showed a slight tendency for lower main peak levels and higher acidic variants from the culture with low cell density to high cell density, but with acidic levels still lower compared to the originator drug. The acidic variants for Trastuzumab are considered to be the main reason for the loss of potency of the binding towards the HER2 receptor [229]. Therefore, low levels are desirable to maintain a high potency of the mAb. The variation of charge variants can be introduced during the cell culture as well as during the purification through temperature and pH. The last quality attribute, the N-Glycosylation, revealed similar glycosylation distribution throughout all the runs with a tendency for lower maturation for the high cell density culture compared to the low cell density culture. Most likely the culture condition influenced this slight difference in the maturation [230]. Overall, the N-Glycan

distribution was in comparable range with the originator and a biosimilar of trastuzumab [229, 231].

The application of the magnetic separation technology downwards of the intensified fed-batch process based on UF-HF cell retention showed excellent performance with mild mechanical conditions. Especially, the very low HCP levels in the eluates coincided with the range given by health authorities [46]. This provides a substantial advantage over the traditional cell clarification step which is known to cause an increase in HCP level due to mechanical stress [71]. In conclusion, the different parameters for all three runs were highly comparable, indicating that the magnetic separation is not influenced by the cell density in the range between ≈ 20 – 120 x 10^6 cells/mL. In view of the present study in combination with the pilot-scale separation in paper I, it can be expected that the cell density has no negative influence in the overall operation time.

	Run_01	Run_02	Run_03
Total cell density at culture harvest [total cells/mL]	17.5× 10^6	56.8× 10^6	103.9× 10^6
Cell viability at harvest (%)	84	86	96
mAb titer at harvest [mg/mL]	0.8	6.6	10.8
HCP concentration in the harvest [ppm]	507	630	1020
Feed volume of the purification process [mL]	300	200	200
Volume of settled magnetic beads [mL]	17.5	35	55.5
Total elution yield (%)	80	90	94
Concentration factor (v/v)	3.9	3.3	1.8
HCP concentration in the eluate [ppm]	0.6	3.5	5
HCP reduction	3.0	2.3	2.3

Table 6.2: Process parameters

mAb capture process performed on the harvest from cult_01, cult_02 and cult_03 using magnetic separation.

6.2 Large scale tool for cell isolation in allogenic therapy approaches

Besides the capture and purification of monoclonal antibodies, magnetic separation can be utilized for many other capture applications. The isolation of cell populations has been used already since the end of the 80's. Selection and isolation of a cell subpopulation play a key role in research and manufacturing. In research it is common to select rare cells to identify diseases or phenotypes in a patient. On the other hand, in manufacturing of cell therapy applications such as autologous or allogenic approaches, cell separation needs to be performed to isolate the right cell type from the cell collection and/or purify the processed cells prior to administration. Although, cell therapy treatments have increased in the recent years and more and more treatments have been approved by the authorities, large-scale manufacturing is still a bottleneck, especially for allogenic cell therapies. In papers III and IV, the magnetic bead-based separation technology has been developed for isolation of cell subpopulations obtained from production-like cell cultures.

Paper III - Proof-of-Concept of a Novel Cell Separation Technology Using Magnetic Agarose-Based Beads

In this paper the magnetic separation based on agarose magnetic beads conjugated with a linker Protein A was used to develop a new technique for the isolation of cells after cultivation. In the recent years Advanced Therapy Medicinal Products (ATMP), for example treatments that are derived from human cells, have gained significant interest as a new approach for fighting diseases often assumed as incurable. The isolation of cells for the envisioned large-scale production needed for commercialization should be robust, efficient, operationally simple and generic while maintaining the cell's biological functionality and ensuring the safety of the product. Particularly, for therapies derived from pluripotent sources the treatment's safety is crucial, where undifferentiated cells present a major concern. The isolation of cell subpopulation is commonly accomplished by two different methods: immunomagnetic

separation (IMS) or immunofluorescence-based isolation, normally referred to as FACS, as described in Chapter 1.3. Both methods are currently not suitable for processing large quantities of cells nor liquid. In the presented work large magnetic beads (≈ 100 μm) providing a higher magnetization have been used, compared to commercial IMS beads in the nm range that provide a much lower magnetization. The large magnetic beads have in essence a different mechanism of action compared to the small IMS beads. The large beads here are covered by several cells, while for the other small IMS beads the mechanism is the opposite with many beads covering one cell. Additionally, the beads used here are conjugated with a Protein A ligand, providing the possibility to freely change the surface receptor recognizing antibody leading to a high flexibility usually only obtained in FACS. The Protein A beads have been tested on a model system that included human breast cancer cells (SKBR3) and human mesenchymal stem cell (hMSC). The SKBR3 cells used here are expressing the human epidermal growth receptor 2 (HER2) on their surface, commonly found in different types of cancers. In the study this surface receptor was targeted by the anti-HER2 mAb know as Trastuzumab, that was conjugated to the Protein A magnetic beads. A schematic representation of the process is shown in Figure 6.7.

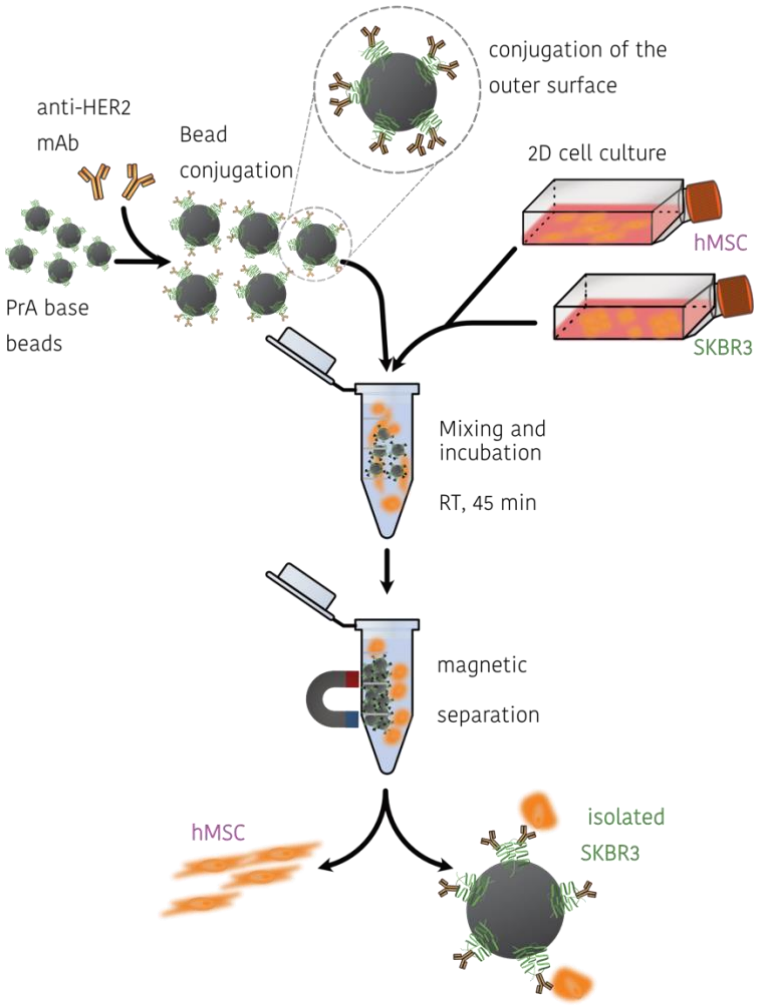

Figure 6.7: Protein A magnetic bead-based negative selection of hMSCs from heterogenous populations

Representation of the Protein A magnetic bead-based negative isolation of hMSC subpopulations from heterogenous populations of HER2+ SKBR3 cells and hMSCs. The process is divided into several steps with (I) bead conjugation where the anti-HER2 mAb recognizing the HER2+ SKBR3 are conjugated to the PrA beads. (II) Magnetic labelling facilitated by incubation and mixing of the bead cell suspension to collect receptor positive SKBR3 cells while hMSCs remain untouched. (III) magnetic separation and isolation of the different cell subpopulations.

The paper addresses firstly the biocompatibility of the commercial magnetic Protein A beads prior to cell isolation, as this is important for cell sorting. Any kind of toxicity provided by the sorting system might not only compromise the sorting but also the cell quality and therapeutic efficacy. In order to investigate the toxicity, the PrA magnetic beads were prepared in two conditions: either conjugated via Protein A with an anti-HER2 mAb or unconjugated. This was tested on the more sensitive cell type, which in this study were the hMSCs. Both conditions were soaked in cell culture medium for 24h in comparison to untreated medium used as positive control and a negative control that did not include cells. 24 h after seeding the medium was exchanged for the assay conditions until 72 h (Figure 6.8). After the termination of the biocompatibility assay the cell morphology and metabolic activity of the different cultures were compared. Condition one and two showed high similarity to the positive control with regards to cell growth and morphology as well as metabolic activity. No signs of the release of cytotoxic compounds from the beads were detected proving the non-toxic behaviour of the sorting system towards hMSCs.

A cell isolation system in which the magnetic beads are larger than the cells, leads to a single bead that is covered in a large number of cells, the outer beads surface is particularly important. The cell attachment with high probability will only occur on the outer surface. Therefore, the coupling of the mAb to the Protein A is different compared to a mAb capture processes, in which the mAb is the product of interest. A high occupation of all Protein A ligands is not desirable, since not all Protein A ligands are found on the outer surface. A large proportion of the Protein A ligands are within the cavities of the beads. Hence, these Protein A ligands will not be accessible to the cells, as they cannot penetrate the bead's cavities due to their size. Therefore, full conjugation with anti-HER2 mAb of all Protein A ligands on the bead will not increase the sorting efficiency and preferably only the Protein A ligands on the outer surface should be conjugated. However, the mAb capture onto the Protein A cannot be directed to only bind to the Protein A on the outer surface. But with the mAb transport into the cavities being mainly depended on diffusion the occupation of Protein A ligands in the cavities will take longer time compared to the occupation of Protein A ligands on the outer surface, as the uptake here is mainly

related to the mixing and therefore faster. This leaves a smaller amount of mAbs to travel inside the cavities and bind to the Protein A there. Additionally, in case of the high and multivalent expression of the HER2 receptor and large size of the cells, a lower occupancy is even more feasible, leading to a decrease of mAbs needed for the sorting lowering the economic pressure of the expensive commercial sorting antibodies. For the tested proof-of-concept of cell sorting, the influence of the mAb coverage on the cell adhesion was evaluated with 100 %, 50 % and 15 % occupation. The low occupation of 50 % and 15 % provided identical excellent cell isolation compared to the full occupation. This indicated that efficient cell attachment occurred on the outer surface of the PrA magnetic beads and fully mAb occupation was not needed for efficient cell isolation.

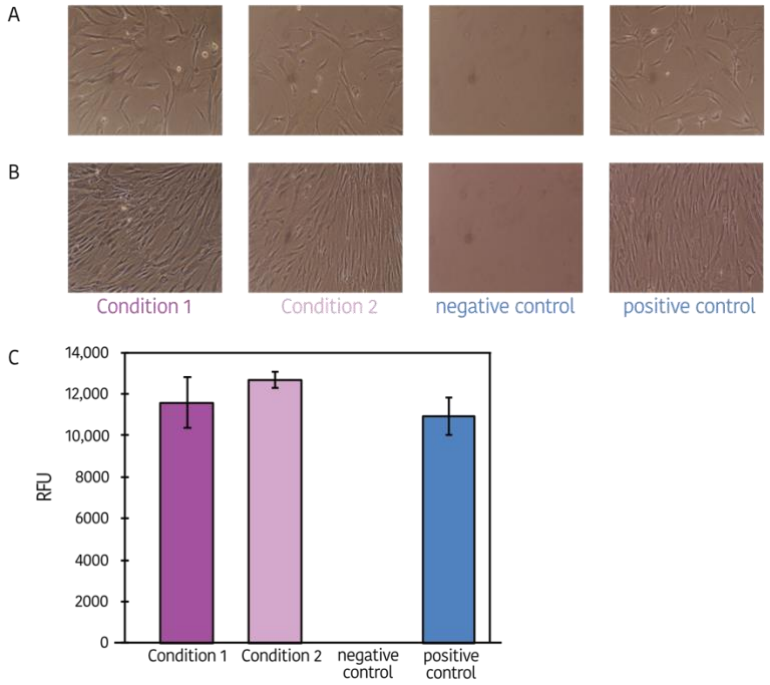

Figure 6.8: Biocompatibility assay

Biocompatibility assay of the magnetic particles MAGicBeads for human mesenchymal stem cell. hMSCs were cultured in medium where MAGicBeads bound to anti-HER2 mAb's had been soaked for 24 hours for condition 1, or in medium where MAGicBeads alone had been soaked for 24 hours for condition 2, in comparison with positive control using untreated medium - negative control did not include cells. (A) Cells cultured in untreated medium for 24 hours, accounted as start of the biocompatibility assay, after which the media were exchanged for conditions 1, 2 or positive/negative controls. (B) Cell cultures after 72 hours. (C) Metabolic activity shown in relative fluorescence units (RFU) measured with alamarBlue® stain.

For further assessment, the effect of mechanical stress and influence of EDTA were evaluated on the PrA magnetic system in comparison with beads with different conjugations. As a matter of fact, until now in this thesis, the used magnetic beads were based on Protein A interaction. However, in the new frame of cell separation, it could have been so that another binding would be more advantageous. The study addresses this question. For this assessment two types of beads were used, the known PrA beads, using PrA as an intermediate linker for the anti-HER2 mAb, and

magnetic beads with anti-HER2 mAb covalently coupled to the surface. The covalently coupled beads were prepared in two different mAb occupations named cc12 and cc1.2. Whereby cc12 had an occupation of 40 % which results in 12 mg mAb/mL beads, while cc1.2 beads provided a coverage of 1.2 mg mAb/mL beads which is an occupancy of 4 %. The mechanical stress was evaluated with three different setting: low shaking (7 rpm), high shaking (50 rpm) and pipette mixing (Figure 6.9). All settings were applied during incubation and wash steps with pipette mixing to be considered the harshest condition. Furthermore, the influence of EDTA at high (7 mM) and low (2 mM) concentration on all bead configurations was also evaluated, as it is a common buffer additive to prevent cell adhesion. In order to support a large-scale manufacturing a robust method is necessary. In preparation for this, the study focused on the impact of mechanical stress during the homogenization, which is highly important for larger operation as pumping and homogenization can have a large influence on the success of the cell isolation. Furthermore, EDTA addition was evaluated as it is a common buffer additive to prevent cell adhesion. Overall, the PrA system showed superior performance compared to the covalently coupled systems (Figure 6.9). The different mechanical stress scenarios provided a similar cell isolation for the PrA system of 96 – 97 %, while both covalently coupled system were lagging behind with isolation efficiencies of 64 – 73 % and 57 – 77 %, respectively. It is essential to understand the mechanical stress on the cell-bead construct in order to develop a process for large-scale. Interestingly the PrA construct provided higher resistance against mechanical stress compared to the covalently coupled beads. A reason could be that covering the conjugation of the PrA beads with the mAb, the binding occurred mainly through the Fc region and therefore, the recognition site of the mAb points outwards ensuring optimal display for the interaction of the antibody with a cell receptor. On the contrary, the mAb covalently coupled is randomly oriented on the bead, thus restricting the accessibility to the binding sites. Additionally, covalent immobilization may disrupt the function of the antibody, as well as the linker, the distance between the bead and mAb, can have an important effect on the sorting efficiency. Compared to the PrA linker which will always display a functional antibody.

The performance of PrA beads under the influence of EDTA was similar for the 7 mM and 2 mM concentration. The PrA system provided higher cell isolation compared to the covalently coupled systems. But in comparison to conditions without EDTA the cell isolation was lower (Figure 6.9). In all three systems the formation of cell aggregates was observed, but to a lesser extend for PrA beads.

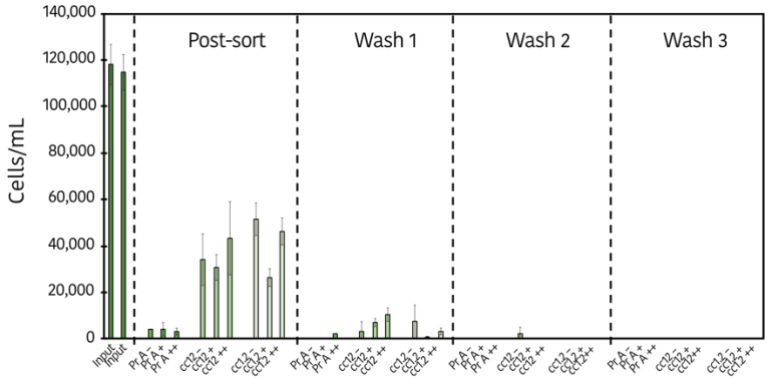

Figure 6.9: Mechanical stress conditions on the cell isolation efficiency for three bead conjugations, PrA, cc12 and cc1.2

Cell isolation efficiency for 3 bead conjugations, Pr A, cc12 and cc1.2, in presence of different mechanical stress conditions during incubation and wash steps in relation to the HER2$^+$ SKBR3 cell concentration prior cell sorting (input); (-) low shaking at 7 rpm, (+) high shaking at 50 rpm, (++) pipette mixing. The first input cell density referrers to the pre-sort population for the PrA and cc12 conjugations, and the second input for cc1.2 conjugation. The cells before isolation (input) are referred to as "pre-sort", while "post-sort" refers to the cells after isolation.

Lastly, sorting of heterogenous cell mixtures of hMSCs and HER2$^+$ SKBR3 cells was studied. The evaluation of HER2$^-$ cells showed negligible unspecific binding for all bead types. This confirmed that the cells are bound via the antibody and not the beads. The model system of hMSCs and HER2$^+$ SKBR3 cells was negatively sorted with the elimination of HER2 expressing SKBR3 cells, leaving the desired population of hMSCs untouched. Several population ratios of 80:20, 70:30 and 30:70 hMSC:SKBR3 were sorted with the PrA system (Figure 6.10). The system provided an excellent removal of HER2$^+$ SKBR3 cells on average of 86 % and a hMSC enrichment of ≈ 90% confirming that the PrA system was excellent for cell separation in the proof-of-concept study.

Overall, the PrA system proved to be advantageous for the isolation of cells. It offers a high flexibility for the antibody used for the cell sorting thanks to the PrA binding. The PrA beads can be produced in large amounts lowering manufacturing costs and are potentially available from various sources reducing monopoly situations, while presenting economical and regulatory advantages.

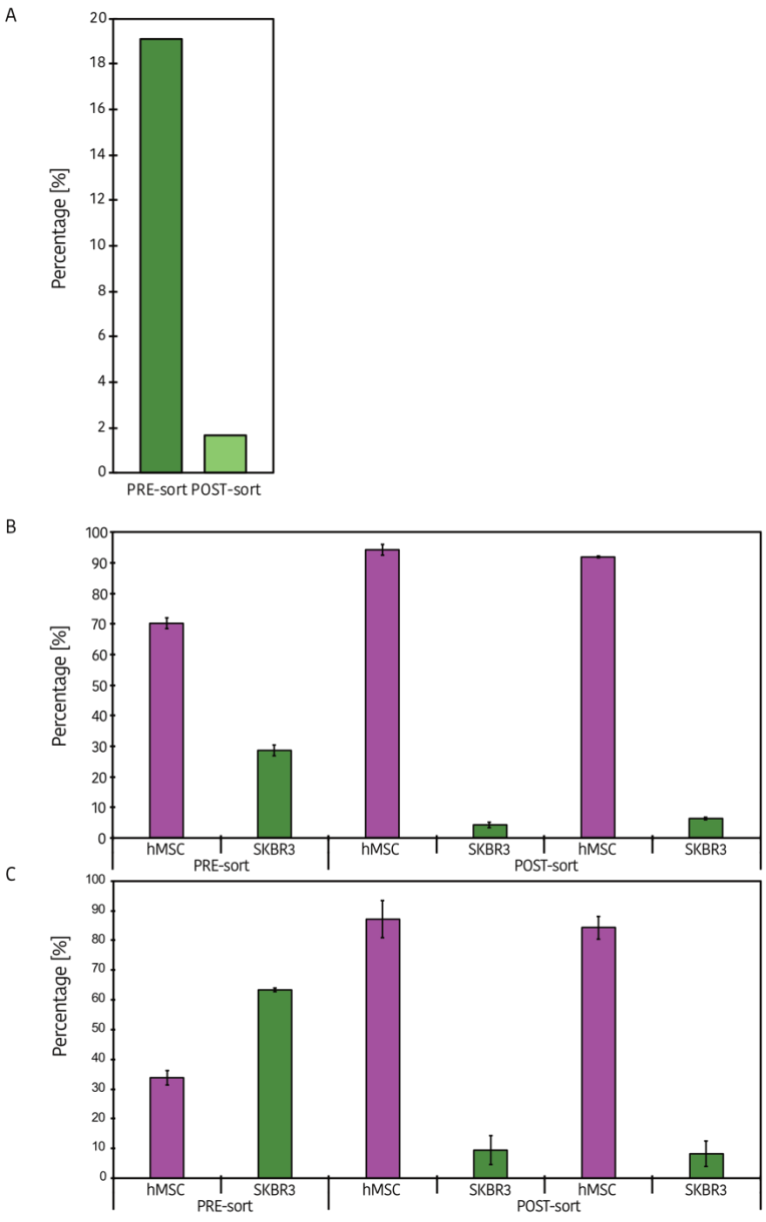

Figure 6.10: Negative selection of HER2⁺ SKBR3 cells from heterogenous cell populations

Negative selection of HER2⁺ SKBR3 cells from heterogenous populations of hMSCs and SKBR3 cells (hMSCs:SKBR3) measured by FC showing the PRE- and POST-sort population percentage; (**A**) 80:20; (**B**) 70:30; (**C**) 30:70

Paper IV – Negative selection of human induced pluripotent stem cells (hiPSC)

In Paper IV, the highly magnetized magnetic beads introduced in paper III were used for the negative selection for human pluripotent stem cells (hiPSCs). Herein, the magnetic beads were also covered with a new protein linker, Protein G. Together with the previous configuration of PrA, the magnetic beads can cover a large range of antibodies against receptors, among others for pluripotent surface receptors. The advances of off-the-shelf cell therapies, commonly in form of allogenic approaches, have put greater interest in pluripotent cells as they can serve as a uniform cell line. hiPSCs are preferred for this task, as they provide fewer ethical concerns compared to embryonic stem cells and are generally easy to generate from somatic tissue. These hiPSCs build a basis in the form of a pluripotent cell line that can be differentiated in any cell, except reproductive cells. This off-the-shelf production brings many advantages compared to individualized cell therapies such as lower cost of the therapy, highly controlled and uniform starting material, and easy accessibility of the starting material. However, hiPSC based allogenic therapy does not only provide advantages. Beside the possibility that hiPSCs can provoke an immune response, undifferentiated cells provide an even higher safety risk. Remaining undifferentiated cell can lead to the risk of teratoma formation [96-101]. Therefore, prior to transplantation undifferentiated cell need to be eliminated.

The targeted isolation of unwanted cell is therefore crucial to ensure the safety of the graft. This type of isolation is commonly referred to as negative selection, because the desired cell type remains untouched and the system selects for the cell type that should be removed. Beside the fact that the desired cell remains untouched and not influenced by the sorting method, the negative selection also provides the advantage that the captured cells do not need to be released from the magnetic beads. Negative selection should therefore be the method of choice for these kinds of applications. Although, it is missing the selectivity of positive selection, the risk of bead transfer into the graft is non-existing.

In a test similar to the one performed with hMSCs, the biocompatibility towards the highly delicate hiPSCs was firstly evaluated, as it is pivotal for the system to be non-toxic towards the

cells to preserve their integrity and ensure the effectiveness of the therapy. It was shown that the magnetic beads did not show signs of cytotoxic release for the hiPSCs.

Following the biocompatibility assay, different selective antibodies were evaluated for their sorting ability. Besides PrA linker, Protein G, PrG, is interacting to enlarge the classes of antibodies to be used during the cell separation. The main constraint for the selective antibodies to be used in this setup, beside their target selectivity, is the free available Fc region to bind to the PrA/PrG linker. This for example excludes antibody classes like IgM. Herein, two suitable antibodies were identified recognizing either the stage-specific embryonic antigen-4 (SSEA-4) or the tumour related antigen2-49 (TRA2-49). Both antigens are commonly expressed in pluripotent stem cells, making them excellent targets.

The magnetic beads were conjugated either with anti-SSEA-4 or anti-TRA2-49 mAbs and used with a varied ratio of cells to beads ranging from $5 \times 10^4 - 5 \times 10^5$ cells/µL beads. Figure 6.11 A showed equally high reduction for the different cell to bead ratios using the SSEA-4 system. Similar behaviour was observed for the TRA2-49 system (Figure 6.11 B). It proved that both systems could reduce pluripotent cells on the basis of different surface makers. Beside the high cell removal both systems also provided great performance with regards to the different cell to bead ratios, indicating that higher cell to bead ratios were feasible for this system. Using a higher ratio can reduce the cost for scale-up operations as the amount of beads and antibodies is lowered. Especially, the commercial antibodies can generate significant cost for the scaling of the system.

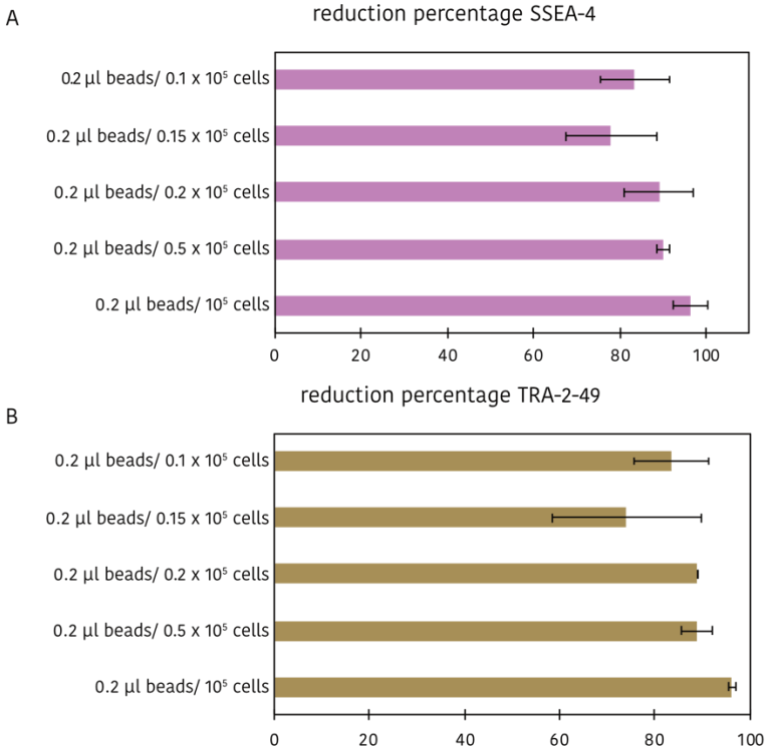

Figure 6.11: Antibody evaluation of selective pluripotent monoclonal antibodies

Removal efficiencies using beads conjugated with anti-stage-specific embryonic antigen-4 (SSEA-4) mAb or with anti-tumour related antigen2-49 (TRA2-49) mAb with cell to bead ratios from 5 x 10^5 cells/ µL beads to 0.5 x 10^5 cells/ µL beads. (A) removal percentage anti-SSEA-4 magnetic beads. (B) removal percentage anti-TRA2-49 magnetic beads.

Furthermore, an unspecific binding assay was conducted, revealing insights if the cells interacted via the selective antibody or randomly with the bead. The latter case would result in non-successful isolation as potentially all cell could interact with the magnetic beads. Initially, no unspecific binding could be observed for hiPSCs towards the PrG beads. Moreover, a screening of different cell types (hiPSC, HUVEC, SKBR3), two bead types PrA and PrG and four buffer combination including an inhibitor towards the Rho-associated kinase (ROCKi) and fetal bovine serum (FBS).

Interestingly, unspecific binding occurred for almost all conditions containing ROCKi independently from the cell and bead type. This showed that the addition of ROCKi in the sorting buffer clearly had negative effects on the sorting which might originate from the complex Rho pathway. Although, ROCK1 and ROCK2 are inhibited by the ROCKi, Rho proteins have another effector in mDia (mammalian homolog of diaphanous) that also promotes stress fiber formation resulting in adhesion [232]. With the addition of ROCKi the survival of single cells is enhanced but on the other hand the adhesion might also be promoted via the mDia pathway. Additionally, ROCKs mediate the disassembly of adherens junctions [233, 234] and with their inhibition the mediation might be lowered leading to more adherens junctions. This might lead to the observed unspecific binding. For the HUVECs a high trend of unspecific binding was seen. Especially, large aggregation formation was observed. The underlying effect cannot be fully explained but potentially the ROCKi mediated effects other than adhesion and fiber formation. A potential down regulated KDM2B enzyme in endothelial cells can increase the tube formation within the cell and lead to adhesion.

However, the process involved in the formation of adhesion and cell aggregation onto the magnetic beads is highly complex and its origin cannot be fully comprehended.

Following, a 100 %. iPCS sorting was conducted (Figure 6.12) on PrA-anti-SSEA-4 and PrG-anti-SSEA-4 magnetic beads. Biological duplicates of both systems provided significant cell removal showing selective isolation via the mAb.

The magnetic microbead system based on different linker proteins showed excellent removal of hiPSCs based on SSEA-4 and TRA2-49 pluripotent receptors. Beside the removal, the system proved to be gentle enough to allow for re-seeding of the cells. The larger magnetic beads, compared to their smaller counter parts, provide the possibility of large-scale application for allogenic manufacturing. In combination with the high flexibility of the different linker proteins the system provides interesting possibilities to individualize the manufacturing process.

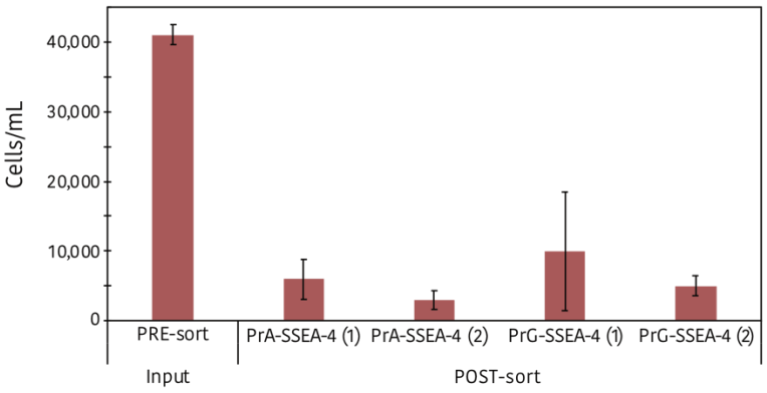

Figure 6.12: hiPSC sorting with PrA/G-anti-SSEA-4 mAb

Removal of SSEA-4+ hiPSCs shown in percentage compared to the PRE-sort population of 100% hiPSC, using beads conjugated with anti-SSEA-4 mAb via PrA linker, PrA-SSEA-4, or PrG linker, PrG-SSEA-4, measured as biological duplicates.

6.3 Concluding remarks and future outlook

Purification represents an essential part in all pharmaceutical manufacturing, reducing impurities and ensuring the therapeutic's safety. With higher quantities needed for existing modalities and new arising ones, purification methods need to adapt rapidly to match the innovation and intensification in the field. Currently, for biological therapeutic modalities, a large amount of intensification is directed towards the cell culture, most prominently monoclonal antibody manufacturing but also cell therapy manufacturing. This however requires innovation and development of the following downstream steps in order to match with the productivity accomplished on the upstream side. Otherwise, the gained productivity and efficiency will be lost in the later part of the process. Utilizing already existing methods and knowledge in a different context is an efficient way to improve processes. As highlighted in this thesis improvement of the DSP becomes more and more a topic of research in both academia and industry with, for example, the implementation of PCC capture steps. Besides the implementation of continuous processes, a reduction in operational steps is another avenue to improve the DSP in order to improve the manufacturing and match the demand.

This thesis aims to contribute to the field of downstream development by designing a strategy to reduce operational steps for the capture of high value modalities such as monoclonal antibodies and induced pluripotent stem cells.

Protein A based antibody capture has been known for decades as an excellent tool for the capture of mAbs from complex liquids (cell culture supernatant). However, its use has been almost entirely restricted to column-based chromatography making it highly susceptible to clogging. Therefore, intensive clarification steps are commonly required, including centrifugation for the removal of larger solids and filtration for the removal of smaller particles. This presents a bottleneck of prolonged process time that, depending on the batch size, can stretch from a few hours to days. Another bottleneck identified here are the multiple process steps, which can lead to a drastic reduction in product yield. Although a single operational step provides superb yield two steps would lead to a loss of already 10 %. Therefore, in Papers I and II magnetic separation, a technique also known for decades, has been designed as integrated method for the purification of monoclonal antibodies. In combination with magnetic beads conjugated with a Protein A ligand, a capture process has been developed and verified. The process was developed to cope with solid containing liquids by integration of cell clarification and capture step into a single step. Reducing the operational steps and reducing product loss. It is worth to note that this system can be applied on very high cell density cell broth (> 1 x 10^8 cells/mL). Currently, very high cell density is challenging for the centrifugation resulting in insufficient removal of solids or cell disruption through harsh conditions. Cell disruption can also have a negative effect on the clarification efficiency and can cause product degradation. The feed-stream is also challenging for the following filtration steps, commonly depth filtration, resulting in filter clogging or poor utilization of the filter membrane. Attempts have tried to improve these clarification steps with the implementation of feed pre-treatments. For instance, the addition of flocculation agents or low pH to increase the clarification, but on the downside new components or unsuitable low pH for the following capture steps are added. Furthermore, intense clarification steps can lead to a higher release of HCP due to the mechanical stress. This eventually leads to even more DSP steps in order to comply with the regulations for biopharmaceuticals.

In manufacturing sites where process intensification is performed in form of an intensified fed-batch generating high cell density, magnetic capture of monoclonal antibody can play a crucial role to fight these bottlenecks. Magnetic separation based on magnetic beads conjugated with Protein A has shown excellent performance in the capture of monoclonal antibodies for cell broth with different cell densities (> 10^8 cells/mL) and culture volumes (up to 16 L). This can improve process designs based on intensified fed-batch cultures in which the single harvest is challenging. Secondly, the magnet separation, performed at different scales, has proven to be gentle towards the cells almost eliminating the release of new HCPs. This leads to final HCP levels below 10 ppm, after a single step, which is in compliance with regulations for the whole DSP. Magnetic separation not only facilitates the reduction of process steps but also provides highly purified material, which is in some key indicators (HCP level) already in compliance with the regulation set by authorities for the removal achieved in the whole DSP.

Besides the implemented magnetic capture of mAbs downstream of intensified fed-batch cultures another application is of interest. Currently, continuous processes based on perfusion cultures are maintained at a certain target density, commonly associated with a steady-state. This is done to provide predictive conditions for the following operations in order to time the different operational cycles. Otherwise, large fluctuations in the culture could lead to potential interference in the continuous set up. In order to maintain the cell density at a certain level, a fraction of the cell mass is commonly pumped out of the reactor, known as cell bleed. Leading to a volume and product loss of 10 - 30% daily, as the cell bleed is normally not purified [235, 236]. For large manufacturing cultures this could result in significant loss of product over the duration of the culture. Magnetic separation can be an excellent tool to purify the non-clarified cell bleed, providing an additional tool to lower process related product loss and increase productivity and throughput.

Future work can be directed towards a higher degree of automation for intermediate and large-scale magnetic separators. But to understand the full potential of the magnetic capture from very high cell density culture and its feasibility towards

implementation into manufacturing scale, studies matching this scale should be conducted in the future.

Magnetic separation, beside the capture of monoclonal antibodies, have another interesting application with the isolation of cells. Cell sorting based on magnetic separation has been known since the 80's and is used in research and industrial applications. The magnetic beads are conjugated with antibodies that are specific towards certain surface receptors expressed by the desired cells. Therefore, this method is also referred to as immunomagnetic separation (IMS). Commonly, immunomagnetic beads are much smaller than the cells resulting in a single cell labelled by multiple magnetic beads. Small immunomagnetic beads have been the preferred size for the sorting of cells. This works very efficiently in small scale applications for research or production of single autologous batches. However, it has its limitation with regards to larger commercial manufacturing. Small beads provide two potential problems, associated with their size. Firstly, it has been shown that small magnetic beads can be internalized by the cells and are found in the intra-cellular spaces and on the cell's surface with retained magnetic properties of up to two weeks after the sorting [237]. Secondly, and even more important for commercial manufacturing the small magnetic particles provide low magnetization, which prevent the processing of larger quantities of liquid. The low magnetization would require a high local magnetic field gradient to facilitate the processing of larger liquids.

The large magnetic beads studied in this work on the contrary are several times larger (≈ 100 μm) compared to a cell, reversing the mechanism to one bead labelling several cells. Therefore, internalization of beads can be prevented. In combination with the preferred negative selection, the risk of retaining magnetic properties on the cell was eliminated. Aside from the safety perspectives above, the larger magnetic beads provide other properties for cell sorting that can be of interest for the industry. Especially, the high magnetization the beads show is crucial for further scale up. Currently, cell therapy manufacturing, mainly allogenic approaches, are on the verge to become off-the-shelf products. However, to realise commercial manufacturing, large quantities of liquid and cells need to be processed via a cell sorting method. The large magnetic beads presented, proved to be gentle towards cells and showed excellent performance to process

large volumes of cell suspensions. Furthermore, the proof-of-concept cell separation showed that the isolation of cells is feasible with larger magnetic beads from various cell types including pluripotent cells. The magnetic sorting system can be implemented in large scale manufacturing providing a more specific purification of therapeutic cells compared to centrifugation and washing steps currently used in larger manufacturing. Additionally, a highly selective step removing pluripotent cells from allogenic cells originating from hiPSCs also ensures extra safety.

Future work should be dedicated to further characterise the systems in large-scale setups. Especially, the sorting efficiency needed to prove similarity in large-scale compared to small-scale. Furthermore, the development of a release system should be implemented to enable positive selection providing a higher selectivity and the implementation of a two-step sorting approach to further improve the sorting efficiency. Additionally, benchmarking the system towards a commercial IMS system would help to understand its full potential.

In conclusion, the alternative approach based on well-known methodologies described in this thesis contributes to the field of downstream processing for different modalities. The alternative capture can not only contribute as a solution to some of the current production bottlenecks in mAb manufacturing but also to the future cell therapy manufacturing by providing a flexible solution for commercial manufacturing.

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