Purification of Monoclonal Antibodies by Aqueous Two-Phase Systems

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„Nichts auf dieser Welt, das sich zu haben lohnt, fällt einem in den Schoß!“

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“Nothing in this world that's worth having comes easy.“

Bob Kelso (Scrubs; Season 4, Episode 20)

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Zusammenfassung

Monoklonale Antikörper (engl. monoclonal antibodies, mAbs) gewinnen als biopharmazeutisches Produkt immer mehr an Bedeutung. Die hohe spezifische Affinität der mAbs wird zur Therapie zahlreicher Krankheiten, wie etwa Autoimmunerkrankungen, Infektionen und vieler Arten von Krebs erfolgreich eingesetzt. Um als Medikament genutzt zu werden, müssen mAbs nach erfolgter biotechnologischer Produktion durch Zellkultivierung, aufgearbeitet werden. Dabei gelten zum Wohle der Patientensicherheit hohe Qualitäts- und Reinheitskriterien, welche es am Ende des Herstellungsprozesses einzuhalten gilt. Durch die in den letzten Jahrzenten stetig steigende Nachfrage in Kombination mit Verbesserungen innerhalb der Zellkultivierung, gelangen bestehende Anlagen und konventionelle Aufarbeitungsmethoden an ihre Kapazitätsgrenzen. Um diesen Herausforderungen zu begegnen rücken neue, alternative Methoden, wie die wässrige Zweiphasen Extraktion (engl. aqueous two-phase extraction, ATPE) verstärkt in den Fokus der Forschung.

Im ersten Teil dieser Doktorarbeit wurde mit Hilfe einer statistischen Versuchsplanung (engl. Design of Experiments, DoE) die Zusammensetzung des wässrigen Zweiphasensystems (engl. aqueous two-phase system, ATPS) bezüglich Ausbeute und Reinheit des verwendeten mAb optimiert. Im Anschluss wurde eine neue Methode entwickelt die mAb-haltige Produktphase des ATPS membranbasiert von Verunreinigungen abzutrennen. Dadurch konnte die Zellabtrennung mit der Isolierung und ersten Aufarbeitung des mAb erfolgreich in eine Grundoperation integriert werden.

Im zweiten Teil der Arbeit wurde die Integration der ATPE in den nachfolgenden Aufarbeitungsprozess untersucht. Nach erfolgter Optimierung der einzelnen Grundoperationen konnte ein Herstellungsprozess des mAb, basierend auf ATPE, mit hoher Ausbeute, Reinheit und konstanter Produktqualität abgebildet werden.

Im dritten Teil der Doktorarbeit wurde die Anwendung von ATPE auf Hochzelldichte (engl. high cell density, HCD) Verfahren untersucht, welche eine Prozessintensivierung und produktivere mAb Herstellungsverfahren ermöglichen. Der Einfluss der Zelldichte wurde anhand verschiedener ATPS untersucht. Auch für HCD Kultivierungen konnten Zellabtrennung sowie mAb Isolation und Entfernung von Verunreinigungen erreicht werden. Damit tragen die Resultate dieser Arbeit dazu bei, bestehende Engpässe in der Herstellung von mAbs, als wichtige biopharmazeutische Produkte, zu überwinden.

Schlagwörter: Monoklonale Antikörper, wässrige Zweiphasen Extraktion, wässrige Zweiphasensysteme, Hochzelldichte Kultivierung

Abstract

Monoclonal antibodies (mAbs) are becoming increasingly important as biopharmaceutical product. The high specific affinity of mAbs is successfully used for the therapy of numerous severe diseases, such as autoimmune diseases, infections and many types of cancer. In order to be used as a drug, mAbs must be processed after biotechnological production by animal cell cultivation. For the benefit of patient safety, high quality and purity criteria apply, which must be met at the end of the manufacturing process. Due to the steadily increasing demand, in combination with improvements in the cell cultivation over the last decades, existing facilities and conventional purification methods are reaching their capacity limits. To meet these challenges, new, alternative methods such as aqueous two-phase extraction (ATPE) move into the focus of research.

In the first part of this PhD thesis, the composition of the aqueous two-phase system (ATPS) was optimized with respect to yield and purity of the used mAb by means of a Design of Experiments (DoE) approach. Subsequently, a new methodology was developed to separate the mAb-containing product phase of the ATPS from the impurity-containing phase using a membrane-based approach. Thus, the clarification with the capture and first purification of the mAb was successfully integrated in one unit operation.

In the second part of the thesis the integration of the ATPE into the subsequent downstream process was investigated. After optimization of the individual unit operations, the entire mAb production process based on ATPE was successfully executed with high yield, purity and constant product quality of the mAb.

In the third part of the PhD thesis, the proof of concept for the application of ATPE to high cell density (HCD) processes was investigated, which allow process intensification and increased productivity of the mAb manufacturing. The influence of cell density was investigated using different ATPS. Clarification as well as mAb capture and purification were obtained for HCD cultivation. Thus the results of this work contribute to overcome existing bottlenecks in the production of mAbs, as important biopharmaceutical products.

Key words: monoclonal antibodies, aqueous two-phase extraction, aqueous two-phase system, high cell density cultivation

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1 Introduction and goals

1.1 Introduction

The crucial role of monoclonal antibodies (mAbs) for the treatment of several severe diseases and high success rate resulted in a constantly increasing demand. Since the first approval of a mAb as pharmaceutical drug (Muromonab-CD3) by the FDA in 1986, several mAbs were approved for diagnostic and clinical use generating a multibillion-dollar market.

The principle of producing mAbs was first published 1975 by César Milstein, Georges Köhler and Niels Jerne, who were awarded the Nobel Prize for Medicine in 1984 for their work. In the beginning, there were some setbacks, due to the immunotoxicity of the initially murine mAbs. However, the development of chimeric and humanized mAbs solved this issue and their high specificity resulted in highly successful therapies with few side effects and numerous areas of application. Possible mechanisms of actions are the alteration of signaling pathways, inhibition of tumor growth/proliferation, activating the endogenous immune system or trigger cell death of certain cells.

For the industrial production mAbs are secreted into the medium throughout the upstream process (USP) by mammalian cell cultivation. Significant improvements in cell-line engineering, media and feeding strategies of the USP resulted in increased cell densities and accordingly increased mAb titers. These achievements shifted the bottleneck in mAb manufacturing from the USP to the subsequent downstream process (DSP). Existing technologies for the clarification of the cell broth as well as capture and purification of the mAb reaching their limits. In order to be able to meet the increasing demand for mAbs in the future, the industry is moving towards new alternative approaches. As an additional challenge, even with increased throughput and productivity, the high purity demands must comply with the authorities and accordingly, the benefit of patient safety.

A promising approach to meet these challenges is the application of aqueous two-phase extraction (ATPE), which has been demonstrated as suitable unit operation in the purification of mAbs. In addition to economic and ecological benefits, easy scalability and various possible applications were reported. However, despite several studies dealing with ATPE of mAbs in laboratory scale a transfer to a production process has not yet been obtained due to challenges to integrate the unit operation in the DSP.

1.2 Goals

The aim of this PhD thesis was to overcome the bottleneck of mAb production within the conventional DSP processes by an ATPS based approach. Furthermore, the numerous advantages of ATPE as unit operation offer great potential for process intensification.

As a first step, the ATPS composition needs to be optimized with regard to high yield of mAb and removal of process related impurities such as deoxyribonucleic acid (DNA) and host cell proteins (HCP), from a conventional fed-batch cultivation. For this optimization a design of experiments (DoE) approach is suitable, in order to save time, effort and resources. As a next step, a method is required for the subsequent phase separation. Membrane-based approaches offer the advantage to integrate clarification, including the sterile filtration, mAb capture and first purification in one unit operation for process intensification. For the subsequent purification, the incorporation of ATPE into a platform DSP is necessary with particular focus on product quality attributes like N-linked glycosylation patterns. In addition to sufficient integration, mAb yield as well as the removal of process related impurities needs to be considered throughout the entire DSP. For an even more intensified process, ATPE could be applied for clarification, mAb capture and purification of high cell density (HCD) cultivations.

Novel, alternative purification strategies for mAbs based on ATPE were investigated in this PhD thesis to meet several challenges of conventional platform DSP. Implementation and further optimization of the presented results offers great opportunities for the biopharmaceutical industry.

2. Theoretical background

2.1 Monoclonal antibodies

Antibodies, or immunoglobulins, are components of the adaptive immune system, binding pathogens or its products and recruiting other molecules or cells [1]. The fusion of murine B lymphocytes and human myeloma cells, so called hybridomas, allowed the continuous secretion of a specific antibody by an indefinite cell line [2]. These mAbs have a highly specific binding capability, which allows versatile application in diagnostic or as active pharmaceutical ingredient [3,4]. Thereby the mAb quality is essential for the therapeutic activity. N-linked glycosylation, as post-translational modification, is considered as a critical quality attribute (CQA) with many known physical functions regarding solubility and stability [5,6]. Even more important are the effects for biological activities such as complement-dependent cytotoxicity and antibody-dependent cellular cytotoxicity through their interaction with Fc-receptors [7,8]. Characterization and monitoring of glycosylation patterns of mAbs, used as therapeutic protein, are critical for product consistency [9].

Immunological disorders, inflammatory, cardiovascular as well as infectious diseases and certain types of cancer are addressed by mAb-based therapies [10–12]. Especially for the treatment of several malignant hematological and solid tumors, mAbs are considered as the most promising approach within the last two decades [13]. Thereby, the high specificity is used to intervene on altered key oncogenes or tumor suppressor genes involved in tumor promotion [14]. This targeted therapy was seen as a revolution in cancer therapy, because only altered cancer cells are affected with only minor side effects towards normal cells, compared to conventional chemotherapeutics [15].

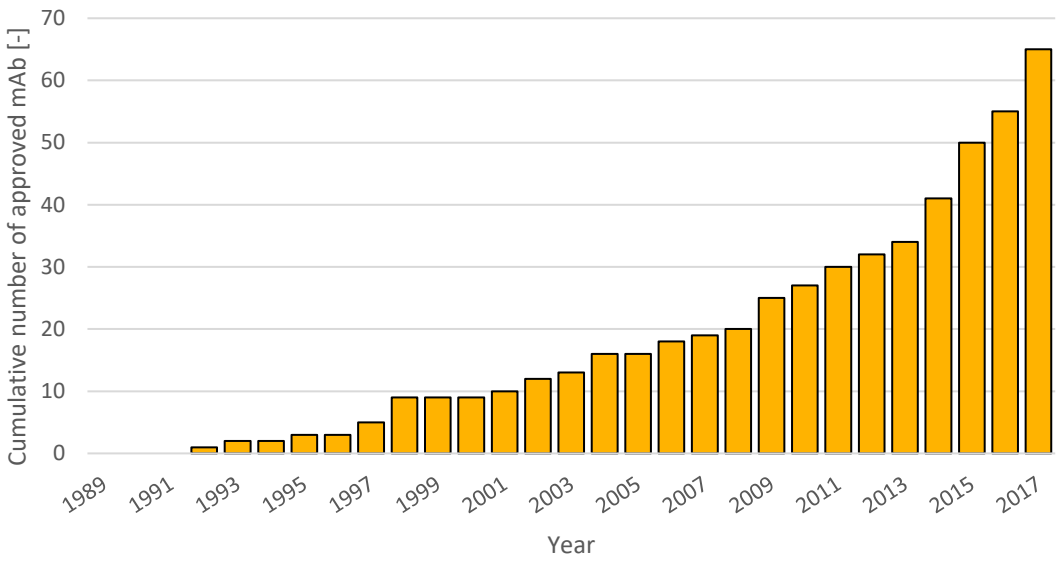

Figure 1. Chronological summary of cumulative EMA/FDA approved mAbs (adapted from [16]).

The European Medicines Agency (EMA) and the United States Food and Drug Agency (FDA) approved more than 65 mAbs for clinical use until 2017 with an increasing number of approvals from year to year (**Figure 1**) [14,16]. At the same time over 300 mAbs were in clinical development [17] resulting in an expected global market of 500 billion US$ by the end of this decade [18]. The growing market perspective and the development of mAb based therapies for additional indications, like Alzheimer diseases and diabetes, strengthen the interest for the production processes of these biopharmaceuticals [19,20].

2.2 Biopharmaceutical Manufacturing

2.2.1 Upstream process

Although various expression systems can be used for the production of mAbs, mammalian cell lines are most common and offer several advantages. They are able to produce complex molecules with post-translational modifications (e.g. glycosylation), which bear a high similarity to the equivalent protein in the human, resulting in a decreased risk regarding immunotoxicity [21]. In addition, the secretion of the protein of interest into the medium allows an easy processing and subsequent purification [22]. Chinese hamster ovary (CHO), baby hamster kidney (BHK21), human PER.C6® and murine myeloma cell lines (NS0 and Sp2/0) are the most common mammalian expression systems [23,24].

During the USP, the cells are cultivated by a controlled production system within a bioreactor. Most commonly stirred tank reactors, plug flow reactors and rocking motion systems are used [25]. After vial thaw, cell expansion is conducted through a series of inoculum steps in seed reactors and the cells are transferred to the production bioreactor [26]. Based on the process different cultivation modes can be

performed. During a batch process, all cell culture and medium components are present from the beginning, resulting in a constant bioreactor volume during the cultivation time. In a fed-batch process nutrients are added to the bioreactor with a specific feeding rate according to the need of the cells and the current growth rate. Thereby the bioreactor volume increases during the cultivation time and a higher cell growth is expected, for what reason this is the most common used process. In a perfusion process, the media is exchanged continuously, while the cells are hold back by a retention device. This offers the advantage of a continuous harvest, high cell growth and productivity [25].

Ongoing research and optimization in biopharmaceutical manufacturing, regarding bioreactor equipment, media as well as feeding strategies and intensified operation modes, like perfusion processes, resulted in significant improvements of the upstream process. Focus was placed on highly productive cell lines, with the right growth characteristics as well as a high specific growth rate, cell density and viability [27]. HCD cultivations with more than 100 million cells/mL [28,29] and mAb titers up to 25 g/L were reported [30–32].

2.2.2 Downstream process

The DSP is focused on separation and purification of the mAb from the cell culture media and can represent a large proportion of the manufacturing costs [33]. Important considerations during DSP development are the speed (pressure of time for a therapeutic candidate to enter clinical trials), process throughput, robustness, scalability, yield and, primary, the product purity [17]. Impurities, which should be removed, can be classified into two major groups. Process related impurities consist of HCP, DNA, endotoxins, leached protein A and some cell culture media additives. Product related impurities arises from the mAb itself and consist of dimers as well as high molecular weight aggregates (HMW), clipped or low molecular weight species (LMW) and undesired charge variants [17,34]. As these impurities pose risk for the patient due to immunotoxic effects, the mAb purity display a CQA for the pharmaceutical product and needs to be defined, measured and monitored during the process.

The ability for rapid development of a robust DSP was a key enabler for the success of mAbs as pharmaceutical product [4,17]. Generic platform processes (**Figure 2**), in general employed for all mAb candidates, are most commonly used by the industry due to several advantages. In addition to predictability, reduced experimentation result in a reduction of development effort, costs and speed to clinic [34]. However, due to significant physicochemical differences between the mAb candidates, a flexibility of the process regarding the selection and operating conditions of the unit operations is necessary [17].

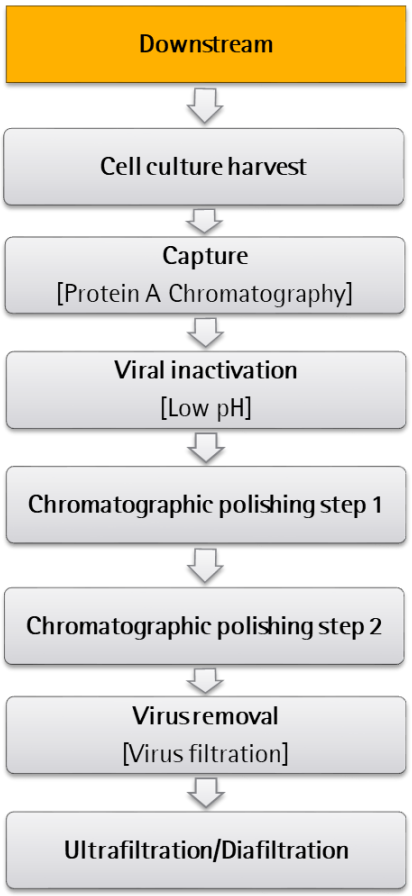

Figure 2. Example of a platform downstream process for mAbs (adapted from [17]).

Since the mAb is secreted into the cultivation medium, removal of cells must be performed at the beginning of the DSP, except for perfusion processes. Due to their economic benefits and scalability, continuous disk-stack centrifuges are commonly used in biopharmaceutical manufacturing as primary clarification step [17,35]. Following this, depth filtration is often used to ensure complete removal of cells and cell debris. They comprise a complex porous matrix of materials to retain particles at both the surface and the internal layers of the medium [36]. Several studies have shown an additional removal of soluble impurities, like HCP and DNA, to some degree [37,38]. The usage of diatomaceous earth (dynamic body feed (DBF filtration) [39] significantly increases the load reduction of these process related impurities. As alternative clarification methods acoustic cell retention devices [40] or continuous single use centrifuges [41,42] are utilized.

For the subsequent mAb capture, protein A affinity chromatography is used in most platform DSP in bind and elute mode. Protein A exhibit a high affinity towards the constant Fc-region of mAbs. Thereby, this

unit operation is widely applicable for mAb purification with high yield > 95 % with little development effort of this unit operation [43]. Binding of the mAb from the cell culture supernatant to the immobilized protein A is enabled under neutral pH conditions. During the washing step unbound impurities are removed resulting in purities of > 99% in many cases. Elution occurs at low pH-values resulting in a concentration of the mAb in the eluate of > 10 g/L [17].

The international conference of harmonization (ICH) as well as the EMA deal with the risk of potential viral contamination and approaches for viral safety [44]. For biopharmaceutical products, derived from mammalian cell lines, two dedicated orthogonal steps for viral reduction are required. A generic acidification is commonly applied for a sufficient inactivation of retroviruses by titration directly after protein A chromatography, due to the already low pH value of the eluate [17,45]. Different studies showed that a robust viral inactivation (VI) requires a pH ≤ 3.8 [46] or ≤ 3.6 [47]. Even though mAbs are most often stable under those low pH conditions for a certain period, a neutralization is performed following the VI for stabilization [17]. As second viral reduction step a filtration through virus retentive filters is currently a key unit operation at the end of most DSP platform processes [34,44].

In most platform processes, at least two additional chromatographic steps are typically employed after VI. For a robust operation and safety of the product a sufficient level of redundancy between both unit operation must be ensured. Must commonly ion exchange chromatography (IEX) executed as cation exchange chromatography (CEX) and/or anion exchange chromatography (AEX), which are used to separate molecules based on their surface charge, are incorporated [48–51]. The net surface charge of a biomolecule, like proteins, depends on the pH of the environment and the isoelectric point (IP) of the molecule. At a pH equal to the IP of the molecule, the net charge will be zero, at a lower pH positive and at a higher pH negative. In addition, the affinity depends on the ionic strength of the environment measured by the conductivity. Charged molecules or ions compete for binding sites of the ligand leading, as a function of the ionic strength, to a displacement of the biomolecule.

CEX is commonly used as first chromatographic polishing step executed in bind and elute mode. Thereby negatively charged ion exchange ligands are used which have an affinity for molecules with a positive surface charge. Conductivity and pH of the feed solution are adjusted to conditions where the mAb binds to the ligands ($pH<IP_{mAb}$) while impurities with a lower IP, like most of the HCP, break through the CEX during the loading phase. The choice of the buffer conditions to subsequent elute the mAb from the negatively charged ligands can further increase the purity. Though there is a considerable variability within the physicochemical characteristics of the impurity spectrum lower pH values as well as lower

conductivities result in a higher purity of the eluate, because a larger fraction of the impurities is positively charged and remain bound on the CEX ligand [52]. The mAb can be eluted either by an isocratic step or by linear gradient elution. Gradient elution can be used for optimization purposes and once the elution profile is established, step elution can be performed for purification in the production.

In most DSP one of the two chromatographic polishing steps, often the AEX, is executed in flow-through mode [17]. Thereby the product of interest remain unbound during the loading phase while the positively charged AEX ligands retain the negatively charged impurities. Predominantly DNA, due to its highly negative charged phosphate-sugar backbone, but also negatively charged HCP, leached protein A and viruses are removed [48,53]. The flow-through mode offers great potential for an increased productivity if the mAb is more abundant compared to the impurities in the feed solution [54]. Since the binding sites on the AEX are only occupied by the impurities, lower total binding capacities, compared to the bind and elute mode, are needed to process the same amount of mAb. As a result, nowadays membrane adsorber (MA) are often used for AEX in flow-through mode. MA consists of microporous membranes containing functional ligands attached to the membrane structure, eliminating the slow mass transfer within long diffusive pores of conventional bead chromatography [55,56]. Advantages are higher flow rates, ensuring increased productivity and reduced capital invest through a single-use approach [56–59].

Alternative chromatography methods recently used for mAb purification are hydrophobic interaction chromatography (HIC), mixed mode chromatography or hydroxyapatite chromatography likewise [48,60,61].

Ultrafiltration/Diafiltration (UF/DF) takes place at the end of the platform DSP prior to the fill and finish step. The pressure driven membrane process is used for mAb concentration and buffer exchange into the formulation buffer [62]. Separation is achieved size-based, while smaller species are able to pass the membrane, larger species are retained [63]. By choosing an appropriate molecular weight cutoff (MWCO) of the membrane, most commonly between 30 and 100 kDa, the mAb remain in the retentate. In addition, impurities like HCP, where the majority has been shown to be smaller than the mAb, are partly removed [64].

2.3 Challenges in the downstream process

The significant improvements in the USP resulted in increasing cell densities and mAb titer in the last decades. However, these achievements has placed the production bottleneck to the downstream process

with major challenges for the clarification and capture steps [34]. Chromatographic steps are inherently throughput limited, resulting in major challenges for processing large volume of mAb solution with high titer. In addition, especially protein A chromatography suffers from the high resin costs, possible leaching of protein A ligands and aggregate formation due to low pH conditions during elution [17]. Existing protein A chromatography units, as current state of the art in mAb production, reach their capacity limits [27,65]. As a result, several investigations into reliable and economic processes, like the operation in continuous mode [66], are being undertaken.

To overcome chromatographic limitations interest has been placed on non-chromatographic unit operations. For instance, selective precipitation using polymers and/or salts is frequently described in the literature. Either the mAb itself, by the usage of polyanionic polymers [67], or impurities like HCP, by the usage of caprylic acid [68], are precipitated. Flocculation represent an extension of precipitation conducted at the beginning of the DSP. By the usage of flocculation agents, like polydiallyldimethylammonium chloride and a low pH value, cell and cell debris as well as a significant amount of impurities like DNA and HCP can be removed [69,70]. However, there are numerous reservations against these precipitation methods, as they may lead to aggregation or impair important CQA of the mAb. As a potential approach for mAb purification directly from the cell culture, ATPE moves into the focus of research [71].

2.4 Aqueous two-phase extraction

2.4.1 Aqueous two-phase systems

Aqueous two-phase systems (ATPS) were first described by Martinus Willem Beijerinck in 1896, who mixed starch and gelantin in an aqueous solution [72]. The most common biphasic systems are polymer-polymer systems (e.g. polyethylene glycol (PEG) and dextran) and polymer-salt systems (e.g. PEG and phosphate, sulfate or citrate) [72,73]. Other types are alcohol-salt [74,75], micellar [76,77] and ionic based ATPS [78].

The mixture of two polymers result in the formation of large aggregates and due to steric exclusion, two immiscible phases are formed. A similar exclusion effect can be applied to high salt concentrations, which capture a large proportion of the present water. ATPS are formed by two hydrophilic components mixed over a certain concentration threshold in aqueous solution, resulting in two immiscible liquid phases [79]. The critical concentration of the components is dependent on the component itself, the pH value, the ion-

strength and temperature of the solution [80]. The phase diagram represents a unique fingerprint of a given ATPS, which delineates the potential working area (**Figure 3**).

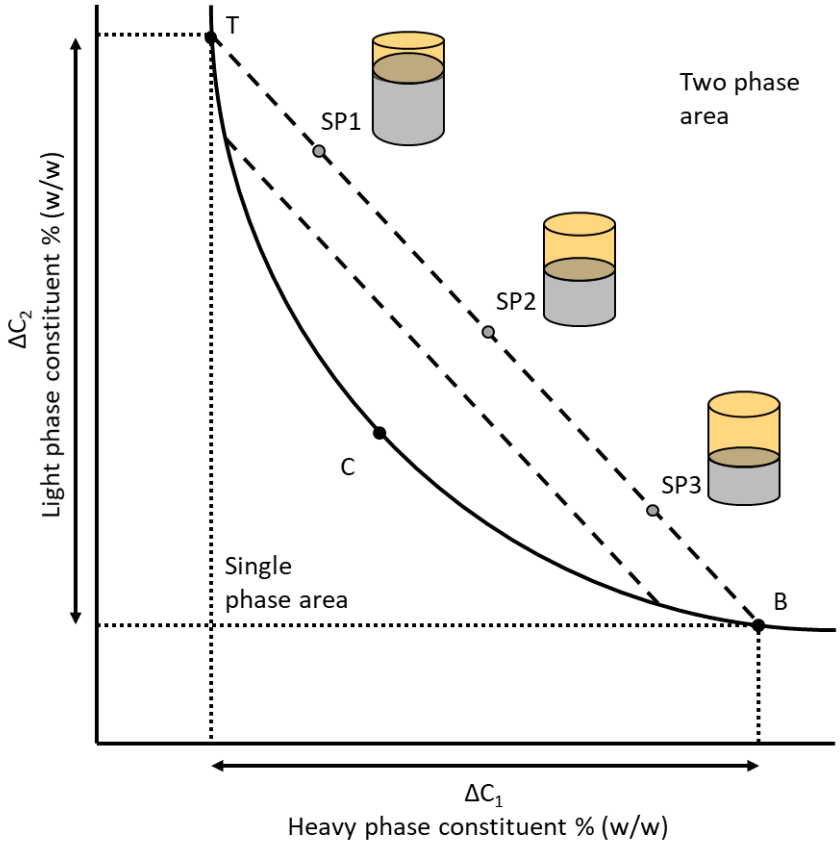

Figure 3. Phase diagram of ATPS. Heavy phase composition is plotted on the abscissa; light phase composition is plotted on the ordinate (adapted from [72]).

The phase diagram gives the exact composition of the light (LP) and heavy phases (HP). For every constitution below the bimodal curve (TCB) a homogeneous, monophasic solution is formed, while constitutions above the curve form aqueous two-phase systems [81]. The final concentration of the phase forming components in both phases is represented by the tie-line length (TLL, **Figure 3** line TB). The TLL is calculated according to equation (1), where ΔC1 and ΔC2 are the absolute concentration differences of the phase forming components, in the light and heavy phase.

$$TLL = \sqrt{\Delta C_1^2 + C_2^2} \quad (1)$$

The parallel TLLs are commonly used to express the influence of the system composition on the partitioning effect. The critical point (C) represents TLL=0, where the composition and volume of both phases are almost equal [82]. All system points (SP) on the same TLL exhibit the same light and heavy

phase composition with a different volume ratio (V_R) according to equation (2), where V_{LP} and V_{HP} are the volumes of the light and heavy phase, respectively.

$$V_R = \frac{V_{LP}}{V_{HP}} \tag{2}$$

Albertson was the first who discovered in 1986, that the different physicochemical properties of both phases allow a partitioning of solutes [83]. The partitioning of a molecule within an ATPS in equilibrium can be described by the partition coefficient (K) according to equation (3), where C_{LP} is the concentration of the solute in the LP and C_{HP} the concentration of the same solute in the HP.

$$K = \frac{C_{LP}}{C_{HP}} \tag{3}$$

According to Albertsons model six different factors exert a different driving force for the partitioning, where K is affected by electrochemical properties (K_{elec}), the hydrophobicity (K_{hphob}), specific affinity ($K_{affinity}$), size (K_{size}) and conformation (K_{conf}) of the molecule as well as environmental factors of the ATPS itself (K^0) [83].

$$\ln K = \ln K^0 + \ln K_{elec} + \ln K_{hphob} + \ln K_{affinity} + \ln K_{size} + \ln K_{conf} \tag{4}$$

Since superficial charge interaction with the ions in solution is a major factor for the solubility of a molecule, ionizable species like salts and ionic liquids as well as the pH value are very important parameters in ATPE [84]. For biomolecules and proteins, a pH value above the IP may induce a higher affinity towards the PEG-rich phase in PEG-salt systems due to electrostatic interactions [85].

Although both phases of an ATPS are predominantly hydrophilic, the LP usually appears slightly more hydrophobic. Hydrophobicity is considered as one of the most important factors for the partitioning of proteins in ATPS [84,86] due to two different effects. Firstly, the phase hydrophobicity, which increases with the polymer molecular weight (presence of extensive hydrophobic areas) and the addition of displacement agents like sodium chloride (less available water). Secondly, the salting-out effect results in a favored partitioning in the more hydrophobic phase by a higher salt concentration, due to a reduced hydration of the solutes [85].

In addition, specific affinity partitioning can be achieved by the usage of ligands, attached to the phase forming polymer or free in the solution. Thereby an increased selectivity can be obtained, without modification of the ATPS composition [87].

Due to a defined size and conformation of the solute steric effects are imposed by the ATPS components as a function of the available volume for the molecule to be fractionated into a particular phase (free volume effect) [88]. This effect especially applies to polymer-polymer and polymer-salt systems, where concentration and molecular weight of the polymer exert considerable influence, due to the limited free volume in the polymer-rich phase. In addition, in polymer-salt systems salting-out effects occur, which enables a partitioning of the molecule at the interphase through volume exclusion effects [89].

All of the factors described above influence the extraction of a specific target molecule out of a mixture with several impurities. To evaluate the performance of the ATPE with regard to a specific separation task, different parameters must be considered. The yield of the product by the extraction into the preferred ATPS target phase (TP), according to equation (5), is one of the most important, where c_{TP} and c_{feed} are the concentrations of the product in the target phase and the feed solution, while V_{TP} and V_{feed} represent the respective volumes.

$$yield = \frac{C_{TP} * V_{TP}}{C_{feed} * V_{feed}} * 100\ \% \tag{5}$$

In addition, the removal of defined impurity species ($removal_{imp}$) can be calculated according to equation (6), where $C_{imp,TP}$ is the concentration of the impurity species in the target phase and $C_{imp,feed}$ concentration of the respective impurity in the feed solution.

$$removal_{imp} = \left(1 - \left(\frac{C_{imp,TP} * V_{TP}}{C_{imp,feed} * V_{feed}}\right)\right) * 100\ \% \tag{6}$$

2.4.2 Application of aqueous-two phase systems

ATPS are used for the extraction of numerous biomolecules for several reasons. While other liquid-liquid extraction methods are inappropriate for the recovery of biological products, due to organic solvents and process conditions, ATPS offers a gentle environment for biomolecules (water content 85-90 % [90]), maintaining the biological activity and stabilize the structure [83]. In addition, ATPE is considered as low cost [91], environment-friendly [92] and easy to scale up method [93], which is also capable to be operated in continuous mode [94].

ATPE is reported in the DSP of several biological products like proteins, enzymes, nucleic acids, virus, virus like particles and cells [72]. Nowadays ATPE is applied for the purification of valuable biopharmaceutical

products like growth factors, hormones and most commonly mAbs. In 1992 Sulk *et al.* were the first to use ATPE for mAb purification [95], and numerous studies followed [18,96–98].

However, despite the structural similarities between different mAbs and due to the diverse impurity spectrum of the producing mammalian cell lines, ATPS composition optimization must be performed for every product independently. Although the underlying mechanistic effects of ATPE are known (section 2.4.1) a model based prediction remains difficult and often insufficient. On the other side, a conventional optimization strategy alternating one variable at a time (OVAT) is often time and effort consuming, due to the numerous influential factors. Therefore, a statistical DoE approach is nowadays commonly used [97,99], alternating all significant process factors at the same time. In addition, this method offers the advantage to examine the interactions between the different factors. As a result, statistical models of the examined responses, like mAb yield and purity in the target phase, can be created. Accordingly, combination of these models allows a trade-off between the responses, to achieve the desired extraction performance.

3. Experimental results

In order to examine a holistic DSP strategy for mAb purification by ATPE the work of this PhD thesis was divided into three closely related parts. Together they demonstrate an alternative DSP, comprising clarification, high yield and consistent quality of the mAb as well as removal of process related impurities, enabling process intensification. Each part was published separately in a peer-reviewed journal.

ATPE was reported in different studies for clarification of mammalian cell cultures due to steric effects, resulting in accumulation of the cells in the interphase [100,101]. However, a subsequent phase separation technique is still needed to remove the cells and to separate the mAb containing target phase, in this work always the polymer-rich LP, from the HP and interphase for the further DSP. In the first publication entitled "*Integrated Clarification and Purification of Monoclonal Antibodies by Membrane Based Separation of Aqueous Two-Phase Systems*", focus was placed on the phase separation after ATPE. Established phase separation methods make use of the density differences between LP and HP, like gravity based separation in mixer-settler devices [101], column [98] or centrifugal extractors [102]. However, for ATPS with only minor density differences of the phases this method can be time consuming and thus expensive [98,103].

Aim of the study was to develop a membrane-based method for phase separation, in order to integrate clarification, mAb capture and purification in one unit operation for process intensification. Riedl *et al.* have demonstrated a surface interaction through a hydrophobic membrane between already separated ATPS phases by surfactant mediated hydrophobization of the PEG-rich LP [104]. However, no passage of a phase through the membrane was realized.

In this PhD thesis, an actual selective phase separation was accomplished by the use of a modified hydrophobic membrane with surfactants. Within the study, a membrane separator with increased membrane area was designed based on the newly developed phase separation method. A complete cell free LP was obtained without the use of any additional sterile filter.

In order to examine the applicability of the new established unit operation for mAb purification, the ATPS composition was optimized by a DoE approach for a conventional fed-batch CHO cultivation broth. Due to this method, an optimal operating point, in terms of mAb yield as well as purity, was predicted with minimal effort, time and resources. The particularly high yield in combination with the removal of DNA and HCP, for the validation of the predicted ATPS, reveals the applicability of ATPE for mAb capture and purification.

The combined use of the optimized ATPS with the newly developed membrane separator was investigated in an application study in lab-scale. In order to process the complete volume of the broth and according the higher amount of ATPS, membrane separators were connected in parallel. In accordance with the preliminary experiments, a selective phase separation was obtained with a complete absence of cells in the LP and similar high values for mAb yield and purity.

Despite the initial purification of the mAb by ATPE, the LP must be further processed, in order to achieve the required purity of a biopharmaceutical product. Although a lot of publications demonstrate the applicability of ATPS for mAb extraction, only minor studies have been conducted to further integrate this method into a complete DSP [94,105]. Therefore, the second publication of this PhD thesis, entitled "*An Alternative Downstream Process Based on Aqueous Two-Phase Extraction for the Purification of Monoclonal Antibodies*", focused on the direct implementation of ATPE and the alignment of subsequent unit operations within the DSP. A complete elimination of the most expensive protein A chromatography step has been achieved, offering a remarkable potential for cost savings. In addition to clarification and extraction of the mAb by an ATPS, a combined diafiltration/VI approach, CEX and AEX were used for the alternative DSP.

For each unit operation, a holistic optimization in terms of mAb yield and purity was performed. Furthermore, it was shown that the critical quality attribute of the N-linked glycosylation profile remained unimpaired after ATPE and throughout the complete alternative DSP. Based on the consistent product quality ATPS has been affirmed as suitable tool for mAb purification.

A diafiltration approach was used for removal of phase forming components as well as LMW impurities. A membrane screening was conducted to select the optimal MWCO as a trade-off between mAb yield and purity. In addition, a simultaneous buffer exchange for the subsequent purification step was conducted, which offered the possibility of an integrated VI step by the selection of a suitable acidic buffer. Further processing was conducted similar to a common platform mAb DSP (**Figure 2**). Thereby existing plants, equipment and optimized process conditions for the respective unit operation can be further used ensuring fast and simple process adaption. Two chromatographic polishing steps, in particular CEX in bind and elute as well as AEX in flow-through mode were utilized. After optimization, both unit operations resulted in a remarkable removal of process related impurities with high yields of mAb.

The novel developed alternative process with the holistic optimization was executed as an application study, where the feasibility of an ATPE based DSP was successfully demonstrated directly from a conventional fed-batch cultivation.

Genetic engineering as well as improved media and feeding strategies in the USP resulted in highly productive cell lines with the right growth characteristics and high specific growth rates offering several advantages for the industry. However, due to these achievements, HCD cultivations with more than 100 million cells/ml have been reported recently, further moving the bottleneck within mAb production to the DSP.

The third publication, entitled "*Aqueous Two-Phase Extraction of Monoclonal Antibodies from High Cell Density Cell Culture*", represents a proof of concept study to examine the transferability of ATPE for clarification, mAb capture and first purification from fed-batch to HCD cultivations.

The ATPS with HCD broth as feed component, similar to those with conventional fed-batch broth, showed an accumulation of the cells in the interphase, resulting in a complete cell removal within the LP. Therefore, the applicability of ATPE based clarification methods, like the membrane-based phase separation (section 3.1), could be probably considered as suitable even for HCD cultivation.

The third publication showed an efficient extraction resulting in high yields of mAb in the LP, despite some differences between the examined ATPS and in terms of the viable cell density (VCD) of the used feed component. In addition, DNA, representative for process related impurities, was removed significantly for almost all investigated systems.

3.1 Integrated Clarification and Purification of Monoclonal Antibodies by Membrane Based Separation of Aqueous Two-Phase Systems

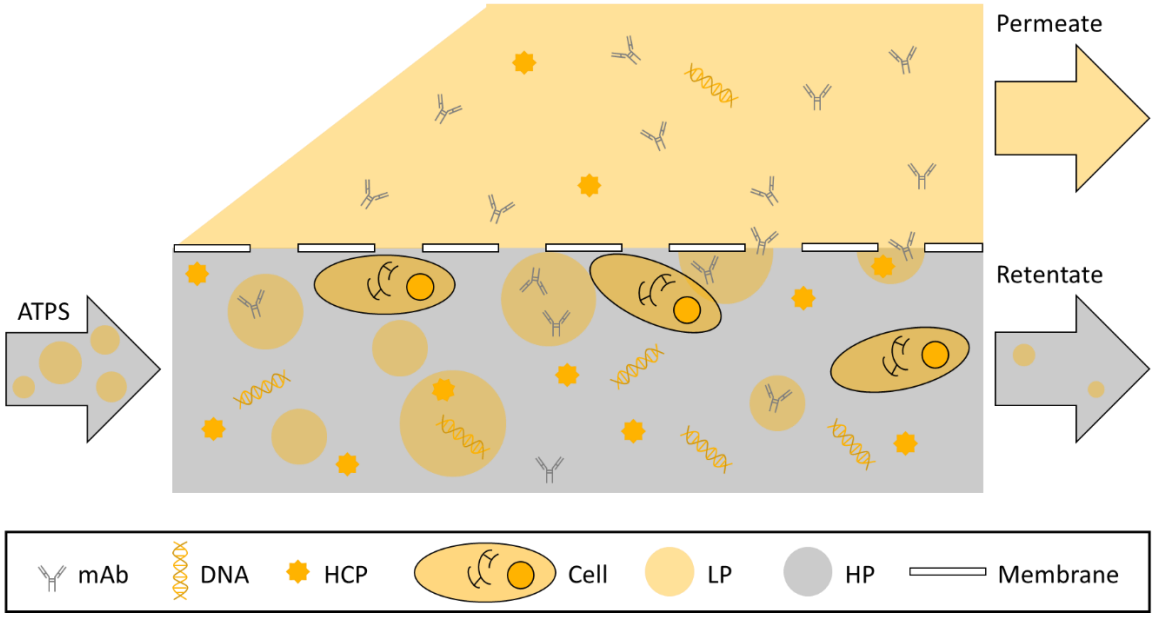

Figure 4. Schematic representation of membrane based ATPS phase separation for clarification and mAb purification from cell-containing cultivation broth.

The first publication focused on the development of a new method for ATPS phase separation based on membrane technology. In order to obtain clarification by removal of the cell-containing interphase as well as allow a further processing of the mAb, a selective separation of the LP as product phase must be obtained. A tangential flow filtration was used to overflow a membrane with the ATPS. Due to a specific membrane modification the mAb-containing LP was able to permeate the membrane, while the HP and cell-containing interphase were retained with the use of an appropriate transmembrane pressure (**Figure *4***). Thereby, an additional integration of the required sterile filtration step, by using a membrane with a corresponding narrow pore size, was obtained for process intensification.

Five different surfactants and two different hydrophobic membrane materials were screened while the modification of a polypropylene membrane with Tween20 provided the best results. Separation performance, in terms of permeate flow rate and phase purity, was investigated for five different ATPS, with water as feed component, with different phase forming components and compositions. Differences between the examined ATPS could be attributed to contact angle differences of the respective LP and HP on the modified membrane. It was shown that a high contact angle difference result in a high yield of LP

until breakthrough of the HP, which allows a prediction whether an ATPS can be efficiently separated by the membrane-based approach.

In order to evaluate the transferability of the preceding phase separation experiments with water to real mixtures, cell-containing cultivation broth with IgG as product and process-related impurities was used as ATPS feed component for the subsequent experiments. In addition, the ATPS composition was optimized for the given broth by a DoE approach. A model for the mAb yield as well as DNA and HCP removal in the LP was created. The simplex or Nelder-Mead method [106] was used for a trade-off between mAb yield and purity. Based on the model the most promising ATPS composition was 36 w% feed, 19 w% PEG400, 16.4 w% phosphate salt (pH=8.0) and 4 w% NaCl as displacement agent. In equilibrium, a mAb yield of 92 % with simultaneous removal of DNA (85 %) and HCP (52 %) was obtained in the LP of the optimized ATPS.

In an application study 400 ml of the optimized ATPS with cell-containing cultivation broth as feed component was processed with a newly developed membrane separator. A complete cell-free LP was obtained at the permeate side and no breakthrough of the HP occurred during the whole process. By the application of an additional flush step 83 % of the LP were recovered in the permeate resulting in an overall yield of 78 % mAb. At the same time 92 % of the DNA and 43 % of the HCP were removed. These results demonstrate the feasibility of ATPE for mAb purification combined with a membrane-based phase separation, offering great potential for process intensification.

antibodies

MDPI

Article

Integrated Clarification and Purification of Monoclonal Antibodies by Membrane Based Separation of Aqueous Two-Phase Systems

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Abstract: Therapeutic monoclonal antibodies (mAb) are used for the treatment of numerous serious diseases, which have led to an increasing demand over the last decades. Increased cell density and mAb titer of the cultivation broth lead to great challenges for the subsequent clarification and capture operations in the downstream process. As an alternative approach to the conventional downstream process, a selective mAb extraction via an aqueous two-phase system (ATPS) directly from the cultivation broth of a mAb producing industrial relevant chinese hamster ovary (CHO) cell line was investigated. An efficient purification of the mAb was accomplished by the ATPS composition. The phase separation was realized by a newly developed membrane based phase separator. Moreover, a complete cell removal was integrated into this process by the used membrane. A selectivity between both phases was achieved by membrane modification. Yields up to 93% in the light phase and removal of process related impurities were obtained after aqueous two-phase extraction (ATPE). Phase separation performance as well as contact angles on the membrane were characterized for different ATPS. ATPE directly from the cultivation broth in combination with the new membrane based phase separation led to a mAb yield of 78% with a simultaneous reduction of deoxyribonucleic acid (DNA) and host cell protein (HCP) load.

Keywords: aqueous two-phase extraction; phase separation; downstream; clarification; membrane

1. Introduction

Due to their enormous importance as active pharmaceutical ingredients for the treatment of numerous severe diseases like immunological disorders [1,2], cancer [2], and inflammatory as well as infectious diseases [1,2], the demand for monoclonal antibodies (mAb) is rising [3]. Improved media and feeding strategies in the upstream process of mAb producing cell lines, like chinese hamster ovary (CHO) cells, have resulted in significantly increased cell density and titers up to 25 g/L [4–6]. However, these achievements have led to high challenges for the subsequent clarification and capture operations, causing a bottleneck in the downstream process (DSP) [7].

As mAb are secreted into the medium, cells must be removed first. Most commonly continuous disk-stack centrifuges are used as a first clarification step at commercial scale due to their economic benefits and scalability [8,9]. Alternative clarification methods are depth filtration [8], flocculation [10–12], dynamic body feed filtration [13] or the usage of an acoustic cell retention device [14]. As subsequent mAb capture step protein A affinity chromatography is used in most platform processes, offering the advantages of high yield and purity as well as a volume reduction [8,12]. However, challenges in DSP are mainly caused by the limited capacity of chromatographic methods and, especially for protein A chromatography, its price [15].

A promising approach to overcome this bottleneck is the application of aqueous two-phase extraction (ATPE). It has been demonstrated that ATPE is suitable as a first purification step in the downstream process of mAb [16–18], has economic benefits and is environmentally sustainable compared to current established platform processes [19]. Formation of aqueous two-phase systems (ATPS) takes place by mixing a variety of components in water. Above a certain concentration of the phase forming components, two immiscible phases are formed [20,21]. As phase forming components, polymer/polymer (usually polyethylene glycol (PEG) and dextran) or polymer/salt (e.g., phosphate, citrate or sulfate) are most commonly used [22]. In the latter case a light, polymer rich (LP) and a heavy salt rich phase (HP) are formed. Based on the composition of the ATPS, a selective extraction of the target molecule (mAb) can be achieved, while impurities like deoxyribonucleic acid (DNA) and host cell proteins (HCP) are enriched in the other phase [18]. Particles like cells, cell debris and other bioparticles accumulate at the ATPS interphase enabling an integration of clarification and first mAb purification [23].

The optimization of the ATPS composition with a high yield and purity of the target molecule has already been shown in many studies using a statistical design of experiments (DoE) approach [18,24,25]. Thereby costs and time consumption are reduced compared to traditional optimization methods like changing one factor at a time (OFAT) [24]. Due to physicochemical differences between different target molecules these investigations must be executed separately for any given separation task (different mAb or other proteins/biomolecules) [22].

For further purification, a separation of the mAb containing target phase (LP in this study) must be ensured. Established methods make use of the different densities of both phases from an ATPS. Phase separation by gravity is often conducted by mixer-settler devices [23]. However, due to the minor density difference between both phases this method is often time consuming and thus expensive [26,27]. Alternatives heretofore are column [27] or centrifugal extractors [28] where the separation is also realized by means of density differences between both phases. However, these methods require an additional sterile filtration step if used as clarification unit operation to ensure complete cell removal [8,27,28]. This sterile filtration step was integrated by the usage of a membrane for phase separation with a narrow pore diameter in this study.

Membrane technology like crossflow filtration is often used for the phase separation of classical liquid–liquid extractions, with organic and aqueous phases [29–31]. Depending on the used type of membrane, hydrophobic or hydrophilic, the organic or aqueous phase can permeate the membrane while the other is held back in the retentate [31]. However, membrane technology has not been reported yet for the separation of ATPS, likely due to the physical similarity of both phases [20,21].

The driving force for both phases to permeate a membrane is the transmembrane pressure (TMP). The TMP is given by Equation (1), where $P_{ret,in}$ and $P_{ret,out}$ are the pressure on the retentate site at the in- and outlet, respectively, and P_{perm} is the pressure on the permeate site [32]. Increased TMP values lead to a higher permeate flux, but from a certain pressure the selectivity decreases by breakthrough of the non-target phase [31].

$$TMP = \frac{P_{ret,\,in} + P_{ret,\,out}}{2} - P_{Perm,\,out} \quad (1)$$

Increased TMP values lead to a higher permeate flux, but from a certain pressure the selectivity decreases by breakthrough of the non-target phase [31].The challenge for ATPS is due to the high physical similarity of both phases (both consist predominantly of water, approximately 80 w%) [20,21], which impedes selective phase separation by a membrane. For an industrial application of this technology, profound examination is required.

It has been demonstrated that non-ionic surfactants mediate a surface interaction between already separated ATPS phases by hydrophobization of the PEG-rich LP. Thereby only the LP was able to permeate the pore of a hydrophobic membrane [33]. This approach could be also used for a membrane based phase separation.

To accelerate process design and to find the knowledge-based optimum operating space, model based methods are increasingly used [34]. However, the scope of this work was to gain fundamental insight into the feasibility of a membrane based ATPS phase separation for mAb purification.

In this work a selective membrane based ATPS phase separation is presented. Flow through of the light target phase was achieved by membrane modification. As modification agents different surfactants were examined for different model ATPS. An integration of clarification and sterile filtration by membrane based phase separation with a first capture and purification step by ATPE was investigated. The results were used for an application study to purify mAb directly from the cultivation broth with a DoE based, optimized ATPS.

2. Materials and Methods

2.1. Cultivation

CHO cells were used for the mAb (immunoglobulin type G, IgG) production in a fed-batch cultivation carried out in commercial serum-free medium. The cells were cultivated for 12 days at 36.8 °C, pH 7.1 and 855 rpm in the Ambr200 single use bioreactor (Sartorius, Göttingen, Germany). At the end of the cultivation the viable cell density was ≥10×10^6 cells/mL and the viability ≥ 80% with an IgG concentration of approximately 2.8 g/L.

2.2. Aqueous Two-Phase Systems

Four different ATPS, which have been reported for mAb purification [18,23,35,36], with different phase forming components and compositions as well as an optimized ATPS (Section 2.7) were examined in this study. ATPS were prepared by weighing the appropriate amounts of the different components. PEG with molecular weights of 400 and 1450 g/mol were purchased (Merck, Darmstadt, Germany). Stock solutions of 40 w% phosphate buffer, 35 w% citrate buffer and solid sodium chloride (NaCl) were used. Sodium phosphate monobasic anhydrous (NaH_2PO_4) and potassium phosphate dibasic anhydrous (K_2HPO_4) were used for the phosphate buffer, citric acid ($C_6H_8O_7$) and trisodium citrate ($Na_3C_6H_5O_7$) for the citrate buffer. All salts were purchased from Carl Roth (Karlsruhe, Germany). The pH value was adjusted by different ratios of the corresponding salts. As a feed solution, reverse osmosis (RO) water, cell containing cultivation broth and cell free culture filtrate were used as indicated. The four different ATPS model systems, with RO water as feed component, were evaluated for their ability to be separated by the modified membrane (Section 2.3). Furthermore, an optimized ATPS for the purification of a mAb from a CHO cell line was investigated. The components of each system are listed in Table 1.

Table 1. ATPS composition and the respective phase ratios. ATPS: aqueous two-phase system.

ATPS	Feed (w%)	Polymer (w%)		Salt (w%)		pH (-)	Displacement Agent (w%)	Phase Ratio (*v*/*v*)
		PEG 400	PEG1450	Phosphate	Citrate		NaCl	
1 [23]	44.5	15.5	-	16	-	6	-	1.43
2 [35]	26.4	19.6	-	-	18.9	6	-	1.02
3 [36]	40.5	-	6	15	-	6	10	0.26
4 [18]	27.2	6.8	-	26.12	-	7.3	0.7	0.24
Opt	36	19	-	16.4	-	8	4	1.13

The phase ratio (PR) was defined as the quotient of the volume of the LP to the volume of the HP in equilibrium.

$$PR = \frac{V_{LP}}{V_{HP}} \quad (2)$$

2.3. Membrane Modification

As hydrophobic membrane materials, polypropylene (PP ACCUREL®, 3M, Wuppertal, Germany), polyvinylidene fluoride (PVDF, Pall, New York, NY, USA), polytetrafluoroethylene (PTFE, Sartorius, Göttingen, Germany) and hydrophobized polyether sulfone (hydrophobized PES, Sartorius, Göttingen, Germany) with a mean pore diameter of approximately 0.4 µm were investigated. For modification, the membrane was incubated overnight in the LP of each ATPS supplemented with different surfactants (Tween20, Tween80, Brij 35 (Carl Roth, Karlsruhe, Germany), TritonX-100 and TritonX-114 (Fisher Scientific, Pittsburgh, PA, USA). The used surfactant concentration (1 w%) was a trade-off between surfactant solubility in the LP and membrane wettability by the solution [37]. After incubation the modified membrane was dried before use for at least 1 h.

2.4. Contact Angle Measurement

ATPS were prepared according to Section 2.2 with RO water as the feed component and both phases were separated by centrifugation for 5 min at 1000× *g*. The LP and HP of each ATPS were examined on modified (Tween20) and non-modified PP membrane. Residual unbound surfactant from membrane modification was removed by washing the membrane three times with the respective LP prior to drying. Contact angles were recorded using a goniometer (OCA 15 EC, Dataphysics, Filderstadt, Germany) and analyzed afterwards (ASC20, Dataphysics, Filderstadt, Germany). The sessile drop method [38] was used and 3 µL of each phase were dispensed on the respective membrane. Contact angles were recorded in triplicates over 5 min or until the droplet was completely absorbed by the membrane. For contact angle differences the values at the last recordable measuring point before one of the phases was absorbed by the membrane were used for both, LP and HP.

2.5. Membrane Based Phase Separation

For phase separation experiments, the ATPS was transferred into a stirred recirculation tank in which the LP was dispersed in the HP. The TMP as well as the pressure difference between the inlet and outlet on the retentate site (dP) was adjusted by the use of the respective valves, while the inlet flow (Q) was regulated by the pump power of the used crossflow device (SARTOFLOW® Smart, Sartorius, Göttingen, Germany) (Figure 1).

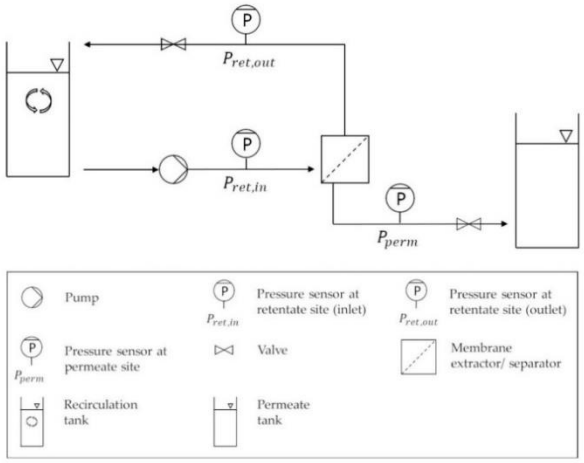

Figure 1. Schematic representation of the setup for phase separation experiments. ATPS was transferred in the recirculation tank. The LP as product phase was collected in the permeate tank. ATPS: aqueous two-phase system. LP: light phase.

Aqueous phase separation experiments were performed by a miniaturized membrane based liquid–liquid extractor for process intensification with an active membrane area of 22.4 cm^2 (Figure 2) [18]. The mixed system was pumped into the membrane based extractor (Figure 2 left). In the meander structure of the extractor both phases came into contact with the modified membrane (Figure 2 middle). Unless otherwise stated, the retentate was recycled in the feed flow while the permeate was withdrawn as product phase (Figure 1, Figure 2 right). For further application experiments with cell containing cultivation broth, newly designed membrane separators with 200 cm^2 active membrane area were used (Figure 3).

Figure 2. Miniaturized device for intensification of liquid-liquid extraction (membrane extractor). Exterior view of the device (**left**); inner channel structure (**middle**); schematic representation of the hypothesized membrane based phase separation (**right**). Feed (F), retentate (R) and permeate (P) channels are shown.

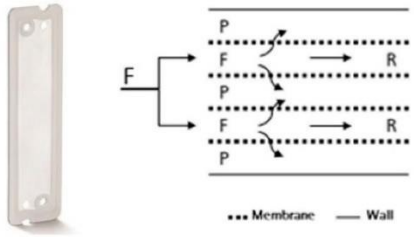

Figure 3. Membrane separator with an increased membrane area (200 cm^2). Front view (**left**); schematic presentation of the feed (F), retentate (R) and permeate (P) channels (**right**).

2.6. Analytical Procedure

For the determination of the IgG concentration, a protein A membrane adsorber (Sartobind® Protein A, Sartorius, Göttingen, Germany) was used because of the relatively high viscosity of the PEG rich samples. The analysis was performed with an Äkta prime plus chromatography system (GE Healthcare, Uppsala, Sweden). Equilibration was carried out with phosphate buffered saline (PBS, pH 7.4) and sample volumes of 500 µL were applied. For elution 0.1 M glycine (pH 3.0) was used. The flow rate was constant at 10 mL/min for all steps. For quantification the measured absorption peak areas (280 nm) were evaluated. The DNA concentration was measured by the Quant-iT™ PicoGreen™ dsDNA Assay Kit (ThermoFisher Scientific, Waltham, MA, USA) with salmon sperm DNA as standard. The HCP concentration was determined with an HCP-ELISA (Cygnus Technologies, Southport, NC, USA).

For equilibrium experiments the appropriate amount of ATPS components were weighed in a 15 mL centrifuge tube to a total mass of 10× *g*. To ensure equilibrium conditions, the tubes were shaken for 5 min at 150 rpm. For phase separation the tubes were centrifuged for 5 min at 1000× *g*. The yield of IgG was determined by the mass in the LP after extraction compared with the mass in the used feed. Removal of DNA and HCP were determined by the removed mass of the respective biomolecule in the LP after extraction compared to the mass in the feed. Cells were counted using a microscope in a Neubauer counting chamber (Brandt, 0.1 mm depth and 0.0025 mm^2).

2.7. Design of Experiments

The design of the experiments as well as the analysis was accomplished with the software MODDE (MODDE Pro, version 12, Sartorius, Göttingen, Germany). Factor areas were chosen by prior knowledge and the state of the art from the literature [18,21,28]. A D-optimal design was used because process factors (e.g., pH-value) were combined with mixture factors (e.g., feed, PEG 400, phosphate buffer and NaCl) with three center points [39].

3. Results and Discussion

3.1. Surfactant and Membrane Screening for Phase Separation

For the desired phase separation, a permeation of the LP as target phase through the membrane is elementary. Due to the hydrophilic nature of both ATPS phases [20,21] none of them were able to wet an unmodified hydrophobic membrane (Section 3.2.1). Therefore, five different non-denaturating surfactants were used for membrane modification prior to phase separating experiments. All investigated surfactants, except Brij35, were able to mediate a wettability of the hydrophobic PP membrane with the used modification method.

In the next step the selectivity of the modified membrane between LP and HP was examined. The phase purity (Z) is defined according to Equation (3), where V_x is the volume of the LP in the permeate or HP in the retentate and V_{total} the overall volume in the respective outlet.

$$Z = \frac{V_x}{V_{total}} * 100\% \quad (3)$$

The course of the permeate purity as a function of the TMP was investigated (Figure 4). For a TMP value of 0 mbar, a high permeate purity was achieved for all examined surfactants. The membrane with TritonX-114 as modification agent exhibited a strong decrease of the permeate purity even at low TMP values (Figure 4). For Tween20, Tween80 and TritonX-100 the permeate consists of pure LP up to 80 mbar TMP. Further pressure increase resulted in a decreased purity by breakthrough of HP in the permeate. Among all tested modification agents Tween20 showed a slightly higher purity even at a TMP higher than 80 mbar. Therefore, membrane modification with Tween20 as surfactant was used for further studies because the highest permeate flow rate and purity was desired for an efficient phase separation.

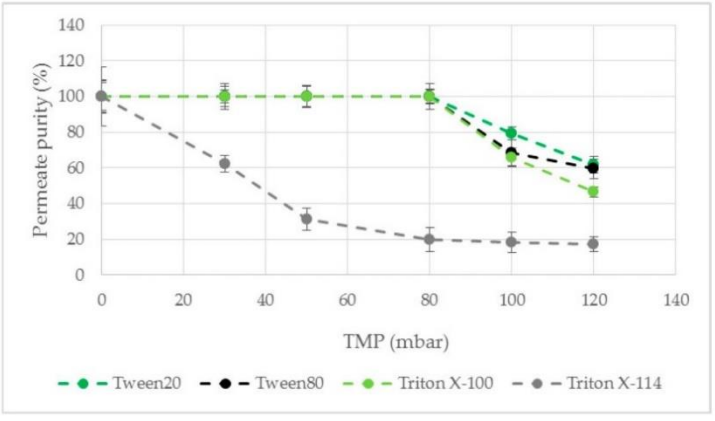

Figure 4. Permeate purity as a function of the TMP. A hydrophobic PP membrane was modified. ATPS4, ΔP = 30 mbar, Q = 16.7 mL/min. PP: polypropylene.

None of the surfactants used as modification agents were able to wet the used PTFE and hydrophobized PES membranes so that no permeate was obtained. PP as well as PVDF led to a pure permeate flow up to 80 mbar TMP (Figure 5). At increased pressure PP showed a higher permeate purity and was therefore used for further experiments.

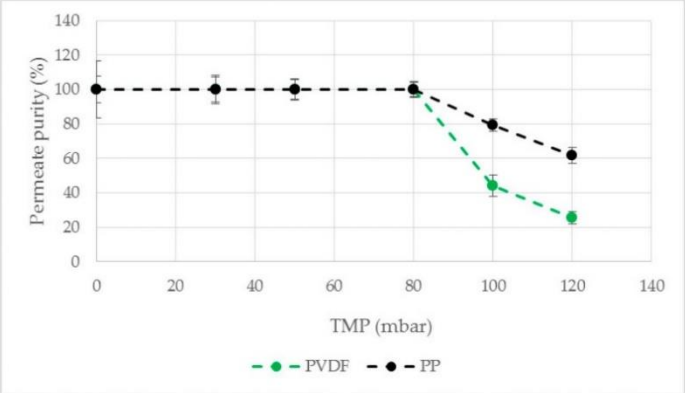

Figure 5. Permeate purity as a function of the TMP. PP and PVDF were used as membrane material for membrane modification with Tween20. ATPS4, ΔP = 30 mbar, Q = 16.7 mL/min. PP: polypropylene. PVDF: polyvinylidene fluoride.

In order to examine the long term stability of the membrane modification the permeate as well as the retentate purity were observed for 24 h process time, while both flows were recirculated. The permeate purity was 100% and the retentate purity between 71% and 79%, without a significant change during the whole experiment (Figure 6), suggesting a stable membrane modification. Possible leached traces of this surfactant entering the process medium are not an issue of concern as Tween20 is also one of the most commonly used surfactants in the formulation of pharmaceutical mAb products [40].

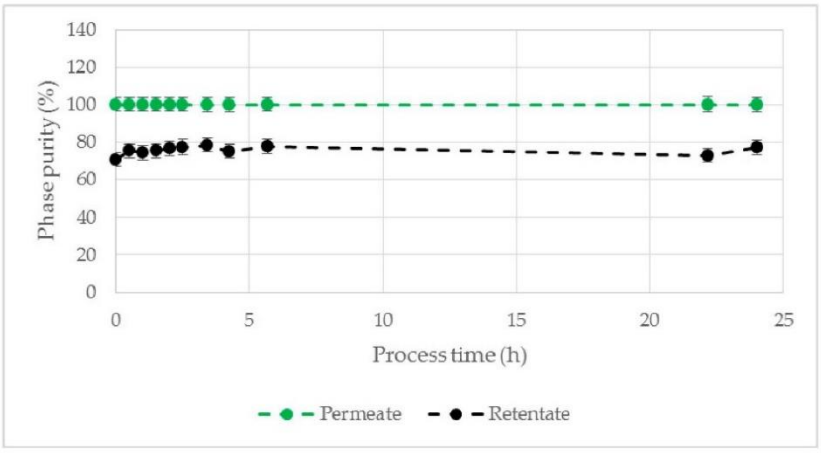

Figure 6. Phase purity of permeate and retentate (both recirculated) for a process time of 24 h. Hydrophobic PP membrane was modified with Tween20. ATPS4, TMP = 10 mbar, ΔP = 30 mbar, Q = 16.7 mL/min. PP: polypropylene.

3.2. Characterization of ATPS Phase Separation

In order to evaluate whether this new process is applicable for different ATPS and to select a first suitable model ATPS for the membrane based phase separation, four model systems with different phase forming components, concentrations, molecular weights of PEG, pH-values and NaCl as displacement agent were investigated (Table 1). First the interaction of both ATPS phases on modified as well as unmodified membranes was examined by contact angle measurements.

3.2.1. Contact Angle Measurements

According to the Young–Laplace equation (Equation (4)) [41,42], the capillary pressure (P_{cap}) needed for a fluid to permeate a membrane pore is dependent on the interfacial tension (γ), the radius of the pore (r) and the contact angle of the fluid on the membrane material (θ).

$$P_{cap} = \frac{2\gamma \cos\theta}{r} \quad (4)$$

Accordingly, optimal phase selectivity is ensured if the capillary pressure of the LP is lower while the value for the HP remains higher than the TMP. Thus a maximal high difference between the contact angles of LP and HP ($\Delta\theta = \theta_{HP} - \theta_{LP}$) on the modified membrane is desired for a selective phase separation.

Due to their high water content, the LP as well as the HP of all investigated ATPS showed a contact angle of approximately 135° (Figure 7) and were thus not able to wet the unmodified hydrophobic PP membrane. The minor decrease of the contact angle over time was due to evaporation of the droplet during the experiment. After membrane modification the contact angle of both phases decreased significantly. The decrease over time observed here was a result of the membrane wetting (contact angle below 90° [43]). The biggest difference between the contact angles was observed for ATPS1 ($\Delta\theta = 21.1 \pm 1.1°$), ATPS4 ($\Delta\theta = 25.1 \pm 1.1°$) and ATPS Opt ($\Delta\theta = 20.4 \pm 2.6°$, introduced in Section 3.3) suggesting the ability for a selective membrane based phase separation.

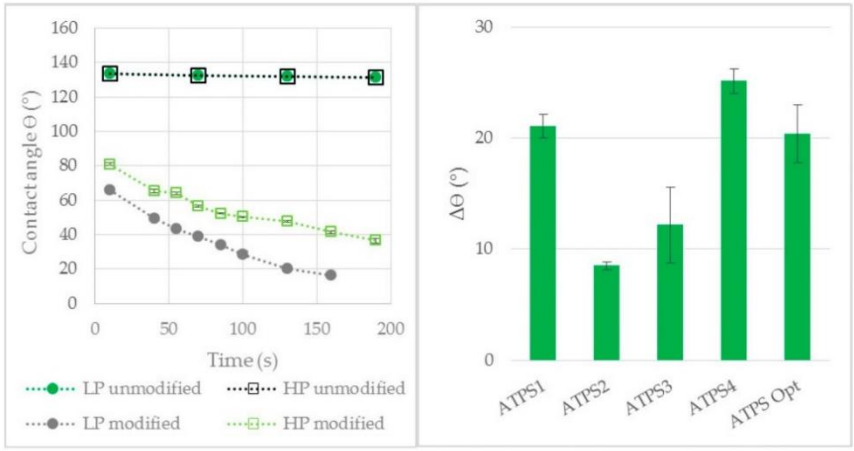

Figure 7. Contact angle of LP and HP on modified and unmodified PP membrane exemplified for ATPS4 as a function of time (**left**) and the contact angle differences (**right**) of ATPS1-4 and ATPS Opt (introduced in Section 3.3). LP: light phase. HP: heavy phase. PP: polypropylene.

3.2.2. Aqueous Phase Separation Experiments

In order to elucidate the applicability of the contact angle measurements for a selective membrane based phase separation the different systems were processed with the miniaturized membrane extractor.

To ensure maximum permeate purity in the experiments, a TMP of only 10 mbar was used for all ATPS with a total volume (V) of 150 mL.

The flow rate as well as the purity of the permeate after the membrane based phase separation were examined (Figure 8). The highest flow rate at the beginning of the phase separation was observed for ATPS1. The value decreased during the separation, for ATPS4 right at the beginning and for ATPS1 stepwise a short time later. ATPS2 and ATPS3 remained at a nearly constant flow rate for approximately 110 min, after which a decrease was also observed. Only ATPS4 showed a 100% pure permeate throughout the whole process. ATPS1 and ATPS2 showed a pure permeate at the beginning of the separation but a decrease was observed after a few minutes. For ATPS3 only a 60% permeate purity was achieved.

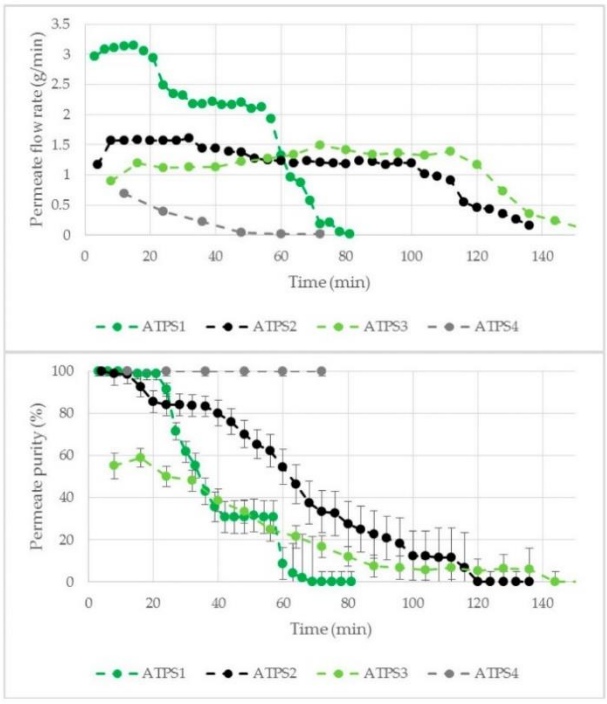

Figure 8. Flow rate (**above**) and purity (**below**) of the permeate by membrane based phase separation for ATPS1–4. Water was used as feed component. V = 150 mL, TMP = 10 mbar, ΔP = 30 mbar, Q = 16.7 mL/min.

For ATPS with a high LP content like ATPS1 (Table 1), it is more probable that the dispersed LP droplets interact with the modified membrane and flow in the permeate channel, resulting in a high permeate flow at the beginning of the process. As the LP was withdrawn as product phase, the amount of LP, which could interact with the membrane, decreased. This could be an explanation for the reduced permeate flow of all investigated ATPS during the experiment. A reduced amount of LP in the ATPS could also be a reason for the decreased permeate purity during the phase separation process. Although the LP showed higher affinity towards the modified membrane, the HP was also able to wet the membrane (Figure 7). With increasing HP proportion, the probability of an interaction of HP with the membrane and its permeation increases.

To further examine these observations, aqueous phase separation was performed with ATPS1 until a HP breakthrough was observed. At this time point LP was added to the recirculation tank. Then the

purity as well as the flow rate of the permeate almost increased to the initial values (Figure 9), which is in accordance with the previous findings and confirms the hypothesis of volume dependent permeation.

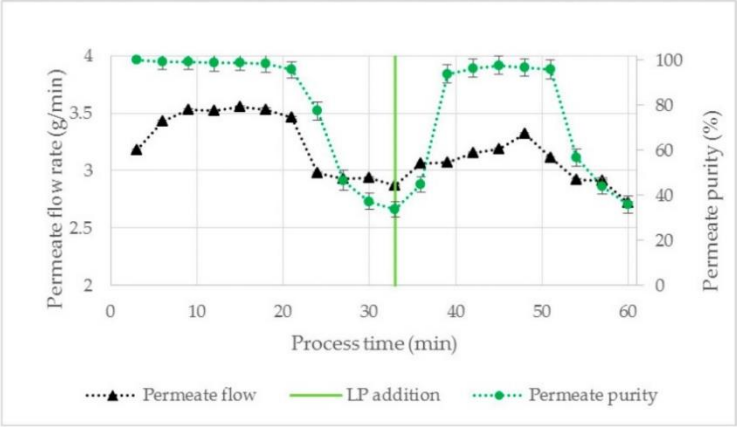

Figure 9. Effect of LP addition (50 mL) on permeate purity and flow rate. ATPS1, V = 150 mL, TMP = 10 mbar, ΔP = 30 mbar, Q = 16.7 mL/min. LP: light phase.

The yield of LP until breakthrough of the HP was higher for ATPS with a high contact angle difference between LP and HP on the modified membrane (ATPS1 and ATPS4) compared to ATPS with lower contact angle differences (ATPS2 and ATPS3, Figure 10). Based on these results the contact angle difference may give an indication, if the LP of an ATPS can be separated by the membrane based approach. This theory was confirmed by the high Δθ value for the optimized ATPS (introduced in Section 3.3) resulting in a high yield of LP for this ATPS (Figure 10).

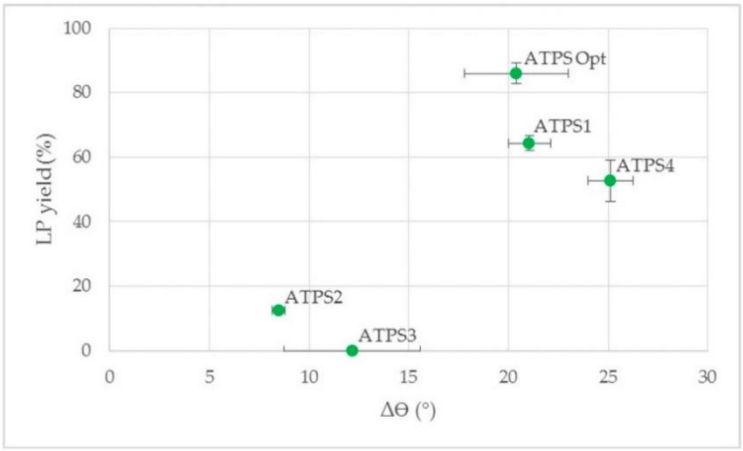

Figure 10. Yield of LP in the permeate until HP breakthrough with respective contact angle differences Δθ for ATPS1–4 as well as ATPS Opt (introduced in Section 3.3). LP: light phase. HP: heavy phase.

3.2.3. Feasibility Study

To evaluate the transferability of the preceding phase separation experiments with water to real mixtures, cell containing cultivation broth with IgG as product, and process related impurities like

DNA and HCP, were used as the ATPS feed component for the subsequent experiments. In order to select a suitable ATPS for this feasibility study and to be able to evaluate the IgG yield and purity after the process, the equilibrium values for the ATPE from all four ATPS models were determined first (Table 2).

Table 2. IgG yield and removal of process related impurities of the investigated model ATPS in the LP. Cell containing cultivation broth was used as the feed component. IgG: immunoglobulin type G. ATPS: aqueous two-phase system. LP: light phase.

ATPS	IgG Yield (%)	DNA Removal (%)	HCP Removal (%)
1	93 ± 1	17 ± 3	−4 ± 1
2	87 ± 3	84 ± 1	21 ± 1
3	32 ± 4	97 ± 1	23 ± 2
4	33 ± 3	66 ± 1	−64 ± 4

High IgG yields were obtained for ATPS1 and ATPS2 in the LP with 93% and 87% respectively, while ATPS3 and ATPS4 showed a lower value of approximately 33%. This is likely due to IgG precipitation in the interphase because no IgG was present in the HP (data not shown). The high molecular PEG for ATPS3 as well as the low phase ratio for ATPS4 are possible reasons (Table 1) [44]. The greatest removal of DNA and HCP was observed for ATPS2 and ATPS3 (Table 2). The negative HCP removal as well as the relatively low reduction of DNA load resulting from ATPS1 and ATPS4 can be explained by cell disruption during extraction. When cell free culture filtrate was used as feed component, a higher DNA (ATPS1: 71 ± 1%, ATPS4: 81 ± 1%) and HCP (ATPS1: 26 ± 3%, ATPS4: 44 ± 2%) removal was observed for both ATPS, while the IgG yield was similar to that obtained with cultivation broth.

Due to the high yield of IgG in the equilibrium experiments together with the high contact angle differences and LP yield from the aqueous phase separation experiments, focus was placed on ATPS1 for the moment as a model for further studies. In the next step, membrane based phase separation was executed with cell containing cultivation broth as the feed component.

Using the miniaturized membrane extractor only, a low permeate flow was obtained, likely due to membrane fouling effects [45]. Therefore, the membrane separator with increased membrane area (200 cm^2) was used for the experiments containing cells.

Preliminary studies with the membrane separator showed a HP breakthrough at 120 mbar TMP. Based on these findings the TMP was adjusted to 100 mbar to ensure a robust process with high permeate purity and flow rate. Until 100 min process time, the LP was separated with a purity of 100%. A breakthrough of the HP was observed after 65 ± 3% LP was separated, resulting in a decreased permeate purity (Figure 11). This is similar to the aqueous phase separation experiments performed with the miniaturized membrane extractor (Figure 10). To increase the yield a flush with 30 mL LP (water as feed component) was executed, indicated by the increase of total LP volume in Figure 11. Due to the higher volume of LP in the feed stream, the permeate purity increased to 100% (Figure 11), which is in agreement with the preliminary aqueous phase separation experiments (Figure 9). At the second HP breakthrough after flushing at 410 min process time, 83 ± 3% of the available LP was collected in the permeate with an overall purity of 95 ± 3%. The mean flux over the whole process was 0.51 $L/m^2/h$.

In the permeate, 81 ± 3% IgG was recovered related to the used amount in the cultivation broth. The IgG loss during phase separation was due to the incomplete LP recovery. The DNA (17 ± 3%) as well as HCP (1 ± 3%) removal was in the same range as the equilibrium data for ATPS1 (Table 2). Due to the small pore diameter of the used membrane, the permeate flow was cell free, as determined by microscopic examination.

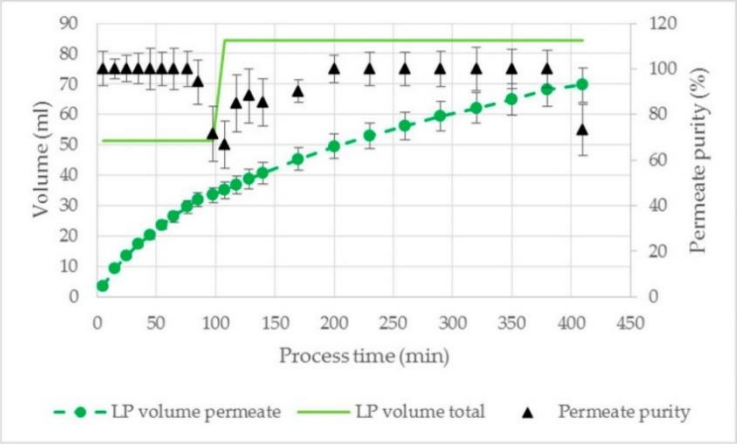

Figure 11. Volume of LP and permeate purity for phase separation of ATPS1 with cultivation broth as feed component. V = 100 mL, TMP = 100 mbar, dP = 250 mbar, Q = 16.7 mL/min. LP: light phase.

3.3. ATPS Optimization by DoE

Despite the high yield of IgG and the feasibility of a membrane based phase separation for ATPS1 only a poor removal of process related impurities was present (Table 2), wherefore a DoE approach was used for system optimization using cell containing cultivation broth as feed to further examine the purification capability of the ATPE. The screened parameters with the respective ranges are shown in Table 3. As responses, the IgG yield as well as the reduction of DNA and HCP load in the LP were chosen.

Table 3. Factor and value range used for the DoE. DoE: design of experiments.

Factor	Value Range
Feed (w%)	20–40
PEG 400 (w%)	8–20
Phosphate salt (w%)	16–24
pH-value	6–8
NaCl (w%)	0–10

Based on the DoE results a model for IgG yield as well as DNA and HCP removal was established. The correlation of the experimental data with the model data showed a high coefficient of determination value (0.86 for the IgG yield model, 0.76 for the DNA removal model and 0.95 for the HCP removal model) suggesting a good model for all three responses.

The simplex or Nelder–Mead method [39] was used to maximize the IgG yield and the DNA as well as HCP load reduction simultaneously. According to the model the most promising ATPS composition was 36 w% feed, 19 w% PEG 400, 16.4 w% phosphate salt (pH = 8.0) and 4 w% NaCl as displacement agent. To confirm the optimized ATPS composition an ATPE with cultivation broth as feed component was executed, and IgG yield as well as DNA and HCP removal were determined. The predicted as well as the experimental results for the optimized system (ATPS Opt) are shown in (Table 4).

The IgG yield as well as the DNA removal were in good agreement with the model prediction, whereas the removal of HCP contaminants was significantly higher than predicted, suggesting that some more investigation of the model is required. This is, however, out of the scope of the work

presented here. ATPS Opt had a similar IgG yield and significantly higher removal of process related impurities compared to ATPS1.

Table 4. IgG yield and removal of process related impurities of the optimized ATPS (ATPS Opt) in LP. IgG: immunoglobulin type G. ATPS: aqueous two-phase system. LP: light phase.

	IgG Yield (%)	DNA Removal (%)	HCP Removal (%)
Model prediction	100	86	15
Experiment	92 ± 3	85 ± 2	52 ± 5

Furthermore, the aqueous phase separation as well as the contact angle difference of the optimized ATPS was examined. ATPS Opt exhibited a high LP yield of 86 ± 3% (no breakthrough of the HP was observed) and also a high $\Delta\theta$ value of 20.4° (Figure 7). Therefore ATPS Opt was used for further phase separation experiments with cell containing cultivation broth.

3.4. Application Study

In order to process a larger volume of cultivation broth for an application study, a fourfold increased membrane area was examined. ATPS Opt with a volume of 100 mL as well as 400 mL were processed using cultivation broth as feed with one (200 cm^2) and four parallel-connected membrane separators (800 cm^2) respectively.

In Figure 12, a similar course of the permeate flux, which consists of pure LP, is shown over the process time. The phase separation with 800 cm^2 membrane area had an overall flux of 0.48 L/m^2/h, which is similar to the flux achieved with 200 cm^2 (0.45 L/m^2/h), suggesting a feasibility of the parallel connected membrane separators.

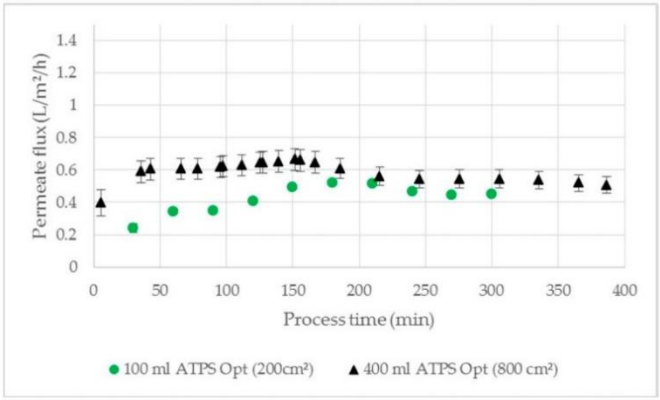

Figure 12. Permeate flux comparison between 200 cm^2 (V = 100 mL) and 800 cm^2 (V = 400 mL) membrane area for membrane based phase separation of ATPS Opt with cultivation broth as feed. TMP = 100 mbar, dP = 250 mbar, Q = 16.7 mL/min.

In Figure 13, the permeate volume and purity as well as the total volume of LP is shown for the process with 800 cm^2 membrane area. During the whole experiment a breakthrough of the HP did not occur resulting in a permeate phase purity of 100%. Similar to the experiments with ATPS1 (Section 3.2.3), a flush with 30% LP (water as feed component, 120 mL) was executed to increase IgG recovery. At the end of the process, 83 ± 3% of the LP was separated resulting in a total IgG recovery of 78 ± 3%. The permeate consisted of 100% LP and was completely cell free.

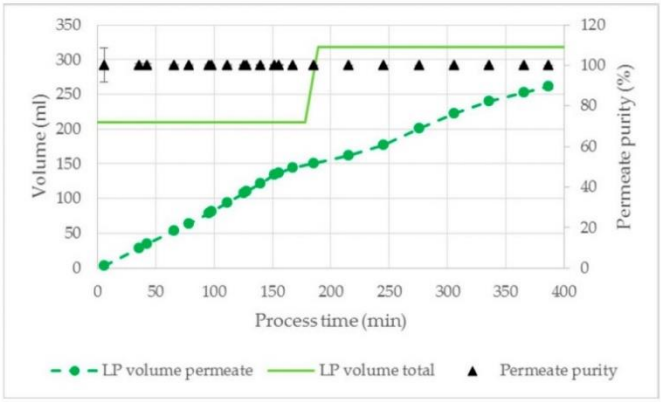

Figure 13. Volume of LP and permeate purity for the application study of the optimized ATPS with cultivation broth as the feed component. V = 400 mL, TMP = 100 mbar, dP = 250 mbar, Q = 16.7 mL/min. LP: light phase.

DNA was removed by 92 ± 3% and HCP by 43 ± 7%. These results were similar to the equilibrium experiments (Table 4), suggesting no interference of the membrane based phase separation with the purity and yield achieved by the ATPE itself. In this application study, ATPS Opt was shown to be the most suitable ATPS for the purification of the investigated IgG from the used cultivation broth. A high IgG recovery together with a high DNA as well as HCP load reduction was achieved not only in equilibrium but also under process conditions.

4. Conclusions

In this study a new method for the separation of aqueous two-phase systems comprising integration of a clarification step with a first capture and purification of mAb has been developed. Phase separation was accomplished by the use of a modified hydrophobic membrane with surfactants. Polypropylene as membrane material in combination with Tween20 showed the best phase selectivity in the surfactant and membrane screening.

Different ATPS models, with different phase building components and compositions, showed a different ability to be separated by the new membrane based method. The ATPS with a high contact angle difference between both phases on the modified membrane also had a high yield of pure LP in the aqueous phase separation experiments. These results suggest that the contact angle measurement is a suitable method for an estimation if a given ATPS can be separated by the membrane.

ATPS1 was used for a first phase separation experiment with cultivation broth as the feed component to purify the IgG. Clarification of the permeate was realized by the narrow pore diameter of the hydrophobic membrane. Most of the total LP (83%) was separated by the use of a newly designed membrane separator. An IgG recovery of 81% was achieved in combination with only a minor removal of process related impurities.

To improve yield and purity of the IgG, a DoE approach was used. An IgG recovery of 78% with simultaneous high removal of DNA (92%) and HCP (43%) was achieved by membrane based phase separation. Taking into account the amount of separated LP, IgG yield and purity were similar to the DoE equilibrium experiments. These results suggest no interference of the membrane based phase separation with the ATPE itself.

In all experiments carried out with cell containing cultivation broth, a complete clarification of the LP in the permeate was achieved in addition to IgG capture and purification by the ATPS. The mean permeate flux for 200 cm^2 and 800 cm^2 membrane area showed a comparable flux course

with approximately 0.5 L/m^2/h. Scaling up may be easily achieved by an increased volume of the ATPS and increased membrane area for phase separation.

This study shows the feasibility of ATPE for mAb purification combined with a membrane based phase separation, offering great potential for process intensification. Future work has to be done in the field of model development and validation for this unit operation to examine the optimum operating space as well as the transferability to different mAbs expressed by different cell lines.

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3.2 An Alternative Downstream Process Based on Aqueous Two-phase Extraction for the Purification of Monoclonal Antibodies

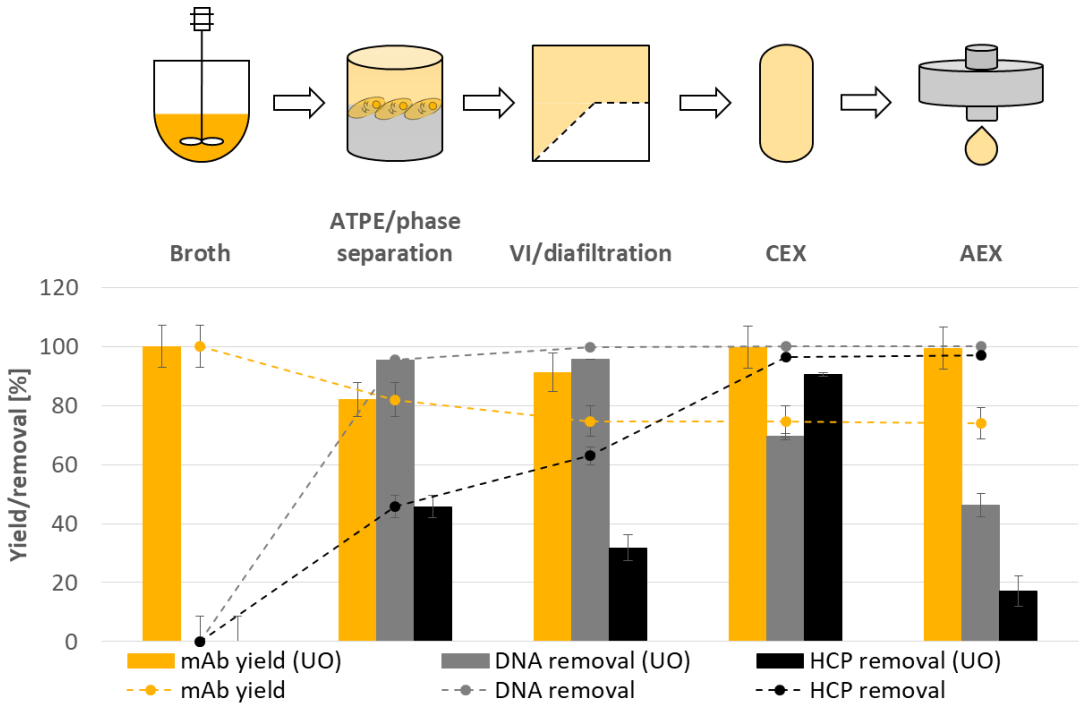

Figure 5. *The alternative downstream for mAb purification based on ATPE. Schematic representation of the conducted unit operations (top). Yield of mAb as well as the removal of DNA and HCP for each unit operation (UO) and throughout the process (bottom).*

The applicability of ATPE as clarification, mAb capture and first purification step was demonstrated in the first publication. However, in order to use ATPE as unit operation for mAb production an integration into the subsequent DSP must be accomplished. To ensure sufficient product quality, N-linked glycosylation patterns as CQA were investigated before and after ATPE as well as throughout the entire DSP. The ratios between the glycan basic structure and glycan structures with one or two additional galactose molecules remained unimpaired. Furthermore, the total amount of fucosylation was determined to be equal, which demonstrates ATPE as suitable mAb purification method with regard to the main CQA.

A combined diafiltration/ virus inactivation protocol was established, which allows an efficient removal of the phase forming ATPS components and acidification of the solution. Membranes with three different MWCO were screened with regards to the achieved yield of mAb as well as removal of DNA and HCP. Thereby the 30 kDa MWCO Hydrosart membrane offered the best trade-off between yield and purity.

For the subsequent purification a CEX column, in bind and elute mode, as well as an AEX membrane adsorber, in flow through mode, were used based on a platform approach for mAb purification. The CEX eluate was directly loaded on the AEX without any additional adjustment, to allow an easy process design. Accordingly, both unit operations must be considered together, in order to determine the best buffer conditions regarding mAb yield and purity. A pH value of 7.5 and a conductivity of 12.5 mS/cm resulted in 99 % yield of mAb with a simultaneous removal of 96 % DNA as well as 91 % HCP and exhibited a high reproducibility.

After the holistic development and optimization of the ATPE-based DSP, an application study was conducted to demonstrate the feasibility of the complete process. Clarification and mAb capture were realized by the ATPS itself, resulting in a high yield of mAb which was maintained until the final polishing step to 74 %. In addition, a continuous increase in purity was obtained throughout the DSP (**Figure 5**). In total, 99.97 % of the DNA and 97.11 % of the HCP were removed. This corresponds to a residual amount of 61 ppm DNA and 6036 ppm HCP.

To conclude, a new innovative DSP was developed, integrating ATPE in a commonly used platform process for mAb purification directly from fed-batch CHO cultivation. Thereby, protein A chromatography as the most expensive unit operation was omitted. The alternative DSP described in this study might contribute to overcome the bottleneck within mAb purification.

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Regular article

An alternative downstream process based on aqueous two-phase extraction for the purification of monoclonal antibodies

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HIGHLIGHTS

- New alternative downstream process based on ATPS without protein A chromatography.
- A holistic development as well as optimization of the individual unit operations is demonstrated.
- 74 % yield of mAb with simultaneous removal of DNA (99.97 %) and HCP (97.11 %).
- mAb glycosylation pattern remained unimpaired after ATPE and throughout the alternative DSP (stable product quality).

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ABSTRACT

An alternative downstream process for the purification of a monoclonal antibody (mAb) based on aqueous two-phase extraction as clarification, capture and primary purification step was developed. For further purification unit operations which are commonly used for mAb platform processes were utilized. A diafiltration approach was used to combine virus inactivation and removal of phase forming components as well as low molecular weight impurities in one step followed by cation and anion exchange chromatography. Starting from cell containing cultivation broth, an overall mAb yield of 74 % was achieved within an application study. The process was optimized regarding mAb yield and the clearance of process related impurities like deoxyribonucleic acid as well as host cell proteins, which were removed to approximately 60 and 6000 ppm respectively. Critical product quality attributes, regarding glycosylation patterns, were also examined and remained unimpaired after the aqueous two-phase extraction. The alternative downstream process presented in this study offers great potential to improve mAb manufacturing.

1. Introduction

Due to their importance for the treatment of several severe diseases like cancer, autoimmune disorders and inflammatory diseases [1–3] the market for monoclonal antibodies (mAb) rises [4]. Ongoing improvements in the upstream process of mAb producing cell lines, like chinese hamster ovary (CHO) cells, have resulted in increased cell densities and high product titers up to 25 g/l [5–7]. However, these achievements shifted the bottleneck in mAb production to the subsequent clarification, capture and purification unit operations in the downstream process (DSP) [8]. Economic drawbacks, mainly due to the high protein A resin costs of the capture step [9,10], and insufficient throughputs of established methods have generated an increased demand for purification methods that can significantly boost productivity [11]. As an alternative method, aqueous two-phase systems (ATPS) have been widely examined for the purification of mAb in numerous laboratory studies [12–14]. Based on these trials, aqueous two-phase extraction (ATPE) is a promising approach addressing the DSP bottleneck challenges.

In most conventional processes the cell removal is realized by centrifugation [11,15], depth filtration [16], flocculation [17] or dynamic body feed filtration [18]. ATPE as alternative method enables a direct clarification of the cultivation broth, since particles like cells and cell debris accumulate at the interphase by steric effects [19,20]. For further processing a separation of the mAb containing target phase must be ensured. Most methods make use of the different phase densities (mixer-settler devices [20], columns [21] or centrifugal extractors [22]) while also membrane based approaches, using physicochemical differences, are available [19].

After clarification, in conventional DSP protein A chromatography is used most commonly as capture step. However, a major drawback of this unit operation is the throughput limitation [16]. Thereby existing

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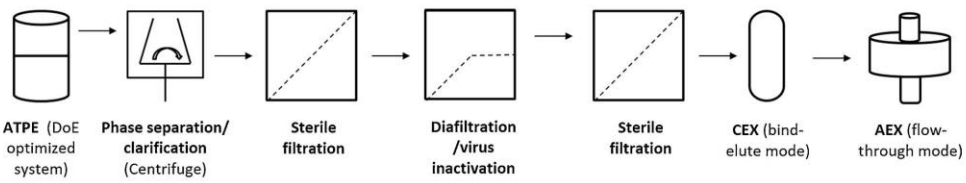

Fig. 1. Schematic representation of an alternative DSP for purification of mAb by integration of ATPE.

chromatography units reach their capacity limit and investigations into a sufficient and economic process by enhancements of the expensive protein A capture step, as current state of the art, as well as several other unit operations, like ATPE, are being undertaken [23,24]. For an efficient extraction of the target molecule the ATPS composition (type and concentration of phase forming components, pH-value as well as additional displacement agents) has to be optimized for each separation task individually [25–27]. For the mAb containing cultivation broth used in this study the ATPS composition was optimized by a design of experiments (DoE) approach previously [19]. Thereby the impurities are enriched in the heavy, salt-rich phase (HP) while the mAb can be extracted into the target phase (the light, polymer-rich phase (LP) in this study).

To ensure an consistent mAb quality, also for ATPE as alternative unit operation, N-linked glycosylation patterns are critical quality attributes (CQA) due to their important role in antibody effector interaction [28]. However, glycosylation analysis of the mAb remained often unconsidered after ATPE so far.

While a lot of studies show the applicability of ATPS for mAb extraction, further process integration has often been neglected and only minor research was done to establish a complete ATPE based DSP for mAb purification [29,30]. However, different approaches have been examined to link to subsequent unit operations. Back and multistage extraction are used to remove phase forming components and impurities [31–33]. Other approaches combine ATPE with precipitation [34] or chromatography [35]. Diafiltration is also often used for removal of phase forming components, mainly polyethylene glycol (PEG) [36]. The molecular weight cut off (MWCO) of the used membrane has to be a trade-off between a narrow pore size for high mAb yield and a large pore size for high purity, by removing low molecular weight impurities [37]. As the majority of the HCP have a low molecular weight [38], compared to mAb, the membrane selection offers great potential for an efficient purification process. Moreover, using a suitable diafiltration buffer, optimal conditions for the subsequent unit operation can be selected, which is usually a viral inactivation (VI) after the capture step.

Two dedicated orthogonal steps for viral reduction are required for safety of products which are produced by mammalian cell culture [16]. For mAb, which are most often stable under low pH conditions for a certain timeframe [16], a generic low pH condition for a sufficient inactivation of retroviruses can be selected [39]. To move the mAb into a more stable pH range the solution is neutralized after VI. Despite the requirement, due to the regulatory demands for biopharmaceutical products, a feasible approach for a direct virus inactivation of the LP after ATPE has not been reported yet.

To further remove HCP, DNA, high molecular weight (HMW) aggregates and low molecular weight (LMW) clipped species at least two subsequent chromatographic steps are typically employed in mAb DSP [16,40]. Robust operation of the entire process is assured by a sufficient level of redundancy between them [16]. Generally cation (CEX) and anion exchange chromatography (AEX) are incorporated, although hydrophobic interaction chromatography (HIC), mixed mode chromatography or hydroxyapatite chromatography are used likewise [41–46].

CEX is often used as first polishing step in bind and elute mode [16]. Dependent on the conductivity and the pH-value the mAb bind to the negatively charged ligand, while a certain amount of impurities break through during the loading phase [47]. Higher purity of the eluate can be achieved by the selection of a suitable elution buffer to ensure an efficient elution of the mAb from the ligands while bound impurities are retained.

Typically, one of the two chromatography steps is operated in the flow-through mode [16]. This offers potential for an increased productivity if the mAb is more abundant compared to the impurities in the feed solution [48]. If the pH of the feed is between the isoelectric point (IP) of the product and the impurities, DNA as well as the majority of HCP, with a lower IP than the mAb, are negatively charged while the mAb is positively charged. Because of that the mAb does not bind to the positively charged AEX ligands whereas impurities are retained [49]. Nowadays often membrane adsorber (MA) are used in flow-through mode in the biopharmaceutical industry, offering increased productivity and reduced capital investment through a single use approach [50–53]. In addition, AEX flow-through processes have been demonstrated to achieve high viral clearance capabilities [54].

In order to further use existing plant and equipment as well as optimized process conditions for the respective unit operations, in this study an integration of ATPE into a platform DSP was examined (Fig. 1) to ensure fast and simple process change. ATPE was used for clarification, capture and first purification of the mAb directly from cell containing cultivation broth. To integrate ATPE into the subsequent DSP diafiltration was investigated and optimized as unit operation for PEG removal and a direct integration of virus inactivation by pH shift. Further purification was obtained by CEX and AEX. The product purity, in terms of deoxyribonucleic acid (DNA) as well as host cell protein (HCP) removal and product quality was examined. Emphasis was placed to the N-linked glycosylation patterns of the mAb as CQA. The feasibility of the alternative DSP was demonstrated by an application study.

2. Materials and methods

2.1. Cultivation

A mAb (immunoglobulin type G, IgG, IP = 8.4) was produced in a fed-batch cultivation of CHO cells with a commercial serum-free medium. Cells were cultivated in an Ambr®250 single use bioreactor (Sartorius, Göttingen, Germany) for 12 days at 855 rpm, 36.8 °C and pH 7.1. At harvest time point the viability was ≥ 80 % with a viable cell density ≥ $10*10^6$ cells/ml and an IgG concentration ≥ 2.8 g/l.

2.2. Aqueous two-phase extraction

ATPS composition (Table 1) for a direct extraction from the cultivation broth was adopted from a previous study [19] based on a DoE approach for highest mAb yield and purity, regarding DNA and HCP, in the LP. PEG with a molecular weight of 400 g/mol was purchased from Merck (Darmstadt, Germany). Sodium phosphate monobasic anhydrous (NaH_2PO_4) and potassium phosphate dibasic anhydrous (K_2HPO_4) were used for the preparation of the respective stock solutions (40 w% phosphate buffer). The pH-value was adjusted by titration of both stock solutions. All salts including NaCl as displacement agent, were

Table 1
ATPS Composition [19].

Component	w.-%
PEG400	19
40 w.-% PO_4 buffer (pH 8.0)	41
Feed	36
NaCl	4

purchased from Carl Roth (Karlsruhe, Germany).

The appropriate amount of the ATPS components were weighed in a Schott flask. As feed cell containing cultivation broth or reverse osmosis water was used as indicated. To ensure equilibrium conditions the ATPS was stirred by a magnetic stirrer for 10 min at 500 rpm. For a quick and easy phase separation, centrifugation was used. Therefore, the ATPS was transferred into 500 ml Corning® centrifuge tubes (Sigma-Aldrich, St. Louis, USA) and centrifuged for 5 min at 538 x g. The LP as mAb containing phase was withdrawn by pipetting. To ensure a complete cell removal, when cell containing cultivation broth was used as feed component, a sterile filter with a nominal pore size of 0.2 µm (Sartopore 2® XLG, Sartorius, Göttingen) was used with a constant pressure of 1 bar for LP filtration.

2.3. Virus inactivation

As a titration approach for acidification based virus inactivation the LP was titrated with 1 M HCl to a pH of 3.4, incubated for 30 min and afterwards neutralized to pH 5.5 by the addition of 1 M NaOH. Titration was performed under constant stirring and all chemicals were purchased from Carl Roth (Karlsruhe, Germany).

In the diafiltration approach, virus inactivation was implemented by the low pH-value of the diafiltration buffer (DF-buffer; 50 mM sodium acetate ($C_2H_3NaO_2$), pH 3.4) which was kept constant at pH 3.4 for 30 min after the diafiltration process (see section 3.4; 30 kDa MWCO Hydrosart®) in the recirculation tank. The samples for the VI optimization experiments were taken afterwards (Fig. 3). Process parameters for the diafiltration process are given in section 2.4.

For neutralization the diafiltrate was adjusted to pH 5.5 and a conductivity of 5 mS/cm (based on an in-house standard protocol) by titration with NaOH and NaCl prior to sterile filtration (Sartopore 2® XLG, Sartorius, Göttingen) before loading on the CEX. This adjustment was assigned to the VI/diafiltration as neutralization step in the application study. All used chemicals were purchased from Carl Roth (Karlsruhe, Germany).

2.4. Diafiltration

Diafiltration was performed with a benchtop crossflow system for process optimization (Sartoflow® Smart, Sartorius, Göttingen, Germany). Two ultrafiltration cassettes (Sartocon® Slice 200, Sartorius, Göttingen, Germany) with a total membrane area of 400 cm^2 and different nominal MWCO of 5 kDa (Hydrosart®), 30 kDa (Hydrosart®), 50 kDa (Polyethersulfone, PESU) and 100 kDa (Hydrosart®) were used.

To analyze the PEG400 removal during the diafiltration process the LP of an ATPS with water as feed component was diafiltrated with KPi as DF-buffer (10 mM, pH 7.0, K_2HPO_4 and KH_2PO_4 were purchased from Karl Roth, Karlsruhe, Germany). Because PEG400 was the only carbon containing component in the used ATPS, total organic carbon (TOC) values were used to determine the PEG400 concentration. Samples were taken from the retentate and analyzed for the TOC content.

In order to improve the PEG removal and to avoid mAb precipitation, when cell containing cultivation broth was used as feed component, the LP was diluted five times with DF-buffer (50 mM sodium acetate ($C_2H_3NaO_2$), pH 3.4) before the solution was transferred to the recirculation tank of the crossflow system. The ultrafiltration cassettes were used according to the manufacturer`s instructions with an inlet pressure of 2 bar and a transmembrane pressure of 1 bar. The PEG400 containing permeate was withdrawn without addition of DF-buffer to the recirculation tank to concentrate the diluted solution down to the initial volume. For further PEG400 removal and buffer exchange, diafiltration was performed with 8 DF-volumes into the DF-buffer at constant retentate volume. Permeate flow as well as conductivity were recorded by the crossflow system.

2.5. Cation exchange chromatography

CEX experiments were performed on an ÄKTA® prime chromatography system (GE Healthcare, Buckinghamshire, UK). A 5 ml HiTrap® Capto S ImpAct (GE Healthcare, Buckinghamshire, UK) column was used for the CEX in bind and elute mode. Buffer chemicals for equilibration/wash (50 mM $C_2H_3NaO_2$, pH 5.5), elution (20 mM Tris − HCl), regeneration (1 M NaCl) and cleaning in place (CIP; 1 M NaOH) were purchased from Carl Roth (Karlsruhe, Germany). Before loading, the diafiltrate (CEX feed) was adjusted according to section 2.3. The conductivity (7.5, 12.5 and 17.5 mS/cm) and pH-value (7, 7.5, and 8) of the elution buffer was adjusted with NaCl and HCl respectively, as indicated. The experimental order of the resulting nine different elution buffers was randomized (N1 to N9) to reduce the chance of time dependent effects which distort the results (Table 2).

For all steps a flow rate of one column volume (CV)/minute was used, according to manufacturer's recommendation. The binding capacity of the used CEX column was determined before by breakthrough experiments, to avoid overloading of the column (first mAb breakthrough after approximately 200 mg mAb under these conditions). Once the column was equilibrated (25 ml), 50 ml (approximately 80 mg mAb) of the CEX feed was loaded for the optimization experiments and 95 ml (approximately 165 mg mAb) for the application study to increase the productivity. A wash step with equilibration buffer (50 ml) was done prior to elution (45 ml). The eluate was fractionated and analyzed. Afterwards regeneration (20 ml) and CIP (20 ml) of the column was performed with additional wash steps (20 ml) in-between, with the respective solutions.

2.6. Anion exchange chromatography

AEX experiments were performed on an ÄKTA® prime chromatography system (GE Healthcare, Buckinghamshire, UK) with a MA in flow-through mode. For the optimization experiments Sartobind® Q membrane (Sartorius, Göttingen, Germany) was cut to a diameter of 30 mm. Three of these cuts (total membrane volume of 0.42 ml, total bed height 0.8 mm) were layered in an in-house screening lab device with a flow distributor. In the application study a Sartobind® Q nano MA (Sartorius, Göttingen, Germany) with 3 ml membrane volume was used.

For all steps a flow rate of five membrane volume (MV)/minute was used. The MA was equilibrated (10 ml) with the respective CEX elution buffer. Afterwards 10 ml of the CEX eluate were loaded on the lab device for the optimization experiments. For the application study, which was executed with another cultivation, the ratio between the loaded mAb mass and the MV was kept equal to the optimization experiments

Table 2
CEX elution buffer screening. Conductivity and pH were adjusted as indicated.

Conductivity [mS/cm]	pH [-]		
	7	7.5	8
7.5	N9	N3	N2
12.5	N6	N1	N8
17.5	N7	N4	N5

(corresponding to 30 ml load of the CEX eluate), with a similar mAb and impurity ratio of the CEX eluate. Afterwards 20 MV of the respective CEX elution buffer were used to flush the lab device as well as the MA. The AEX flow-through fractions of the loading and the flush were pooled according to the UV signal and analyzed afterwards. At the end of each run the AEX membrane was regenerated and cleaned according to the procedure described for the CEX column (section 2.5) using 20 MV for each step with a flow rate of 5 MV/min.

2.7. Analytical procedure

Turbidity was measured by a nephelometer (TL2350, Hach-Lange, Düsseldorf, Germany) using the standard solutions provided by the manufacturer for calibration.

For the determination of mAb concentration and to evaluate the amount of low and high molecular weight species within the ATPS size exclusion chromatography (SEC) was used as high performance liquid chromatography (HPLC) with a Dionex UltiMate 3000 HPLC System (ThermoFisher Scientific, Waltham, USA) and a Yarra 3 µm SEC 3000 column (Phenomenex, Torrance, USA) with a flow rate of 1 ml/min. As SEC buffer 0.1 M Na_2SO_4, 0.05 M NaH_2PO_4 and 0.05 M Na_2HPO_4 (all chemicals purchased from Carl Roth, Karlsruhe, Germany) with a final pH of 6.6 was used. The SEC method was verified with analytical protein A HPLC previously to ensure appropriate mAb quantification (data not shown). Prior to analysis samples were diluted with SEC buffer if necessary and filtered through a Minisart RC4 0.2 µm syringe filter (Sartorius, Germany, Göttingen). Concentration was determined based on the peak area (280 nm) of a concentration calibration curve obtained using a known reference IgG material. Yield of mAb ($yield_{mAb}$) was calculated according to Eq. (1), where $m_{mAb,end}$ is the mass of mAb at the end and $m_{mAb,start}$ the mass of mAb at the beginning of the unit operation.

$$yield_{mAb} = \frac{m_{mAb,\,end}}{m_{mAb,\,start}} * 100\,\% \quad (1)$$

Concentration of DNA was measured by the Quant-iT™ PicoGreen™ dsDNA Assay Kit (ThermoFisher Scientific, Waltham, USA) with salmon sperm dsDNA as standard (Biomol, Hamburg, Germany). Samples were diluted in TE buffer (10 mM TRIS, 1 mM EDTA, 0.1 % SDS; all chemicals purchased from Karl Roth, Karlsruhe, Germany) if necessary. Measurement was performed with a FLX 800 plate reader (Bio-TEK instruments, Bad Friedrichshall, Germany) with a limit of detection of 31.25 ng/ml.

For the determination of the HCP concentration a HCP-ELISA (Cygnus Technologies, Southport, USA) was used according to the manufacturer`s instructions. Samples were diluted in ELISA buffer (20 mM TRIS, 50 mM NaCl; all chemicals purchased from Karl Roth, Karlsruhe, Germany) if necessary. Measurement was performed with a µQuant plate reader (Bio-TEK instruments, Bad Friedrichshall, Germany) with a limit of detection of 1 ng/ml.

Removal of DNA and HCP ($removal_{imp}$) were calculated according to Eq. (2), where $m_{imp,end}$ is the mass of the respective impurity at the end and $m_{imp,start}$ the respective mass of impurity at the beginning of the unit operation.

$$removal_{imp} = \left(1 - \left(\frac{m_{imp,\,end}}{m_{imp,\,start}}\right)\right) * 100\,\% \quad (2)$$

For analysis of mAb N-linked glycosylation patterns gel electrophoresis via laser induced fluorescence (LabChip® GXII Touch, PerkinElmer, Waltham, USA) was used with a ProfilerPro® Glycan Profiling assay (PerkinElmer, Waltham, USA) according to the manufacturer`s instructions. Glycosylation ratios and the degree of fucosylation of the mAb were evaluated as quality attributes.

To determine the TOC content a TOC analyzer (TOC-V CSH, Shimadzu, Kyoto, Japan) was used. The calibration range was automatically set by the instrument (500 µg/l to 30 000 mg/l). Dilutions were carried out if the concentration was out of the calibration range (≥ 20 %).

The kinematic viscosity was determined with an Ubbelohde type viscometer (SI Analytics, Mainz, Germany) according to the manufacturer's instructions.

Table 3
Analytic results of the cell broth and LP after ATPE, phase separation and sterile filtration. The yield of mAb was referred to the preceding unit operation. DNA and HCP removal were only analyzed after sterile filtration. ATPE values based on the measured total volume of LP and mAb concentration in the LP. Error values represent the standard deviations from the means of experimental triplicates.

	Broth	ATPE	Phase separation	Sterile filtration
Turbidity [NTU]	2370 ± 16	N/A	12.5 ± 2.3	8.5 ± 1.6
mAb concentration [mg/ml]	3.1 ± 0.2	2.4 ± 0.1	2.4 ± 0.1	2.4 ± 0.1
mAb yield [%]	–	97 ± 4	80 ± 5	100 ± 5
DNA removal [%]	–	–	–	97 ± 3
HCP removal [%]	–	–	–	42 ± 8

3. Results and discussion

3.1. ATPE

ATPE was performed as clarification and capture step with cell containing cultivation broth as feed direct from the fermenter. After centrifugation for phase separation of the ATPS the cells formed an interphase resulting in a strongly decreased turbidity of the LP (12.5 NTU) and HP (22.3 NTU) compared to the broth (2370 NTU) (Table 3). The LP was filtered through a sterile filtration capsule to ensure complete cell removal (final flux of approximately 180 l/m²/h) resulting in a turbidity of 8.5 NTU (Table 3). The filtrate served as feed stock for subsequent unit operation optimization experiments.

The mAb was extracted into the LP of the ATPS resulting in a defined peak with a retention time of approximately 7.7 min in the SEC chromatogram, while none was detected in the HP (Fig. 2). The mAb was slightly diluted into the LP of the ATPS with a yield of 97 %. 80 % of the mAb was recovered after phase separation where withdrawal of the LP offers potential for further optimization. During the sterile filtration no product loss occurred (Table 3). Specially designed mixer-settler devices could be examined as alternative phase separation method to further increase the yield of LP and mAb [20]. In addition, recently developed membrane-based approaches offer the potential to directly include the sterile filtration step during the phase separation [19]. However, focus of this study was the integration of the ATPE and the further DSP development for mAb purification.

97 % DNA and 42 % HCP of the broth were removed by ATPE (after

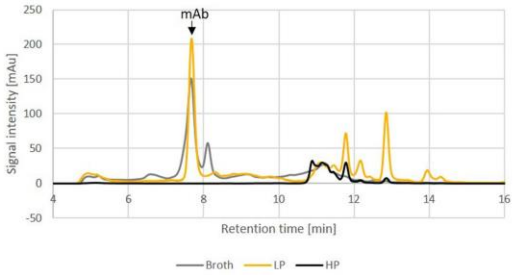

Fig. 2. SEC Chromatogram of the cultivation broth as well as LP and HP after ATPE. The mAb has a retention time of approximately 7.7 min.

sterile filtration of the LP, Table 3). This was confirmed by the SEC chromatogram, where a removal of HMW (retention time < 7 min) and LMW (retention time 8 – 10 min) impurities was observed compared to the cultivation broth (Fig. 2). Phase forming components of the LP and HP or media components for the cell broth probably represent the majority of the signals with a retention time > 10 min.

HMW and LMW impurities were also not detected in the HP (Fig. 2), probably due to precipitation likely caused by the polymer or salt components of the ATPS [55]. The advantage of using ATPE instead of precipitation is the direct combination with clarification in one single step, resulting in an intensified process. In addition, an extraction of low impurity amounts, not detectable in the SEC chromatogram, or small DNA fragments, with a higher retention time, into the HP also contribute to the purification by ATPE.

3.2. Virus inactivation and diafiltration

3.2.1. pH shift

Virus inactivation was examined as subsequent unit operation of the alternative DSP. Acidification by pH shift below pH 4 has been shown as reliable method for virus inactivation [56] and was therefore investigated for the LP after ATPE. Preliminary titration experiments were performed for the acidification of the LP from ATPS with cell containing cultivation broth as feed component (Fig. 3).

Addition of HCl caused a strong increase in the turbidity (up to 240 NTU) likely due to precipitation of mAb, HCP or DNA. As a result, the yield of mAb after acidification was only 8 % (Table 4). An additional performed neutralization of the solution re-dissolved some of the mAb precipitates to some extent, resulting in a mAb yield of 16 %. Although process related impurities were removed to some extent by the titration approach (Table 4), the low mAb yield was insufficient.

Therefore diafiltration into an acidic buffer was examined to realize the pH shift (Fig. 3). Based on this approach a mAb yield of 76 % was achieved with simultaneous removal of DNA (71 %) and HCP (65 %; Table 4). This method offered the advantage to incorporate virus inactivation, buffer exchange and removal of phase forming components (section 3.2.2) as well as mAb purification in one step and was therefore used for the further process.

3.2.2. Buffer exchange and removal of phase forming components

For further processing the removal of the phase forming components, mainly PEG400, is crucial. To investigate the feasibility of a sufficient PEG400 removal, an ATPS with water as feed component was prepared and diafiltration of the LP was performed using membranes with a different MWCO (100 and 5 kDa; Fig. 4). To evaluate these experimental results the theoretical unrestricted permeation was calculated according to Eq. (3), where c_{PEG400} is the concentration of PEG400, DF is the DF-volume and c_0 is the PEG400 concentration at the beginning of the diafiltration [57].

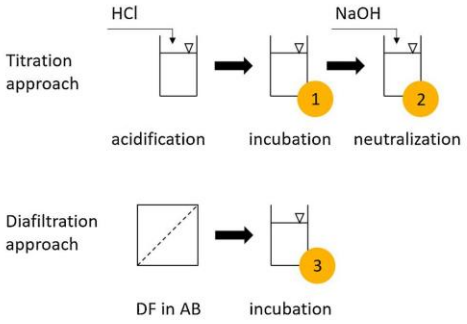

Fig. 3. Schematic representation of the performed VI approaches. The sampling points (1–3) are indicated. For diafiltration a 30 kDa MWCO membrane was used. AB = acidic buffer (50 mM sodium acetate, pH 3.4).

$$c_{PEG400} = \frac{1}{e^{DF}} * c_0 \quad (3)$$

The PEG400 concentration as a function of the DF-volume was nearly the same for the theoretical unrestricted permeation of the polymer and the membrane with a MWCO of 100 kDa, while the 5 kDa MWCO membrane displayed a slight retention (Fig. 4). However, for both membranes the polymer concentration was reduced by > 99 % after 8 DF-volumes (Fig. 4).

As a result of the PEG400 removal the permeate flow increased during the diafiltration process from approximately 5 g/min up to 98 g/min at the end of the process (Fig. 4). This is due to the high viscosity of the LP at the beginning of the process (4.17 mm^2/s) which decreased with proceeding removal of PEG400 (approximately 1 mm^2/s after 10 DF-volumes).

In addition to PEG400, phosphate salts and sodium chloride are the major components even for the LP, resulting in a high conductivity of 113 mS/cm. The salt removal for the 100 kDa MWCO membrane is shown by the decrease of the conductivity in the retentate during diafiltration (Fig. 4). After 6 DF-volumes a conductivity of approximately 8 mS/cm was obtained (Fig. 4). This value was only slightly higher compared to the value of the used DF-buffer (approximately 7 mS/cm), indicating that most of the salt was removed. To ensure an almost complete buffer exchange and removal of phase forming components, 8 DF-volumes were used for further experiments with LP derived from ATPE with cell containing cultivation broth as feed component.

3.2.3. Membrane selection

In order to retain the mAb in the retentate (high mAb yield) with simultaneous high permeation of impurities (high mAb purity) three membranes with different MWCO (30 kDa, 50 kDa and 100 kDa) were investigated for the diafiltration process.

The yield of mAb after diafiltration was remarkably decreased for the 100 kDa MWCO membrane (60 %) compared to the 50 kDa and 30 kDa MWCO membranes (77 % and 80 % respectively, Fig. 5). This was due to incomplete retention of the mAb for the 100 kDa membrane, confirmed by the occurrence of mAb in the permeate (data not shown). No mAb was detected in the permeate for the both membranes with a narrower pore size of 30 kDa and 50 kDa MWCO (mAb loss only due to the void volume of the crossflow system and potential adsorption on the membrane).

A HCP removal of 89 % was obtained with the 100 kDa membrane, while the 50 kDa (65 %) and 30 kDa (63 %) MWCO membranes exhibited a lower value (Fig. 5). Also the DNA removal was the highest for the 100 kDa MWCO membrane (79 %). Both impurity species exist in a broad range of molecular weights resulting in a higher removal for membranes with a high MWCO. Thereby the majority of the impurities, probably the LMW species, were removed. A larger amount of DNA was removed by the 30 kDa MWCO (72 %) compared to the 50 kDa MWCO (37 %) membrane, despite the smaller pore size. This is potentially due to the different membrane material. The 50 kDa MWCO membrane is composed of more hydrophobic PESU [58] which seems to retain a larger amount of the DNA in the retentate compared to the highly hydrophilic (according to manufacturer's specifications) stabilized cellulose Hydrosart® membranes (30 kDa and 100 kDa MWCO).

To ensure a high yield of mAb with simultaneous removal of DNA and HCP the 30 kDa MWCO membrane was used for the diafiltration of the LP in an acidic buffer for virus inactivation prior to the CEX experiments.

3.3. Chromatography optimization

3.3.1. CEX

After diafiltration with the 30 kDa MWCO membrane, the diafiltrate

Table 4
Yield of mAb as well as removal of DNA and HCP for different virus inactivation approaches. For diafiltration a 30 kDa MWCO membrane and 50 mM sodium acetate (pH 3.4) as acidic diafiltration buffer was used. The initial concentration of the LP was 2.14 mg/ml mAb, 3.5 µg/ml DNA and 94.0 µg/ml HCP. Error bars represent the standard deviations from the means of experimental triplicates.

Sample point	Sample	mAb yield [%]	DNA removal [%]	HCP removal [%]
1	Acidification	8 ± 1	60 ± 3	41 ± 10
2	Neutralization after acidification	16 ± 2	46 ± 5	2 ± 16
3	Diafiltration	76 ± 10	71 ± 5	65 ± 4

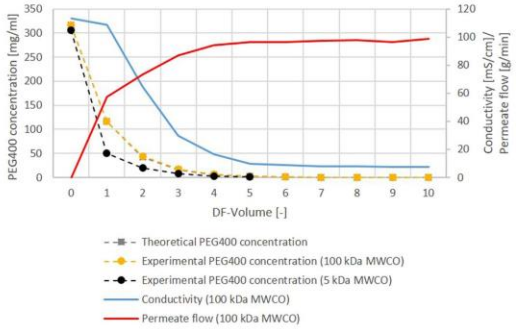

Fig. 4. PEG400 concentration, conductivity and permeate flow as a function of the DF-volume. The experimentally determined PEG400 concentration for a 5 kDa MWCO and a 100 kDa MWCO as well as theoretical values for an unrestricted permeation are shown. The course of the conductivity and permeate flow is shown for the 100 kDa membrane. For all experimental diafiltrations a 10 mM KPi buffer (pH 7.0) was used. Error bars represent the standard deviations from the means of experimental duplicates.

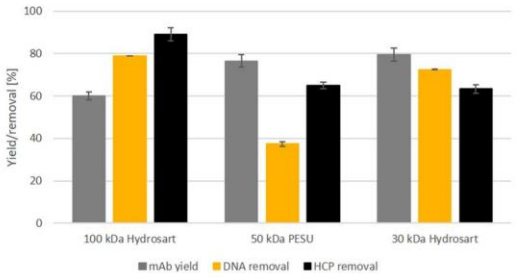

Fig. 5. Yield of mAb as well as removal of DNA and HCP by diafiltration for membranes with different MWCO. 50 mM sodium acetate (pH 3.4) was used as diafiltration buffer. Error bars represent the standard deviations from the means of experimental triplicates.

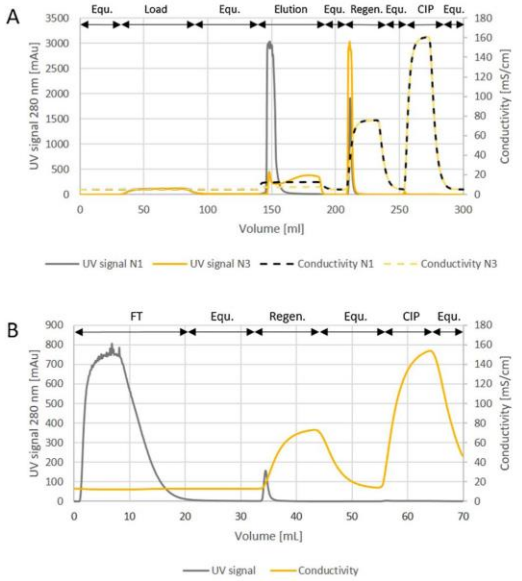

Fig. 6. Chromatograms of CEX and AEX. Chromatogram of the CEX (A) for CEX elution buffer N1 and N3.Chromatogram of the AEX (B) for CEX eluate N1. The UV signals as well as the conductivity are shown. Equ. = equilibration, Load = loading, Regen. = regeneration, CIP = cleaning in place, FT = flow-through.

was adjusted to the pH (5.5) and conductivity (5 mS/cm) of the CEX load conditions and sterile filtered. The influence of the pH-value and conductivity of the elution buffer was examined to obtain an optimal trade-off between a high mAb yield and purity (Table 2).

Two exemplary chromatograms (N1 and N3) are shown in Fig. 6A. While the elution performed with N1 exhibited a well-defined peak directly at the beginning of the elution, only a small peak with a large signal plateau afterwards was observed for N3. This is likely due to an insufficient elution of the mAb for N3 because of a lower conductivity (7.5 mS/cm) compared to N1 (12.5 mS/cm). Accordingly, in the elution fraction the yield of mAb was relatively low (24 %; Fig. 7A). The retained mAb was found in the regeneration fraction (Data not shown), which resulted in an increased UV signal compared to N1 (Fig. 6A).

The pH of the used elution conditions had only a minor effect on the yield, probably because the pH of all elution buffers was below the IP of the examined mAb (IP = 8.4), resulting in a positive charge of the mAb for each examined condition. Only for the lowest investigated conductivity of 7.5 mS/cm a higher pH-value (N2) resulted in a remarkably higher yield of mAb compared to a lower (N3) and moreover the lowest pH (N9; Table 2, Fig. 7A). The high pH-value of N2 results in the least positive surface charge and consequently better elution from the negatively charged ligand (Table 2).

The removal of DNA for N7 (pH 7) was greater than for N4 (pH 7.5), which had higher DNA removal than N5 (pH 8), at the same conductivity of 17.5 mS/cm. Furthermore at a conductivity of 12.5 mS/cm, N6 with pH 7 resulted in a higher removal of DNA compared to N8 with pH 8. For the lowest investigated conductivity of 7.5 mS/cm no significant effect of the pH on the removal of DNA and HCP was observed (N2, N3 and N9, Fig. 7A).

The net charge of the impurities is influenced by the pH due to protonation-deprotonation of functional groups. Due to lower pH-values during the CEX elution more impurities remain bound to the negatively charged CEX ligands. Thereby lower pH-values of the elution buffer usually lead to a higher mAb purity by CEX, even if there is a considerable variability within the physicochemical characteristics of the impurity spectrum [47,59].

Elution conditions with lower conductivity (N9, N3, N2) exhibited a higher removal of DNA and HCP compared to those with higher

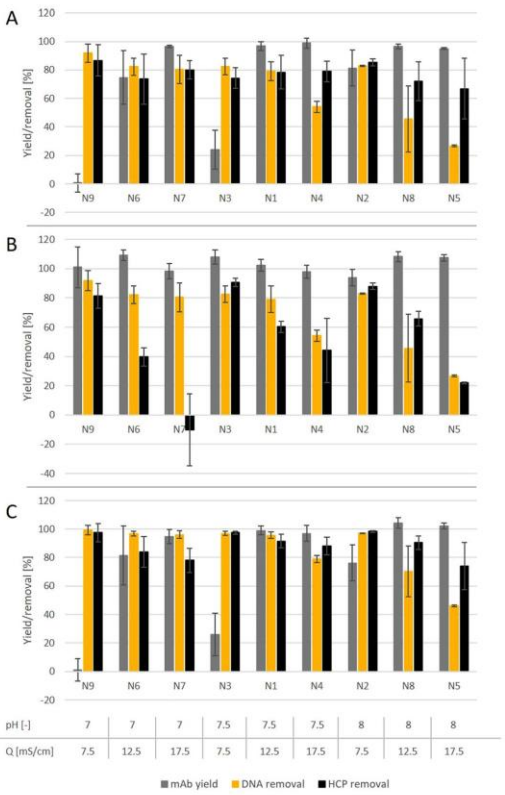

Fig. 7. Yield of mAb as well as removal of DNA and HCP by CEX (A), AEX (B) and combined after both steps (C) for different CEX elution buffer. Q = conductivity. Error bars represent the standard deviations from the means of experimental triplicates.

conductivity at the same pH-value (N7, N4, N5; Table 2, Fig. 7A). Increased conductivity of the elution buffer results in greater displacement of impurities from the ligands by positively charged salt ions and thereby in a reduced purity of the mAb eluate [47,59].

CEX elution conditions N1, N2 and N7 exhibited the most promising trade-off between yield and purity of mAb. For these elution conditions a mAb recovery between 81 % and 97 % with simultaneous high removal of DNA (79–83 %) and HCP (78–85 %) was obtained (Fig. 7A). However, to determine optimal CEX elution condition for the overall process, performance of AEX, as subsequent unit operation, was examined.

3.3.2. AEX

In order to reduce process complexity, which come along with expenditure of time, labor and cost, CEX and AEX were directly combined without further buffer adjustment. AEX was used as last polishing step for a final removal of impurities in flow-through mode. The CEX elution fractions with different pH and conductivity values (Table 2) were examined as AEX load. The flow-through of the membrane adsorber was collected as the mAb containing product phase (Fig. 6B).

All examined pH and conductivity conditions resulted in a high mAb yield of approximately 100 % (Fig. 7B). The high yield was due to a low interaction of the mAb with the positively charged ligands. This was obtained by the used conductivities, where the salt ions displaced the mAb from the ligands, as well as the used pH-values, at which the mAb surface was positively charged according to its IP [47]. Nevertheless, even the unlikely event of a mAb binding to the AEX ligands is not an issue of concern as long as the impurities have a higher affinity to the ligand and are removed from the AEX feed solution.

For conductivities of 12.5 mS/cm a higher HCP removal was obtained with increasing pH (N8 > N1 > N6; Fig. 7B). Also for a conductivity of 17.5 mS/cm the HCP load reduction was remarkably increased for higher pH-values (N4, N5) compared to the lowest examined pH (N7; Fig. 7B). In contrast to the CEX elution a higher pH-value resulted in a higher HCP removal for AEX, because a larger fraction of HCP was negatively charged and interacted with the AEX ligands. For a further increase of the mAb purity and to increase the productivity of this unit operation, a potential overloading of the AEX MA with process related impurities should be considered. If the capacity is exceeded impurities break through the membrane even if the used buffer conditions would have resulted in an adsorption.

However, a high purity of mAb was obtained with low conductivities, similar to the CEX experiments, for all examined pH conditions (pH 7: N9 > N6 > N7, pH 7.5: N3 > N1 > N4, pH 8: N2 > N8 > N5; Fig. 7B).

3.3.3. Combined evaluation of CEX and AEX

The direct serial connection of CEX and AEX offers potential for process intensification. Accordingly, aim of the CEX and AEX experiments was to optimize the buffer condition for both chromatography steps to obtain a high yield of mAb as well as high removal of DNA and HCP.

Conditions with low pH and low conductivity are inappropriate due to their low mAb yield (N3, N9), while high pH and conductivity values resulted in low mAb purity after CEX and AEX (e.g. N5, Fig. 7C). A pH-value of 7.5 and a conductivity of 12.5 mS/cm resulted in 99 % yield of mAb with a simultaneous removal of 96 % DNA as well as 91 % HCP and exhibited a high reproducibility of the results (N1, Fig. 7C). For this reason the N1 condition was chosen for the following application study.

3.4. Process integration and application study

In order to evaluate the optimized conditions and to demonstrate the integration of the different unit operations an application study of the alternative DSP based on ATPE was performed. A total volume of 500 ml cell containing cultivation broth was processed according to Fig. 1 and the IgG yield as well as the removal of DNA and HCP was determined after each unit operation (Fig. 8).

After ATPE and phase separation 81.9 % yield of mAb was obtained. The mAb was slightly diluted into the LP (2.2 mg/ml) compared to the

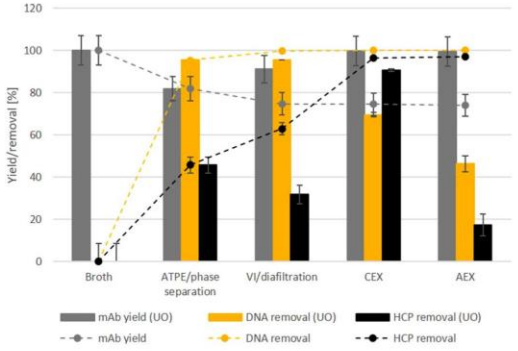

Fig. 8. Yield of mAb as well as removal of DNA and HCP for each unit operation (UO) and throughout the application study. Error bars represent the error propagation from the means of analytical triplicates.

Table 5
Concentration (c) of mAb as well as DNA and HCP and the respective amount in parts per million (ppm; related to the mAb concentration) obtained from the application study. Error values represent standard deviations from the means of analytical triplicates.

Unit operation	c_{mAb} [mg/ml]	c_{DNA} [µg/ml]	c_{DNA} [ppm]	c_{HCP} [µg/ml]	c_{HCP} [ppm]
Broth	2.9 ± 0.2	389 ± 3	132936	452 ± 27	154565
ATPE/phase separation	2.2 ± 0.1	16.8 ± 0.1	7557	228 ± 8	102467
VI/diafiltration	1.7 ± 0.1	0.64 ± 0.01	371	133 ± 7	76745
CEX	4.6 ± 0.2	0.52 ± 0.02	113	33.3 ± 1.3	7247
AEX	2.9 ± 0.2	0.18 ± 0.01	61	17.6 ± 0.9	6036

concentration of the cultivation broth (2.9 mg/ml, Table 5). For the integrated VI and diafiltration approach a mAb yield of 91.2 % was obtained, which was slightly higher compared to the membrane selection experiments (80 %) in section 3.2.3, likely due to a decreased effect of mAb adsorption on the membrane owing to a higher ratio between the processed volume and membrane area. After diafiltration and neutralization a mAb concentration of 1.7 mg/ml was obtained. Both chromatography steps resulted in a high mAb yield of 99.7 % and 99.4 % for CEX and AEX respectively (Fig. 8). Due to the used process mode CEX (bind and elute mode) increased the mAb concentration to 4.6 mg/ml while the value was decreased after AEX (flow-through mode with equilibration and flush of the MA) to a final concentration of 2.9 mg/ml (Table 5). The alternative DSP resulted in a total mAb yield of 74 %.

The majority of DNA (95.3 %) and 45.7 % of the HCP were already removed after ATPE and phase separation, as first unit operations. For the combined VI and diafiltration step DNA removal was higher and HCP removal lower compared to the membrane selection experiments (section 3.2.3, Fig. 5, Fig. 8). This is probably due to the pH and conductivity adjustment after the diafiltration process which was assigned as neutralization to the VI/diafiltration unit operation in this application study (Fig. 8). During the adjustment the turbidity increased, probably due to DNA precipitation (decreased DNA concentration after adjustment, Data not shown) resulting in an increased removal. Further purification was achieved by the two chromatography steps (Fig. 8). DNA as well as HCP removal for the CEX were quite similar to the results of the respective CEX elution optimization (N1 Fig. 7A, Fig. 8). For the AEX a removal of 46.2 % DNA and 17.2 % HCP was obtained (Fig. 8). However, these values were lower than those obtained from the optimization experiments (Fig. 7B, Fig. 8). This seems to be due to an overloading of the AEX membrane adsorber although a similar ratio between the mAb, DNA and HCP mass and AEX MV was used for the application study. This was likely caused by an insufficient scalability or the different procedure between the in-house membrane screening lab-device and the Sartobind® nano MA. In total 99.97 % of the DNA was removed to a concentration of 0.18 µg/ml corresponding to 61 ppm and 97.11 % of the HCP were removed to a concentration of 17.6 µg/ml corresponding to 6036 ppm (Table 5).

Despite the enormous impact on patient safety, there are no appropriate standard values for residual impurity amounts for mAb given by the regulatory authorities, resulting in a case-by-case assessment for every product [60]. However, common reported values are below 100 ppm HCP [60,61] and below 0.01 ppm residual amount of DNA [61] in the final drug. An approach to improve the mAb purity after the investigated chromatography steps is an optimization of the CEX loading conditions to decrease the amount of bound impurities to the CEX ligand for instance by a higher conductivity and pH-value. This could result in a higher mAb purity already during adsorption on the CEX. However, such optimization must be considered as trade-off between the mAb yield and purity. In addition, the effective buffer range of the used buffer solution must be taken into account.

In addition, an adjustment of the pH-value and conductivity of the CEX eluate, prior to AEX loading could result in a higher removal of DNA and HCP. Based on the optimization experiments the CEX elution conditions of N1 with the AEX conditions of N2, N3 or N9 for the AEX are promising combinations to further increase the mAb purity (Fig. 7). For the buffer adjustment between CEX and AEX conventional titration as well as diafiltration approaches could be used. However, this would also make the process much more complex.

To avoid overloading of the AEX the ratio between the AEX membrane volume and the CEX eluate should be optimized. In addition, virus filtration as well as final ultrafiltration/diafiltration before the fill and finish step, which were not considered in this study, would probably also increase the mAb purity.

The mAb quality throughout the alternative DSP was examined regarding N-linked glycosylation patterns which have a critical role in antibody effector function [28]. Ratios between the glycan basic structure (G0) and glycan structures with one (G1) and two (G2) additional galactose molecules as well as the total percentage of fucosylation were determined (Fig. 9).

Throughout the entire application study similar ratios of G1/G0 (between 1.23 and 1.27) and G2/G0 (between 0.28 and 0.30) as well as percentage of fucosylation (between 88.5 % and 91.1 %) were obtained, indicating consistent product quality, regarding the N-linked glycosylation pattern. Due to the uniform glycosylation pattern, as a critical quality attribute, ATPE and moreover the entire alternative DSP presented in this study, were considered suitable for mAb purification.

4. Conclusion

In this study a new, innovative DSP, integrating ATPE in a commonly used platform process for mAb purification, without the most expensive protein A chromatography step, is described. The process included extraction of the mAb by an ATPS, an integrated diafiltration and virus inactivation approach, and CEX as well as AEX (Fig. 1). Starting with cell containing cultivation broth directly from the fermenter the individual unit operations were optimized and a holistic process was development which was carried out as an application study.

ATPE, in combination with phase separation by centrifugation, was shown to be feasible as clarification step (Table 3). In addition, a mAb capture (97 % mAb yield; Table 3) as well as a first purification (Table 3, Fig. 8) was achieved. An incorporation of the required virus inactivation step was accomplished by diafiltration into an acidic buffer which enabled a simultaneous removal of the ATPS phase forming

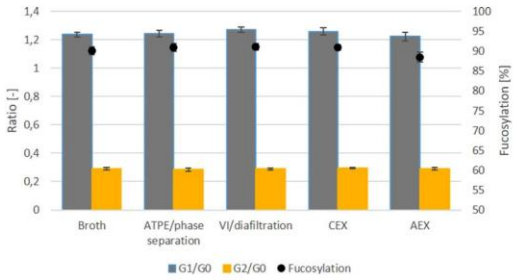

Fig. 9. Glycosylation pattern throughout the application study. Error bars represent standard deviations from the means of analytical duplicates.

components (Fig. 4) and further purification with high mAb yield by the use of the 30 kDa MWCO membrane (Table 4, Fig. 5). The CEX elution conditions (pH and conductivity) were optimized regarding mAb yield as well as purity. For a simple process design the eluate was loaded directly, without any further adjustment, on the AEX membrane adsorber, which was operated in flow-through mode.

In the application study a high mAb yield of 74 % was obtained (Fig. 8). It was shown that the critical quality attribute of N-linked glycosylation of the mAb remained unchanged after ATPE and throughout the entire alternative DSP (Fig. 9). In addition, 99.97 % (61 ppm residual load) of the DNA and 97.11 % (6036 ppm residual load) of the HCP were removed (Fig. 8, Table 5). Further studies must be conducted to assess the impact of the residual impurity levels obtained on patient safety. In order to further increase the mAb purity, additional unit operations could be incorporated. In addition, novel technologies, like the use of new cell lines engineered to produce reduced or less challenging impurities [62,63]. These technologies together with the alternative DSP presented in this study offer great potential to meet the challenge of the bottleneck within the DSP for mAb purification in future intensified processes.

CRediT authorship contribution statement

Thomas Kruse: Conceptualization, Methodology, Investigation, Writing - original draft. **Markus Kampmann:** Writing - review & editing, Supervision. **Ines Rüddel:** Investigation. **Gerhard Greller:** Supervision, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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3.3 Aqueous Two-Phase Extraction of Monoclonal Antibodies from High Cell Density Cell Culture

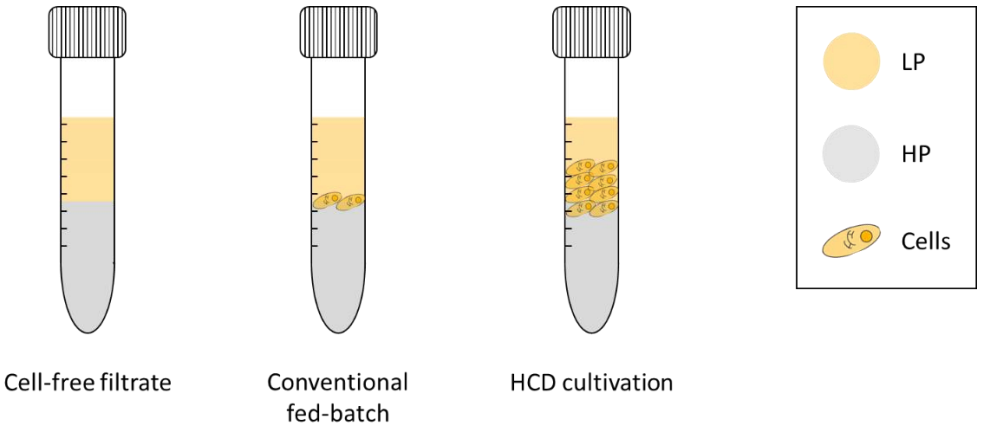

Figure 6. *Schematic representation of phase formation in ATPS with cell-free filtrate (left), cell-containing fed-batch (middle) and high cell density cultivation broth (right) as feed component.*

While several studies have proven ATPS as suitable clarification and capture unit operation from conventional fed-batch broth [71,101], and the feasibility of an ATPE based DSP for mAb purification was shown in the second part of this PhD thesis, an application for HCD has not been reported yet.

Due to the increased biomass of cultivations with higher cell densities, commonly used standard approaches for the clarification like centrifugation and depth filtration are struggling to support sufficient cell removal [107,108]. High costs, turnaround times and decreased capacities with increased VCD are issues of concerns [109,110]. Therefore, ATPE offers great potential for the processing of higher biomass and mAb concentrations.

In the third publication, a proof of concept study was performed to examine the influence of the cell density on cell removal, yield of mAb as well as the removal of DNA as representative for process related impurities. Within the study, six different compositions, based on ATPS reported for mAb extraction in the literature, were investigated with cell-free and cell-containing conventional fed-batch broth (approximately 15 * 10^6 cells/mL) as well as HCD broth (as a model system for HCD cultivations a broth generated by a perfusion approach with a VCD of approximately 70 * 10^6 cells/ml was used).

The formation of an interphase was observed for all examined ATPS with a cell-containing feed component. Due to the increased biomass of the HCD broth, the interphase appears much broader while

the LP volume was slightly reduced compared to the conventional fed-batch broth (**Figure 6**). Due to the complete cell removal in the LP, ATPE was considered as suitable clarification method, also for HCD broth.

With increasing VCD, a lower mAb yield was obtained for three of the examined ATPS, probably due to adsorption effects of the mAbs on the cell surface based on the chemical composition of the respective ATPS. However, no dependency on the VCD was observed for the remaining three ATPS, resulting in high yields of mAb of > 99 % for broth with high VCD. These results confirmed ATPE as potential mAb capture step for HCD cultivations.

In addition, it was shown that ATPE was suitable to remove DNA, as representative for process related impurities. With cell-free feed, a DNA load reduction of > 70 % was achieved for all ATPS. The presence of cells resulted in a reduced purity of some of the examined ATPS. An increased level of cell lysis due to the respective ATPS composition might be a possible explanation. Nevertheless three out of the six examined ATPS were able to remain high DNA removal levels even for HCD cultivation broth as feed component.

For ATPS4 a cell free LP with a mAb yield of 99 % and a simultaneous DNA removal of 68 % was achieved. Even if a sufficient scale-up of the method needs to be proven, the results of the third publication enable a foundation for the industrial application of ATPE for the clarification, capture and purification of mAbs from HCD cell cultures.

Chemie Ingenieur Technik

Aqueous Two-Phase Extraction of Monoclonal Antibodies from High Cell Density Cell Culture

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Improvements of the upstream process for the production of monoclonal antibodies (mAb) has resulted in mammalian cell cultures with significantly increased cell densities. These high cell density (HCD) broths pose severe challenges for established downstream unit operations due to the increased biomass load. To meet these challenges, aqueous two-phase extraction was examined as a potential method for the clarification, as well as first stage purification of a Chinese hamster ovary expressed mAb with cell densities up to $70 \cdot 10^6$ cells mL^{-1}. A clarification with 99 % yield of mAb and 68 % DNA removal was accomplished, which contributes to enable a foundation to the industrial application of aqueous two-phase extraction for manufacturing of mAb from HCD cultivations.

Keywords: Aqueous two-phase extraction, Chinese hamster ovary, High cell density, monoclonal antibody

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1 Introduction

Monoclonal antibodies (mAb) are used for the treatment of numerous important therapeutic areas including cancer [1], immunological disorders, inflammation [1, 2] and infectious diseases [1]. Due to their enormous importance as active pharmaceutical ingredient the market for mAb therapies continues to expand [3].

During the upstream process (USP) mammalian cells are cultivated to express mAb into the process medium. In the USP, process intensification towards highly productive cell lines with the ability to obtain high cell densities with a high viability and specific growth rate using suspension culture with chemically defined medium has been achieved [4]. The improved media and feeding strategies used in industry are now obtaining high cell density (HCD) cultivations with more than 100 million cells mL^{-1} [5, 6].

These achievements provide a significant challenge for the subsequent clarification and purification unit operations, well known as the downstream process (DSP) bottleneck [7–9]. For cell removal as the first DSP step, centrifugation followed by depth filtration is most commonly utilized in platform processes for conventional cultivations [10, 11]. However, due to the increased biomass at higher cell densities, these standard approaches are struggling to support sufficient clarification [12].

As an alternative, aqueous two-phase systems (ATPS) have proven to be suitable for direct clarification of conventional fed-batch cultivation broth with cell densities of 10–20 million cells mL^{-1} [13, 14]. Cells and cell debris accumulate in the ATPS interphase due to steric effects, enabling separation from the mAb-containing target phase. However, an application of ATPE for the clarification of HCD cultivation broth (> $50 \cdot 10^6$ cells mL^{-1}) has not been reported yet.

As a result of the increased cell density and productivity, higher mAb concentrations of up to 25 g L^{-1} are achieved. Simultaneously, at high cell densities often more impurities are obtained during the cultivation process [8, 12, 15–17]. These impurities generally consist of product- (e.g., aggregates, fragments and charge variants) as well as process-related impurities (e.g., deoxyribonucleic acid (DNA), host cell protein (HCP) and endotoxins) [12]. Thereby existing protein A affinity chromatography units, as most established capture step [18], reach their capacity limit. Given the key significance of protein A chromatography high efforts have been undertaken to improve its understanding and mitigate limitations [19, 20]. Throughput boundaries as well as the limited lifetime and costs of the resin are issues of concern [19, 20]. To overcome these limitations, non-chromatographic technologies were brought into focus [19, 21]. ATPS have been successfully used in several studies as mAb capture and first purification step from mammalian cell cultures [22, 23]. Thereby ATPE aims to separate the mAb from process-related impurities such as DNA [24, 25].

For the purification of mAb, ATPS consisting of the phase forming agents, a polymer such as polyethylene glycol (PEG) and a salt such as phosphate or citrate are most commonly used [24]. In addition to these phase-forming components, displacement agents as well as the pH are used

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to influence the distribution of biomolecules within the immiscible light (LP) and heavy phase (HP) of the ATPS [26].

Given the great potential of ATPE for an integrated clarification, capture and first purification, a proof of concept study for HCD cultivations was conducted. Six different ATPS with different phase-forming components and compositions reported for mAb extraction were used. The performance of cell removal, mAb yield and removal of DNA, as example for process-related impurities, for ATPE procedures were examined with cell-free filtrate, conventional fed-batch and HCD model broth systems.

2 Experimental

2.1 Cultivation

The cultivation of an mAb-producing (immunoglobulin type G) Chinese hamster ovary (CHO) cell line was carried out in commercial serum-free medium (FMA/FMB, Sartorius Stedim Cellca GmbH).

For conventional fed-batch cultivation, an Ambr®250 single-use bioreactor (Sartorius, Göttingen, Germany) was used for 12 days at 36.8 °C, pH 7.1 and 855 rpm. At the time of harvest, the cells exhibited a viability ≥ 80 % with a viable cell density (VCD) of approx. $15 \cdot 10^6$ cells mL^{-1}, a wet cell weight (WCW) of approx. 80 g L^{-1} and mAb concentration of approx. 2.8 g L^{-1}.

As a model solution for HCD cultivations a broth generated by a perfusion approach was used. A BIOSTAT® RM bioreactor (Sartorius, Göttingen, Germany) with a minimum cell-specific perfusion rate of 50 pL cell^{-1}d^{-1} was used. The permeate was withdrawn by a membrane at the bottom of the bag with a mean pore size of 1.2 µm while fresh medium was added to maintain a constant volume. During the cultivation for 6 days, a temperature of 36.8 °C and a pH value of 6.95 was maintained. A rocking cascade starting at 30 rpm with a rocker angle of 10° maintained a pO_2 value of 60 %. At harvest time, the cells exhibited a viability ≥ 80 % with VCD approx. $70 \cdot 10^6$ cells mL^{-1}, WCW approx. 320 g L^{-1} and mAb concentration approx. 4 g L^{-1}.

2.2 Aqueous Two-Phase Extraction

ATPS were prepared by weighing in the appropriate amount of the different components into 15-mL centrifuge tubes to a total mass of 10 g. Six different compositions, based on ATPS reported for mAb extraction in the literature, were examined in this study (Tab. 1). Solid sodium chloride (NaCl) as well as stock solutions of 40 wt % phosphate buffer or 35 wt % citrate buffer were used. Sodium phosphate monobasic anhydrous (NaH_2PO_4) and potassium phosphate dibasic anhydrous (K_2HPO_4) were used for the phosphate buffer, citric acid ($C_6H_8O_7$) and trisodium citrate ($Na_3C_6H_5O_7$) for the citrate buffer. All salts were obtained from Carl Roth (Karlsruhe, Germany). Different ratios of the corresponding salts were used to adjust the pH value. PEG with an average molecular weight of 400 g mol^{-1} and 1450 g mol^{-1} was obtained from Merck (Darmstadt, Germany).

As feed component a cell-free (broth filtered by Sartopore 2® XLG, Sartorius, Göttingen) or cell-containing conventional fed-batch broth or HCD broth was used as indicated. To ensure equilibrium conditions the tubes were shaken at 150 rpm for 5 min. Phase separation was performed by centrifugation at 1000*g* for 5 min. For determination of the phase ratio, the volume of the LP was divided by the volume of the HP, using cell-free filtrate as feed component. The LP was withdrawn and further analyzed according to Sect. 2.3.

2.3 Analytics

Cell removal was evaluated using a microscope and counting the cells in a Neubauer counting chamber (Brandt, 0.1 mm depth and 0.0025 mm^2 area). Due to the high viscosity of the PEG-rich samples from the LP, a protein A

Table 1. Composition of the examined ATPS.

ATPS	Feed [wt %]	Polymer [wt %]		Salt [wt %]		pH [–]	Displacement agent [wt %]	Phase ratio [–]	Reference
		PEG 400	PEG1450	Phosphate	Citrate		NaCl		
1	44.5	15.5	–	16	–	6	–	1.43	[13]
2	38	9	–	21.2	–	6	–	0.40	modified from [13]
3	26.4	19.6	–	–	18.9	6	–	1.02	[27]
4	28.4	21	–	–	17.7	6	–	1.18	modified from [27]
5	40.5	–	6	15	–	6	10	0.26	[28]
6	27.2	6.8	–	26.1	–	7.3	0.7	0.24	[29]

membrane adsorber (MA; Sartobind Protein A, Sartorius, Göttingen, Germany) was used to determine the mAb concentration. An Äkta prime plus chromatography system (GE Healthcare, Uppsala, Sweden) was used for the analysis. After equilibration with phosphate buffered saline (PBS, pH 7.4), sample volumes of 500 µL were loaded onto the MA, which was then washed with equilibration buffer. Subsequently, elution was performed with 0.1 M glycine (pH 3.0). All steps were carried out at a constant flow rate of 10 mL min^{-1} and all chemicals were obtained from Carl Roth (Karlsruhe, Germany). The measured absorption peak area at 280 nm was used for quantification of the samples with a concentration calibration curve generated based on an IgG stock solution (5 % Cytoglobin human immunoglubin G, Bayer Vital GmbH). The yield of mAb (yield$_{mAb}$) was calculated according to Eq. (1), where $c_{mAb,LP}$ and $c_{mAb,feed}$ are the concentrations of mAb in the LP and the feed component. V_{LP} represents the volume of the LP and V_{feed} the volume of the feed component without cells.

$$\text{yield}_{mAb} = \frac{c_{mAb,LP}\ V_{LP}}{c_{mAb,feed}\ V_{feed}} \cdot 100\ \% \quad (1)$$

The DNA concentration was measured by the Quant-iT PicoGreen dsDNA Assay Kit (ThermoFisher Scientific, Waltham, USA) using salmon sperm DNA as standard according to the manufacturer's instructions. If necessary, the samples were diluted in TE buffer (10 mM TRIS, 1 mM EDTA, 0.1 % SDS; all chemicals were obtained from Karl Roth, Karlsruhe, Germany). Measurements were performed with a FLX 800 plate reader (Bio-TEK instruments, Bad Friedrichshall, Germany) with a detection limit of 31.25 ng mL^{-1}. Removal of DNA (removal$_{DNA}$) was calculated according to Eq. (2), where $c_{DNA,LP}$ and $c_{DNA,feed}$ are the concentrations of DNA in the LP and the feed component.

$$\text{removal}_{DNA} = \left(1 - \left(\frac{c_{DNA,LP}\ V_{LP}}{c_{DNA,feed}\ V_{feed}}\right)\right) \cdot 100\ \% \quad (2)$$

3 Results and Discussion

3.1 Clarification by ATPE

The formation of a cell-containing interphase was observed for all six examined ATPS. Due to the higher VCD and biomass content (less liquid content), the interphase was significantly broader in ATPS with HCD broth, while the LP volume was slightly decreased compared to ATPS with conventional fed-batch broth. However, the LP formed a clear, defined phase above the interphase of all ATPS examined in this study (exemplary shown in Fig. 1).

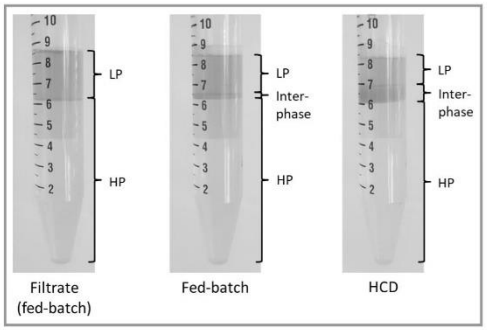

Figure 1. Phase formation in ATPS with filtrate (left), conventional fed-batch (middle) and HCD cultivation broth (right) as feed component. Exemplary shown for ATPS2.

No cells were found in the LP, neither for standard fed-batch nor for HCD broth, suggesting a complete removal of cells. Therefore, ATPE was considered as a suitable clarification method for HCD cultivations.

3.2 Yield of mAb

Using cell-free filtrate of the standard fed-batch broth the mAb yield was > 97 % for ATPS1–4, whereas with ATPS5 and ATPS6 it was approximately 70 % (Fig. 2). However, in

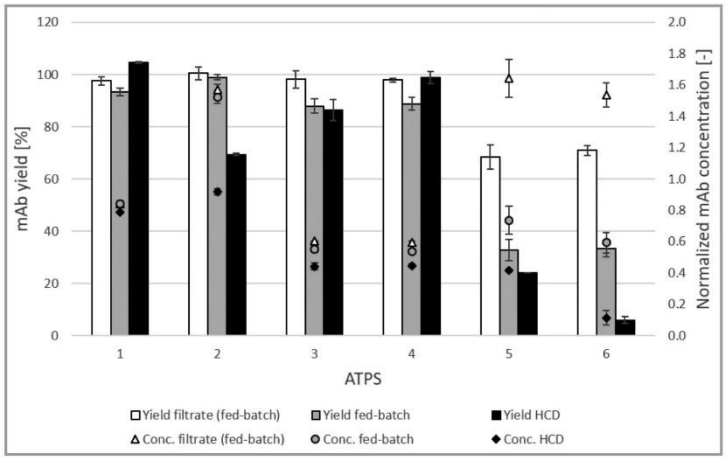

Figure 2. Yield and concentration of the mAb in the LP of ATPS1-6 for conventional fed-batch broth and filtrate as well as HCD cultivation broth as feed component. Concentration (conc.) was normalized to the mAb concentration of the respective feed component.

all ATPE experiments performed, mAb was not detectable in the HP. The possible reason for the lower mAb yields of ATPS5 and ATPS6 could be the high amount of phosphate [30] in combination with a lower PEG concentration, which resulted in a low phase ratio (Tab. 1). This likely caused precipitation of mAb in the interphase due to supersaturation of the LP [31] caused by the relatively high mAb concentration (Fig. 2). The high molecular weight of PEG used for ATPS5 (Tab. 1) might also promote precipitation [32].

Using conventional fed-batch cultivation broth as ATPS feed component, the yield of mAb for ATPS5 and ATPS6 was approximately 33 %. This value was significantly lower than the mAb yield reported in the literature (> 95 % for both ATPS). The differences can be attributed to different physicochemical properties of the examined mAb or different composition of the broth itself, resulting in a changed distribution for the same ATPS composition.

The mAb yield decreased further to 24 % (ATPS5) and 6 % (ATPS6) for the HCD cell culture as feed component, due to the lower mAb concentration in the LP (Fig. 2). A possible explanation for the decreasing mAb yield with increasing cell densities could be an interaction, e.g., adsorption, of the mAb on the cell surface within the respective LP composition. Thereby the mAb was removed from the LP by enrichment of the cells in the interphase and the effect was enhanced with higher cell densities. A similar trend was observed for ATPS2, were the mAb concentration as well as the yield was significantly lower for the HCD cultivation broth compared to the cell-free filtrate and the conventional fed-batch broth (Fig. 2).

For all three, ATPS1, ATPS3 and ATPS4, there was no significant difference in the concentration and yield of mAb in the LP between the three different feed components. ATPS1 and ATPS4 achieved an excellent mAb yield of > 99 % with the HCD broth (Fig. 2). Therefore, ATPE was considered as a suitable unit operation for mAb capture from HCD cultivation broth.

3.3 Removal of DNA

While ATPS have been shown to remove several process-related impurities (DNA, HCP, viruses [24] and endotoxins [33]) from standard fed-batch cultivations, focus in this study was placed on DNA as a representative impurity. All six ATPS were tested for their ability to remove DNA from the different feed components. Using cell-free filtrate as feed component, a DNA removal of > 70 % was achieved with all ATPS, whereas ATPS5 exhibited the highest value of 95 % (Fig. 3).

However, the presence of cells in the used feed component had a strong effect on the DNA removal ability, at least for some ATPS compositions. For example, both ATPS1 and ATPS6 achieved lower DNA removal at a higher VCD (Fig. 3). For ATPS1 even a negative value of –35 % was observed for the HCD broth (Fig. 3). For ATPS2, the DNA removal with fed-batch and HCD broth was significantly lower compared to cell-free filtrate (Fig. 3). An explanation for this effect could be an increased level of cell lysis by the respective ATPS [13], where the higher amount of DNA was released from the cells into the ATPS and LP with increasing VCD.

For ATPS3–5 the normalized DNA concentration as well as the DNA removal was independent of the VCD (Fig. 3). This could be due to a gentler composition of the ATPS, which avoids cell lysis, or to a higher distribution coefficient of DNA to the HP.

4 Conclusion

This study examined ATPE as potential clarification, capture and first purification method to extract mAb directly from HCD cultivation broth. Using six different ATPS compositions (Tab. 1), the influence of the VCD on cell removal, mAb yield as well as the DNA removal in the LP was investigated. With all examined ATPS, clarification by formation of an interphase was achieved. Similar results were obtained for both the conventional fed-batch (VCD ≈ $15 \cdot 10^6$ cells mL^{-1}) as well as the HCD (VCD ≈ $70 \cdot 10^6$ cells mL^{-1}) broth. Due to the proven cell removal ability, ATPE has

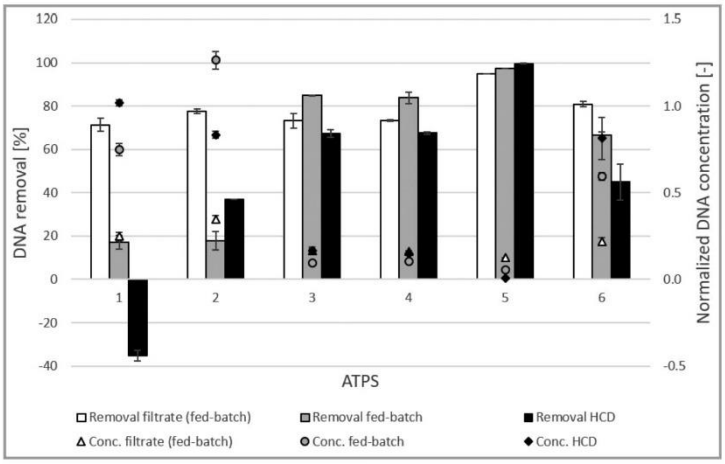

Figure 3. Removal and concentration of DNA in the LP of ATPS1-6 for conventional fed-batch broth and filtrate as well as HCD cultivation broth as feed component. Concentration (Conc.) was normalized to the DNA concentration of the respective feed component.

been shown to be a promising approach for clarification of HCD cultivations.

For three of the examined ATPS, the mAb yield decreased with increasing VCD in the feed component, which could be due to interaction, e.g., adsorption, of mAb to the cells within the chemical composition of the respective ATPS. Nevertheless, > 99 % of the mAb was recovered in the LP of ATPS1 and ATPS4 with the HCD model broth as feed component (Fig. 2). This work has shown that to maintain high yields of future higher titer processes, the solubility of the mAb in the LP should be considered. Such concentrated fed-batch processes can be cultivated, e.g., by HCD perfusion processes with a product retention device.

This work has shown that while all ATPS-containing cell-free filtrate, removed > 70 % of DNA, different extraction properties were observed for both the conventional fed-batch and HCD broth as feed component. It is anticipated this is due to cell lysis during extraction as indicated by the high residual DNA. Nevertheless, three ATPS compositions (ATPS3–5) were found to maintain a high DNA removal including for the HCD culture (Fig. 3). The extent of DNA removal is highly encouraging and supports the need to expand future studies to widen the scope of impurity removal such as host cell proteins and endotoxins.

These results demonstrate that several aspects must be taken into account to switch from an ATPE with filtrate or conventional fed-batch to a HCD broth. Nevertheless, ATPS3 and ATPS4 exhibited similar extraction performances regardless of the cell density examined. These two ATPS allowed efficient mAb capture and purification. Further ATPS optimization could be achieved by design of experiment approaches. For the HCD broth examined in this study, ATPS4 had the most suitable composition of the investigated ATPS, resulting in a clarified LP with a mAb yield of 99 % and simultaneously 68 % DNA removal. Even through the easy scalability of ATPS is one of the most advantageous side of this unit operation [24], the transferability to larger scales has yet to be proven for the usage of ATPE as a suitable unit operation in the downstream process of mAb from HCD cell cultures. However, several studies have already shown ATPS-based methods for mAb clarification and purification even for pilot scales of standard fed-batch cultivations [13]. Therefore, this proof of concept study contributes to enable a foundation to the industrial application of ATPE for clarification and purification of mAb from HCD cultivations.

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Abbreviations

ATPE	aqueous two-phase extraction
ATPS	aqueous two-phase system
CHO	Chinese hamster ovary
DNA	deoxyribonucleic acid
DSP	downstream process
HCD	high cell density
HP	heavy phase
MA	membrane adsorber
mAb	monoclonal antibody
LP	light phase
PEG	polyethylene glycol
USP	upstream process
VCD	viable cell density
WCW	wet cell weight

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Aqueous Two-Phase Extraction of Monoclonal Antibodies from High Cell Density Cell Culture

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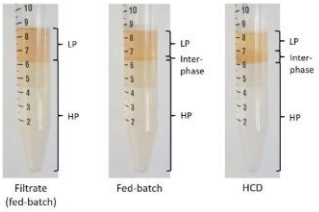
Communication: High cell density approaches in the upstream process offer great potential for an intensified production of monoclonal antibodies (mAb). However, significant challenges are imposed on the subsequent downstream process. In this study, proof of concept for clarification, capture and first purification of mAb from high cell density cell cultures by aqueous two-phase extraction is presented.



4. Conclusion and outlook

Since their application for the therapy of numerous severe diseases, mAbs have become a rising star in the pharmaceutical industry. In order to serve the growing market demand intensified USP processes have been established, resulting in increased productivities. However, these achievements shifted the bottleneck in mAb production to the DSP where established unit operations and facilities for clarification and capture reach their capacity limit. Therefore, a predominant focus is placed on alternative methods to overcome the DSP bottleneck. ATPE has been shown as feasible unit operation for cell removal and mAb capture by several studies. Nevertheless, a transfer into the pharmaceutical production has not been reported yet, due to a lacking process integration and the high product quality and purity demands of the authorities.

The work of this PhD thesis focused on a feasible approach to integrate ATPE in the DSP for mAb purification. The presented membrane-based approach for phase separation and clarification offers several advantages. In addition to high yield and purity of the mAb, a sterile filtration step was directly integrated by using a correspondingly narrow pore size. The new method enables a process intensification by combining clarification, mAb capture and first purification in one unit operation. Phase separator prototypes with increased membrane area were developed, characterized and operated for the separation of ATPS with cell-containing cultivation broth.

Further investigation could deal with model development and validation of this unit operation in order to examine the optimum operating space and to potentially meet the quality by design (QbD) requirements of authorities like the FDA. In addition, the transferability to different cell lines with different mAbs as products are in the focus of future research. For further process intensification a continuous operation of the phase separator is conceivable. Thereby both, the cell-containing bleed as well as the cell-free permeate stream of a perfusion process could be processed by ATPE in combination with membrane based phase separation.

In order to meet the challenge of a lacking integration further focus was placed on the subsequent unit operations within the DSP after ATPE. Due to the fact that most mAb manufacturing processes are based on platform approaches these were used as a basis. This offers several advantages for instance the usage of already existing plants and process parameters as well as an easy process adaptation. A holistic development and optimization of the alternative, ATPE based, DSP was presented. The critical integration of the ATPE into the subsequent unit operation was achieved by a diafiltration approach. In addition to

the removal of the phase forming components, process related impurities like DNA and HCP were removed. Moreover, the usage of a suitable acidic diafiltration buffer enabled a pH shift for virus inactivation. Throughout the entire DSP a high yield of 74 % and increasing purity of the mAb was obtained. In addition, the N-linked glycosylation pattern remained unimpaired after ATPE and every subsequent unit operation of the alternative DSP.

However, in order to meet the high purity requirements of the authorities for biopharmaceutical products and the established mAb platform DSP further focus should be placed on process optimization in particular with regard to purity. While the alternative DSP was not able to achieve the low residual amounts of HCP and DNA often stated in the literature, it must be considered that there are actually no fixed limit values from the authorities. These limits are evaluated for each product, whereby possible immunotoxic effects and the robustness of the manufacturing process itself are important criteria. Therefore, further studies must be conducted to examine the impact of the obtained impurity amount on patient safety to be able to correctly evaluate the obtained purities.

The residual impurity amount at the end of the DSP could be also reduced by an initial lower HCP and DNA amount during the USP. For instance, recent research is focused on cell line engineering based on novel genetic methods like the CRISPR/Cas gene editing system. Aim of some of these studies is the generation of improved host cell lines with a higher cell specific production rate of the product but also a reduced or less challenging impurity level.

The incorporation of further unit operation could be considered to further increase the purity of the product. MA in flow-through mode could be used for example, offering further advantages like high productivities as well as an easy process setup and can be used in addition to AEX as HIC or mixed-mode chromatography. Considering this, the alternative DSP based on ATPE offers great potential for an intensified mAb manufacturing due to the complete removal of the protein A chromatography step as the most expensive unit operation.

While the feasibility of an ATPE based DSP was proven so far for standard fed-batch cultivations in this study, HCD cultivations are increasingly coming into focus in the development of an intensified USP. Even for this challenging cultivation broth, where until today no appropriate clarification method is implemented in a production process, ATPE was shown as reliable method for clarification in this work. A cell free LP was obtained for all examined ATPS for cultivation broth up to 70 * 10^6 cells/mL. On top of that high yields of mAb with simultaneous removal of DNA, as representative for process related impurities, was achieved for some ATPS as a function of their composition. The extent of DNA removal is highly

encouraging. This supports the need to conduct further studies to investigate the removal of other contaminants such as HCP or endotoxins.

Although the results of this small-scale study were promising, the transferability to larger scales has yet to be proven with extensive considerations of productivity as well as economic benefits. However, a successful application of ATPE for a clarification, capture and purification directly from a HCD cultivation broth was shown.

This PhD thesis enables a foundation to the industrial application of ATPE for an intensified production of mAbs and contributes to overcome the DSP bottleneck. Moreover, the results of this work could be used not only for mAb manufacturing but potentially also for numerous additional biologicals with challenging processing such as antibody fragments, antibody drug conjugates or even exosomes.

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List of figures

List of abbreviations

AEX	Anion exchange chromatography
ATPE	Aqueous two-phase extraction
ATPS	Aqueous two-phase separation
BHK	Baby hamster kidney
CEX	Cation exchange chromatography
CHO	Chinese hamster ovary
CQA	Critical quality attribute
DBF	Dynamic body feed
DNA	Deoxyribonucleic acid
DoE	Design of experiments
DSP	Downstream process
EMA	European medicine agency
FDA	Food and drug administration
HCD	High cell density
HCP	Host cell protein
HIC	Hydrophobic interaction chromatography
HMW	High molecular weight species
ICH	International conference of harmonization
IEX	Ion exchange chromatography
IP	Isoelectric point
LMW	Low molecular weight species
MA	Membrane adsorber
mAb/s	Monoclonal antibody/-ies
OVAT	One variable at a time
PEG	Polyethylene glycol
QbD	Quality by design
TLL	Tie-line length
UF/DF	Ultrafiltration/diafiltration
USP	Upstream process
VCD	Viable cell density
VI	Virus inactivatio

Publication and conference contributions

Publications

(1) Thomas Kruse (85%), Axel Schmidt (10%), Markus Kampmann and Jochen Strube. *Integrated Clarification and Purification of Monoclonal Antibodies by Membrane Based Separation of Aqueous Two-Phase Systems.* MDPI antibodies 2019, doi: 10.3390/antib8030040

TK (85%): Conceptualization, methodology, investigation, writing—original draft preparation

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(2) Thomas Kruse (85%), Markus Kampmann, Ines Rüddel (5%) and Gerhard Greller. *An Alternative Downstream Process Based on Aqueous Two-Phase Extraction for the Purification of Monoclonal Antibodies.* Biochemical Engineering Journal 2020, doi: https://doi.org/10.1016/j.bej.2020.107703

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MK, GG (in total 10%): Writing—review and editing, supervision

(3) Thomas Kruse (85%), Markus Kampmann (10%) and Gerhard Greller (5%). *Aqueous Two-Phase Extraction of Monoclonal Antibodies from High Cell Density Cell Culture.* Chemie Ingenieur Technik 2021, doi: 10.1002/cite.202000017

TK (85%): Conceptualization, methodology, investigation, writing—original draft preparation

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Conference Contributions

(1) Thomas Kruse, Markus Kampmann, Franziska Krumbein and Jochen Strube. *Membrane-based separation of aqueous two-phase systems for the purification of monoclonal antibodies.* ProcessNet-Jahrestagung und 33. DECHEMA-Jahrestagung der Biotechnologen 2018, München (Poster and Conference Article; https://doi.org/10.1002/cite.201855341)

(2) Thomas Kruse, Markus Kampmann, Franziska Krumbein, Axel Schmidt, and Jochen Strube. *Membrane based separation of aqueous two-phase systems for the purification of monoclonal antibodies.* SPICA 2018, Darmstadt (Oral Presentation)

(3) Thomas Kruse, Markus Kampmann, Franziska Krumbein, Axel Schmidt, and Jochen Strube. *Membrane based separation of aqueous two-phase systems for the purification of monoclonal antibodies.* 5th Biotech days 2018, Laupheim (Oral Presentation)

(4) Thomas Kruse, Axel Schmidt, Markus Kampmann and Jochen Strube. *ATPS phase separation for integrated clarification and purification.* ESACT 2019, Copenhagen (Poster)

(5) Thomas Kruse, Markus Kampmann and Gerhard Greller. *Integration of aqueous two-phase extraction in the downstream process for the purification of monoclonal antibodies.* ZHAW BioTech 2019, Wädenswil (Poster)

Curriculum vitae

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