Continuous recovery of antibodies with non-interrupted mass flow of the product

Dissertation zur Erlangung des Doktorgrades an der Universität für Bodenkultur Wien

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Wien, August 2019

Danksagung

Ich möchte mich bei der gesamten Boku Downstream Arbeitsgruppe für die vielen interessanten und förderlichen Diskussionen, sowie für die schöne freundschaftliche Arbeitsatmosphäre bedanken. Mein spezieller Dank geht an meinen Doktorvater Professor Alois Jungbauer, der immer mit Hilfestellungen, Ideen und Motivation zur Stelle war, im Besonderen wenn diese mal fehlten. Ein großer Dank geht an meinen Co-Supervisor Peter Satzer für dessen Ratschläge und Hilfe zu jeder Zeit während des Projektes. Danke auch an meine Dissertationskolleginnen Walpurga Krepper für die Unterstützung und hilfreichen Debatten. Ein weiterer Dank geht an das Next-generation Biopharmaceutical Downstream Process Projekt Team für die tolle Zusammenarbeit und der Europäischen Union, welche dieses Projekt im Zuge des Horizon 2020 Programm gefördert hat (Grant agreement number 635557). Sehr inspirierend und motivierend waren Nico Lingg und Petra Steppert. Danke für eure Unterstützung. Abschließend bedanke ich mich noch bei meiner Familie und allen Freunden, die mich während dieser schönen und prägenden Zeit begleitet und unterstützt haben.

Eidesstattliche Erklärung

Ich erkläre ehrenwörtlich, dass ich die vorliegende Arbeit selbständig und ohne fremde Hilfe verfasst habe, andere als die angegebenen Quellen nicht verwendet habe und die den benutzten Quellen wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht habe.

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Abstract

Continuous production increases product quality and product safety, enables flexible production and decreases production costs and facility footprint. A fully continuous primary recovery by flocculation assisted depth filtration was developed. A cationic polymer was continuously added to the process stream of cell culture broth. Incubation and mixing was achieved in a tubular reactor with inbuilt static mixers. Cell flocks were continuously separated by parallelization of depth filtration. With this setup, DNA was almost completely depleted and the necessary filter area was reduced four-fold compared to a standard depth filtration. For continuous product capture a continuous precipitation using polyethylene glycol and zinc ions was developed. A similar tubular reactor with inbuilt static mixers was used for continuous precipitation to achieve mixing and to prevent precipitate from settling. Solid-liquid separation was accomplished by a novel configuration of continuous two-stage tangential flow microfiltration. In a first stage, the precipitate was continuously concentrated by a factor of ten. In a second stage, which was fed by a slow bleed out of the first stage, the precipitate was washed by continuously adding wash buffer and continuously harvested. The best re-solubilization kinetics are at a low pH of 3.5. This facilitates a subsequent viral inactivation at low pH after the product capture step. Yields of 95% and purities of 97% were achieved. High molecular weight impurities as well as the volume was reduced by a factor of five. This product capture step enables a real and fully continuous operation with real continuous product inflow and real continuous product outflow of the unit operation. The process is very stable and do not require complicated online monitoring and control strategies. This method enable a fully continuous process and can be considered as a really continuously operated alternative to chromatography based product capture steps.

Kurzfassung

Kontinuierliche Prozesse in der biopharmazeutischen Produktion erhöhen die Produktqualität und Produktsicherheit und reduzieren gleichzeitig die Produktionskosten und den Platzbedarf der Anlage. Im Zuge der Dissertation wurde eine kontinuierliche Zellklärung durch Flockung der Zellen entwickelt. Dazu wurde ein kationisches Polymer kontinuierlich zum Prozessstrom zudosiert, in einem Rohrreaktor mit eingebauten statischen Mischern inkubiert und kontinuierlich abfiltriert. Mit diesem Setup wurde die DNA fast vollständig abgereichert und die notwendige Filterfläche im Vergleich zur herkömmlichen Tiefenfiltration um den Faktor vier reduziert. Für die kontinuierliche Antikörperaufreinigung wurde eine kontinuierliche Fällung mit Polyethylenglykol und Zinkionen im Rohrreaktor entwickelt. Statische Mischer sorgten für die nötige Mischung und verhindern ein Absetzen des Präzipitats. Die Fest-Flüssig-Trennung wurde durch eine neuartige, kontinuierliche, zweistufige Tangentialflussfiltrationfiltration realisiert. In der ersten Stufe wurde das Präzipitat kontinuierlich um Faktor zehn aufkonzentriert. In einer zweiten Stufe, die durch einen langsamen Produktstrom aus der ersten Stufe gespeist wurde, wurde das Präzipitat durch kontinuierliche Zugabe von Waschpuffer gewaschen und schließlich geerntet. Die beste Lösungskinetik des Präzipitats ist bei pH 3,5. Dies ermöglicht eine direkt anschließende Virusinaktivierung bei niedrigem pH-Wert. Ausbeuten von 95% und eine Reinheit von 97% wurden erreicht. Produktaggregate und Volumen konnten um Faktor fünf reduziert werden. Dieser Produktaufreinigungsschritt ermöglicht einen kontinuierlichen Betrieb mit einem kontinuierlichen Produktzulauf und einem kontinuierlichen Produktabfluss. Darüber hinaus war der Prozess sehr stabil und erfordert keine aufwendige Online-Überwachung und Prozesssteuerung. Dieser kontinuierliche Prozess kann als eine tatsächlich kontinuierlich betriebene Alternative zur Chromatographie gesehen werden.

List of abbreviations

ATPS	Aqueous two-phase systems
CCF	Cell culture broth
CHO	Chinese hamster ovary
CSTR	Continuous stirred tank reactor
DSP	Downstream processing
EMA	European Medicines Agency
FBRM	Focused Beam Reflectance Measurement
FDA	Food and Drug Administration
FT	Flow through
HCP	Host cell proteins
HMWI	High molecular weight impurities
HPLC	High-pressure liquid chromatography
LMH	Liter per square meter per hour
PAT	Process analytical technology
PBS	Phosphate buffer saline
PEG	Polyethylene glycol
pDADMAC	poly(diallyldimethylammonium) chloride
PFR	Plug flow reactor
QbD	Quality by design
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEC	Size exclusion chromatography
SMB	Simulated moving bed
SPTFF	Single-pass tangential flow filtration
TTF	Tangential flow filtration

1 Introduction

In this thesis, a continuous cell flocculation for primary recovery and a continuous antibody precipitation for product capture was developed. The state of the art of a commercial antibody production process and continuous unit operations were reviewed. Moreover, an overview of the regulatory issues about continuous production of biopharmaceuticals was given and how to transfer a batch process into a continuous process. The principle of filtration, flocculation and precipitation were described.

1.1 State of the art

A commercial antibody production process includes several unit operation steps; an upstream process connected to a downstream process. Commercially manufactured monoclonal antibodies are expressed in Chinese hamster ovary (CHO) cells, from which they are secreted into the cell culture supernatant. The cells are cultivated in fed-batch mode in bioreactors up to 25 m^3. Currently, continuous cultivation in perfusion cell culture is rarely applied for commercial full-scale production. The first step after upstream processing is often called primary recovery. Primary recovery is either performed by centrifugation, normal flow filtration or tangential flow filtration to remove cells and cell debris. The cell removal step is followed by a sterile filtration, because primary recovery is not performed under fully closed conditions. In order to reduce potential microbial burden this step is necessary, because the following steps are also not conducted under closed conditions. In the next step - the capture step – the majority of impurities, such as host cell proteins and DNA are depleted and the antibody is concentrated. In a typical antibody production process as depicted in Figure 1 the clarified antibody solution is captured by staphylococcal Protein A affinity chromatography. The antibody is eluted from the protein A stationary phase at low pH and virus inactivation at low pH is therefore reasonable at this stage of the process. To prevent a potential risk of viral contamination of the production cell line, two orthogonal virus inactivation steps have to be integrated into the process. Further antibody polishing is done by at least one chromatographic step. Various chromatography adsorptions and separation principles such as hydrophobic interaction, ion exchange or mixed chromatography mode are employed. Ion-exchange chromatography is very common for this purpose. Anion exchange chromatography is used to reduce residual DNA and HCPs whereas cation exchange chromatography is used to achieve a reduction of the antibody aggregates level, residual HCPs and sometimes protein variants. After polishing or after ultra/diafiltration a second orthogonal virus clearance is required. This is usually achieved by nano-filtration using membranes, which excludes small

virus, but antibodies are passed through. Finally, an ultrafiltration/diafiltration step is required for product concentration and buffer exchange followed by the final product formulation [1, 2].

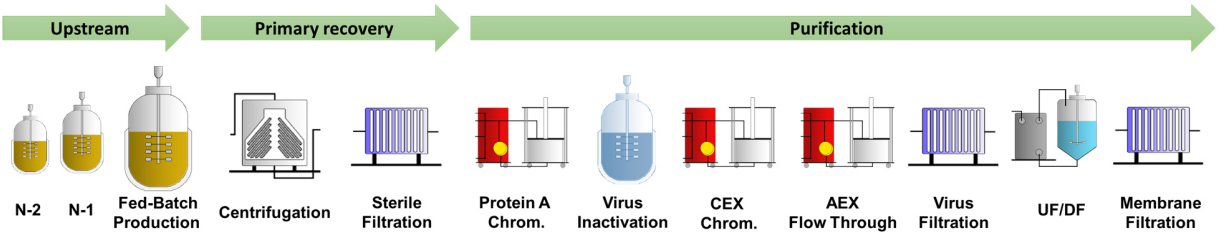

Figure 1: Flow scheme of a usual antibody production process.

1.2 Regulatory issues of continuous biopharmaceutical production

Continuous production describes a production process, where a product is continuously produced without any interruptions. Continuous manufacturing is already well established in the chemical and pharmaceutical industry but biopharmaceutical production lags behind [3]. Continuous production in biopharmaceutical manufacturing would increase productivity, flexibility and product quality while reducing facility size and equipment costs [4, 5]. Despite all the benefits of continuous production, several concerns remain in regard of e.g. regulatory issues, the definition of a batch, process control strategies and process monitoring. Regulatory organizations, industry and academia are already working to solve these issues [6]. The U.S. Food and Drug Administration included continuous manufacturing already in their guidelines to clarify such uncertainties. Continuous production is in line with the regulatory attempt to implement quality-by-design (QbD) and process analytical technology (PAT). This implicates necessary process understanding, risk management and control strategies complemented by real-time measurements and model based data prediction. Hence, product quality should be built-in with the overall objective of a safe product with constant quality [7, 8]. A typical batch is defined as: “Batch means a specific quantity of a drug or other material that is intended to have uniform character and quality, within specified limits, and is produced according to a single manufacturing order during the same cycle of manufacture.” [9]. Hence terms like “cycle of manufacture” are not applicable for continuous production, there is a different definition for continuous production which is termed lot: “Lot means a batch, or a specific identified portion of a batch, having uniform character and quality within specified limits; or, in the case of a drug product produced by continuous process, it is a specific identified amount produced in a unit of time or quantity in a manner that assures its having uniform character and quality within specified limits”[9]. In continuous production a batch or lot is not physically separated. Surge tanks between continuous unit operations and various continuous unit operation itself – such as continuous

stirred tank reactors or chromatographic operations – do not allow plug flow and result in broad residence time distribution (RTD). Therefore, boundaries have to be defined in respect to the residence time distribution. This requires proper process understanding and validation of the RTD. In case of a disturbance, which cannot be handled by process control it has to be clear which lot is effected or might be out of specification. Engisch and Muzzio cover this issue by RTD simulation and disturbance propagation in a continuous pharmaceutical pelleting process [10]. Despite all these concepts and regulations there are still concerns in continuous production in biopharmaceutical production, which have to be debated. Other concerns are, how to deal with startup and shutdown phase, what to do if the process runs out of specification or how narrow such a lot has to be defined. To answer these questions, all parties reaching from industry to regulatory authorities and academia have to be included into scientific discussion.

1.3 How to convert a batch process into a continuous process

The attempt to convert an existing antibody process could be based on a radical strategy where completely new unit operations are employed or a conservative strategy where existing unit operations conducted in batch are modified to continuous operations. The latter strategy will most probably lead to a pseudo continuous process in which the product stream is not continuous but cyclic, as depicted in Figure 2.

The principle of continuous production in biotechnology is a continuous inflow of a feedstock, a continuous reaction and a continuous outflow of a product or intermediate. Bioreactors can be run continuously using continuously stirred tank reactors (CSTRs), packed bed reactors or plug flow reactors (PFR) [11]. For production of antibodies with mammalian cell culture continuous upstream processing can be done by perfusion cell cultures, where cell culture broth is continuously withdrawn through a membrane, whereas cells are retained. This cell retention is usually realized by tangential flow filtration (TFF) [12], alternating tangential flow filtration (ATF) [12, 13], acoustic wave separation [14] and continuous centrifugation [15, 16]. ATF and continuous TFF are the most common methods for continuous antibody production with perfusion cell culture. The advantage for downstream processing is the delivery of an already clarified culture supernatant. Only removal of fines is necessary for further processing. This can be accomplished by membrane or depth filtration. Chromatography can be run continuously by multicolumn chromatography [13, 17, 18], simulated moving bed chromatography [19-21], annular chromatography [22, 23] or chromatography run in flow through mode where usually membrane absorbers are used [24, 25]. Product concentration and buffer exchange can be accomplished

continuously by single-pass tangential flow filtration [26], single-pass diafiltration [27, 28] or countercurrent diafiltration [29]. Continuous virus inactivation can be achieved by tubular or packed-bed reactors, CSTRs or coiled flow inverters [30]. These assure that a certain residence time at distinct inactivation conditions (low pH or exposure to solvent/detergent) are maintained [31]. Alternative unit operations for continuous antibody purification are aqueous two-phase systems (ATPS) [32, 33], antibody crystallization [34] and precipitation [35-37].

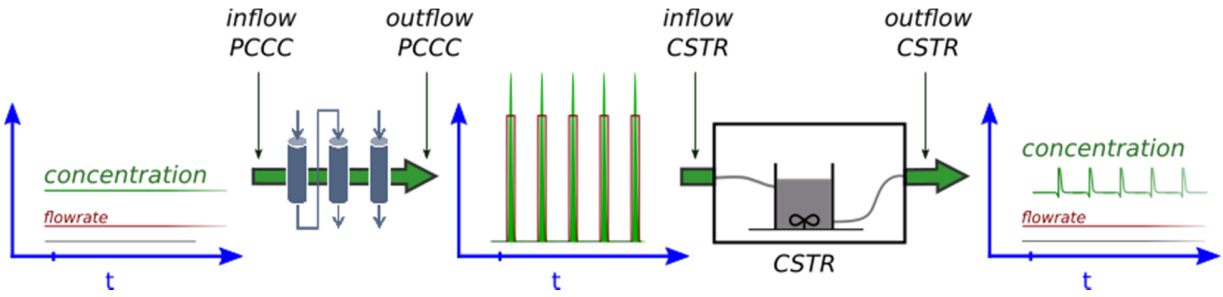

Figure 2: Pseudo continuous process – for example periodic countercurrent chromatography (PCCC) with continuous product inflow into the system but interrupted product outflow because of the bind-elute based operation mode. The discontinuous elution peaks can be collected in a continuous stirred tank reactor (CSTR).

It has to be highlighted that several of this continuous unit operations per se run in a pseudo continuous mode as they have an interrupted product mass flow out of the unit operation. An example is periodic countercurrent chromatography (PCCC). PCCC is a bind-elute based chromatographic method [38]. Hence, PCCC has a continuous product inflow but an interrupted product outflow (Figure 2). These interruptions can be resolved by collecting the elution peaks in a CSTR. However, concentration fluctuations in the outflow of a CSTR may occur due to the fact, that elution peaks have a distribution and do not have an ideal rectangular shape (Figure 2). This causes concentration spikes at the outlet of the CSTR because the concentration of the peak over time varies from low concentrations in the beginning followed by a concentration increase up to a peak maximum and again a concentration decrease to the end of the peak. If an elution peak is collected in a CSTR, this results in a slight dilution at the beginning of the peak because the antibody concentration it the peak shoulder is lower than in the CSTR. At the peak maximum, there will be a concentration spike in the CSTR, as the antibody concentration in the peak is higher at this point than in the CSTR. Indeed, this fluctuations are dependent on various parameters such as CSTR volume, mixing performance, peak shape and so on. Nevertheless, additional surge tanks or vessels are not desired to keep the process simple. The aim is an uninterrupted product mass flow through each unit operation and ideally through the whole production process. To achieve this objective, completely new unit operations and downstream strategies have to be considered.

1.4 State of the art in continuous antibody production processes

Several groups and companies currently work on continuous biopharmaceutical production. Warikoo, Konstantinov and coworkers interconnected a twelve liter perfusion cell culture directly to a continuous four-column PCCC capture step to process monoclonal antibodies as well as a recombinant human enzyme [13]. In additional work, they added an interconnected cation exchange PCCC step and an anion exchange membrane adsorber operated in flow through mode as intermediate and polishing step. They showed, that interconnecting continuous upstream and continuous downstream is feasible and stable product quality over the whole process time was achieved [4]. Steinebach and coworkers developed a similar integrated continuous antibody production process in lab scale. They interconnected perfusion cell culture with alternating tangential flow filtration as cell retention unit with a two-column protein A capture process. This was followed by virus inactivation at low pH in a surge vessel and multicolumn countercurrent solvent gradient purification (in bind-elute mode) plus a flow through chromatography for product polishing. They claim steady state and constant process performance throughout the entire process [39]. Another conceivable concept is a hybrid process, where batch operations are interconnected with continuous unit operations. Klutz et al. showed the economic benefits analyzing the cost of goods of a batch and a continuous antibody production process. They came up with a hybrid process as the most economically beneficial process. A hybrid process combines the benefits of a fed-batch upstream process with lower media costs compared to perfusion cell culture and the continuous downstream process, which comes with better resin utilization compared to batch chromatography [40]. Walther, Konstantinov and others used economic tools to characterize antibody as well as non-antibody manufacturing processes. They compared a conventional batch based production process, an integrated continuous production process and a hybrid production process and found, integrated continuous production is the most beneficial concerning cost savings and flexibility [41]. Several reviews list continuous or semi-continuous unit operations and discuss the advantages and drawbacks of continuous production [5, 11, 42]. Jungbauer additionally raises concern about the downscale-ability and the high material demand for process development as well as the process comparability because of various production scenarios [11]. Somasundaram et al. gives a broad overview of the current challenges in continuous production including valves, probes and column integrity testing within continuous production. Additionally he emphasize the importance of a holistic view on a continuous biomanufacturing process [42]. Anyway, a full-scale integrated continuous pilot scale facility is not reported so far.

1.5 Filtration

The principle of filtration is based on the size of the solid particles or molecules. Solids, particles and molecules that are bigger than the pore size of the filter material are retained either on top or in the filter media, whereas smaller fractions flow through the filter media. Filtration can be performed either by dead-end filtration – the feed flow is orthogonal to the filter layer – or tangential flow (cross flow) filtration, where the feed flow is parallel to the filter membrane (Figure 3).

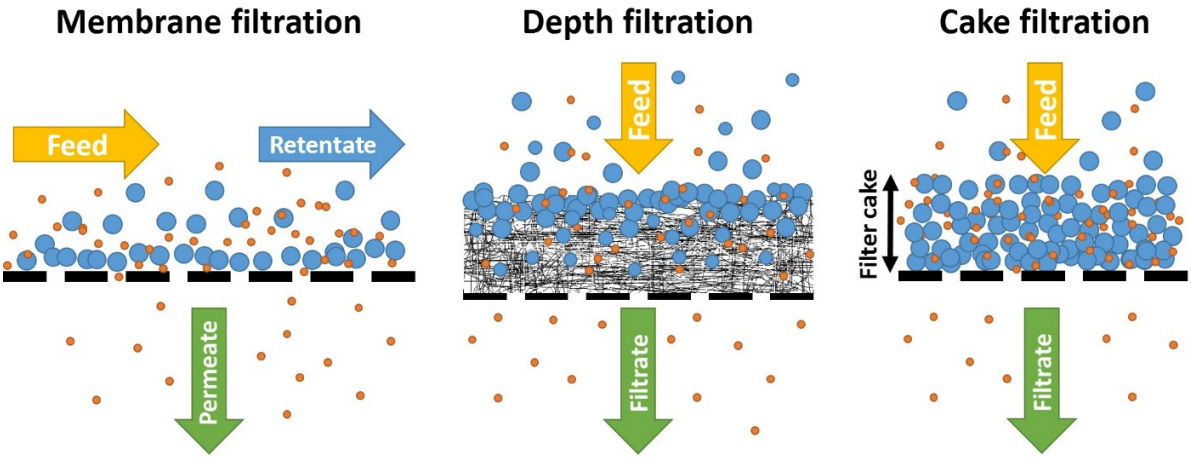

Figure 3: Filtration principles – (left) Membrane filtration: can be run in dead-end (feed flow orthogonal to filtration membrane) and in cross-flow or tangential flow filtration mode (feed flow parallel to filter membrane, filter cake is flushed away); (middle) Depth filtration: feed flow orthogonal to filtration membrane, particles penetrate into the filtration layer (right) Cake filtration: feed flow orthogonal to filtration membrane, filter cake builds up over time and acts as a filter itself.

Dead-end filtration can be subclassified in membrane filtration, depth filtration and cake filtration. In cake filtration, filtered solids and optionally filter aids build up a filter cake over time, which acts as a filter itself. In depth filtration solids can penetrate the filter material dependent on their size. Various filter layers with different cut-offs, permeability or functional materials are commercially available. In this work Clarisolve® filters from Merck were used, which are positively charged filters, composed of polypropylene and cellulose and additional diatomaceous earth which acts as filter aid material. Clarisolve® filters are specially developed for clarifying flocculated cell culture broths. An issue that has to be addressed here are the use of filter aids. Filter aids are insoluble materials, which are added to the filter feed to improve permeability of the filter cake or are incorporated in the filters itself to improve permeability. Commonly used filter aids are diatomaceous earths, a natural compound of the skeletal remains of diatoms (algae) and perlite, a volcanic mineral. Diatomaceous earths composed mainly SiO_2, perlite mainly SiO. They contain significant amounts of Al_2O_3 of approximately 4 to 12 weight %, respectively [43]. Leakage of

substances of this natural compounds cannot be precluded. The use of such filter aids containing filters can cause a leakage of aluminum, which is critical in biopharmaceutical production and has to be taken into consideration.

In dead-end filtration, the flux through the filter membrane declines over time because of an increase of the cake thickness over time. This can be avoided by tangential flow (cross flow) filtration. The feed flow is parallel to the filter membrane and the shear force of the fluid flushes away the accumulated material from the filter membrane surface. This prevents the permeate flux through the filter membrane [43]. The pore size of filter membrane defines the cut-off of the retained solutes and/or solids. In biopharmaceutical processes filtration is classified in microfiltration (cut off 10 µm – 0.1 µm), ultra filtration (100 nm – 10 nm), nano filtration (10 nm – 1 nm) and reverse osmosis (below 1 nm). Continuous operated tangential flow filtration can be realized by single-pass tangential flow filtration, by partial recycling of the retentate and sequentially arranged multiple filtration units [11]. Continuous dead-end filtration can be accomplished by parallelization of filters or e.g. continuous rotary drum or belt vacuum filters.

1.6 Cell flocculation

Flocculation is widely used in biotechnological applications. The most prominent application is wastewater treatment where organic and inorganic matter colloids and microorganisms coagulate and flocculate to gain better sedimentation and filtration properties, hence a better clarification efficiency of the waste water [44]. In biopharmaceutical applications, flocculation is used to boost primary recovery of cells and cell debris. With higher product titers and higher cell densities more efficient separation is required to avoid product loss e.g. due to frequent discharge cycles in centrifugation or increased filter area demand. Flocculation can also provide additional purifying effects such as a reduction of DNA, HCPs [45, 46] and viruses. Akeprathumchai et al. reports a 1000 fold retrovirus reduction by the use of a cationic polyelectrolyte [47]. Flocculation applications are reported for various biotechnological expression systems such as microbial systems [48, 49], transgenic plants [50] and mammalian cell systems [46, 51-54].

The overall net charge of the cell surface (e.g. of a CHO cell) is negative. Due to this negative charge, the cells repulse from each other. Flock formation can be induced by adding charged polymers also named polyelectrolytes. Various cationic polymers are reported for cell flocculation such as polyethylenimine [48], polydiallyldimethylammonium chloride (pDADMAC) [46, 51], chitosan [52] or modified polyallylamine [53]. Flocculation by charged polymers is described by two mechanism, flocculation by bridging and flocculation by electrostatic patches. A precise differentiation between these two

mechanisms is not possible as presumably both mechanisms contribute to flock formation to varying degrees. Polymers have a high molecular weight and therefore a low diffusion coefficient. The addition of charged polymers for flocculation to a cell culture broth requires sufficient mixing. The adsorption of the cationic polymer to the negative charged cells is fast and irreversible at neutral conditions due to multiple attachments of the polymer chain to a cell. The addition of salts and therefore the increase of ionic strength can benefit the adsorption of polymer and cells as it makes the charges of a polymer chain more accessible and may reduce repulsion between particles with the same charges. Bridging behavior, hence flock formation, is dependent on the molecular weight, flexibility and charge density of the polymer. Polymers with high molecular weight can extend their charged loops further into the solution compared to short polymers, which benefits the particle bridging. A more flexible polymer will attach to more binding sites than a rigid polymer; hence, leading to stronger binding and bridging. A high charge density is also beneficial for bridging . This leads to repulsion of adjacent charged groups on the polymer, hence the polymer extends further into solution.

Another essential parameter of flocculation is the polymer concentration. If the ratio polymer to cell is too high, the charges of the whole cell will be covered by polymer chains and shield them from bridging with other polymer-cell-particles and flocks. In contrast, if the polymer-cell ratio is to low, not enough polymer chains are available to bridge between the cells. Mixing is essential to disperse the polymer in solution. The strength of flocks is dependent on the number of bridges that are formed. Thus, strong mixing reduce the bridging effect, as the positive charges of the polymer and negative charges of the cells are hinder to get in sterical proximity. For effective and stable flocculation, a trade-off between these parameters has to be found [55, 56] (Figure 4).

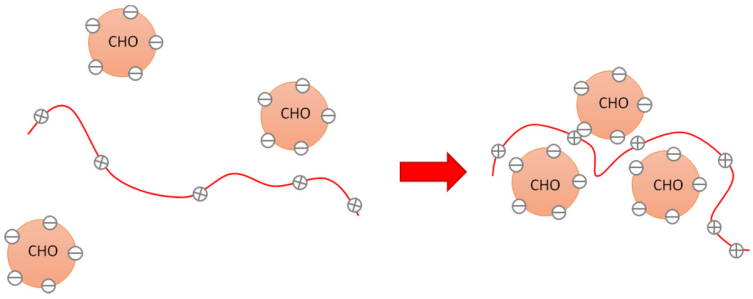

Figure 4 Principle of flocculation by bridging with a cationic polymer (e.g. pDADMAC)

The second mechanism described for flocculation by charged polymers is flock formation by electrostatic patches. Cationic polymer chains tightly adsorbs on the surface of the negative charged cell and forms

positively charged batches. After alignment of negatively charged cells with positively charged batches on their surface, the repulsion of the cells is decreased and flocks can form [55].

Other mechanisms for flocculation without polymer additions are acidification and co-precipitation. A pH shift of the cell culture broth neutralize the surface charge of cells and cell debris and reduce the electrostatic repulsion and therefor cause flocculation [54, 57]. The principle of co-precipitation is based on the addition of salts (e.g. calcium and phosphate) with low solubility, which instantaneously precipitate. Such salt-precipitates additionally co-precipitate large particles, cells and DNA [58].

1.7 Separation by precipitation

Precipitation can be employed for protein purification in two ways. Either impurities are precipitated and the product remains in solution or the product is precipitated and present in solid state. Hence, if the product is precipitated, the supernatant can be withdrawn, which significantly reduces the volume. The main principle of protein precipitation is to reduce the solubility of a protein below its solubility limit and it precipitates. A reduction of the protein solubility is achieved by adding a precipitant such as salts, polymers or solvents or by changing the pH, ionic strength or temperature or a combination thereof. This has been successfully demonstrated from our group [35, 36, 59-64] and others [37, 65-67]. The most famous example which is still in use in large scale is the blood plasma fractionation by Edwin J. Cohn where blood plasma is fractionated by variation of ethanol concentration, temperature and pH [68, 69].

For proteins in aqueous solution, it is energetically more favorable to stay in solution. This is described by two theories, the double layer theory and the hydration layer theory. Proteins in aqueous solutions attract each other by Van der Waals force. This protein-protein attractions are shielded by an electrical double layer consisting of the stern layer and the diffuse layer. The structure and thickness of these layers is dependent on the protein surface structure (electrostatic charge) and the surrounding solution (ion concentration and dielectric constant). To overcome this repulsive forces, ions can be added, which decreases the potential energy and lead to attraction of the proteins, hence the destabilization of the protein solution. The colloidal stability of a protein solution can be determined by measuring of the zeta-potential. A high zeta-potential means that the protein solution is stable. A second theory is that a hydration layer surrounds the protein. The addition of salt detract water from the hydration layer into the bulk and therefore make hydrophobic regions accessible for protein-protein interactions. This mechanism is called salting-out. The solubility of a protein is given by the equation 1.

$$lnS = \beta - K * I \qquad \text{(Equation 1)}$$

The solubility of a protein in solution S is defined by the ionic strength I multiplied by the salting-out constant K, which is protein and salt dependent. The constant β defines the solubility of the pure protein and dependent on the pH and the temperature. High molecular weight proteins precipitate at lower salt concentration. This effect can be exploited for the purification of high molecular weight products such as antibodies. Equation 1 was expanded for polymers by Juckes [70].

$$K = \frac{v}{2.303}\left(\frac{r_s + r_r}{r_r}\right)^3 \qquad \text{(Equation 2)}$$

He expanded the constant K by adding an empirical form factor v, which considers the structure of the polymer. Moreover it includes the radios of the protein r_s and the radius of the polymer r_r.

Protein precipitation with solvents is based on a similar principle. Solvents have a lower dielectric constant than water. Hence, solvents increase the electrostatic permeability of a protein solution. This leads to a combination of two effects; an increase of protein-protein interaction and a reduction of the hydration layer, hence to protein precipitation. Drawbacks of solvent precipitation are, solvents can cause protein denaturation and solvents are often flammable which causes safety issues. Nevertheless, the most popular example, cold ethanol precipitation has been applied highly successfully.

A selective precipitation technique is affinity precipitation where an affinity ligand and target protein complex is formed. The lower solubility of this ligand-target complex leads to precipitation. The reversible ligand-target protein complex has to be dissociated similar as in protein affinity chromatography (by pH shift, addition of a competitor, etc.) [71]. The addition of inorganic metals ions such as Fe^{3+}, Cd^{2+}, Zn^{2+} or Cu^{2+} can have selective effects as well [72]. Another important factor is the isoelectric point of a protein, where the net charge is neutral, hence proteins at the pI have their lowest solubility. This property of a protein allows precipitation by shifting the pH similar to the pI value of the protein.

In my thesis, I focused on precipitation by polyethylene glycol, a linear chained polymer, available in various molecular masses [61]. The precipitation mechanism of PEG is still not clearly understood but two theories were formulated to describe the mechanism: the excluded volume theory and the attractive depletion theory. The excluded volume theory assumes that the addition of a precipitant such as a polymer detracts solvent and consequently reduces the water that is available for solubilization of the protein. This effectively excludes the protein from parts of the solvent. The protein precipitates when the solubility in the water that is still available is exceeded. This effect is usually directly proportional to the molecular size of the protein as well as for the polymer [73, 74]. A second theory is attractive depletion. The protein is surrounded by a depletion zone, where the PEG concentration is lower as in the bulk

solution. If two depletion zones overlap, entropic effects of the polymer lead to an attraction of the proteins which cause precipitation [75, 76].

A phase diagram for precipitation and crystallization plots the protein concentration over the precipitant concentration. The diagram is divided in three zones: the soluble zone, a crystallization zone and the precipitation zone. An additional, metastable zone between soluble and solid phase can be described. After addition of a precipitant to a protein solution, precipitation occurs instantaneously, as far as the protein concentration and precipitant concentration fulfill the requirements and reach the precipitation zone [77, 78]. The precipitation process itself can be described in three stages. The first stage is called nucleation. After addition of precipitant to a protein solution submicron particles are formed. Sufficient mixing is required to avoid spots with high concentration of precipitant and co-precipitation of other proteins. After nucleation, the particles grow. As this process is diffusion driven, mixing is required during this stage of precipitation. In a final stage the precipitate ages where it is getting more dense. Excessive mixing in this stage can cause breakage and erosion of precipitate [72]. These three stages of precipitation occur simultaneously and result in a broad particle size distribution of the precipitate. As precipitant dosing and efficient mixing are critical process steps in precipitation, this may cause issues in large-scale batch process where homogenous mixing becomes a significant problem. Dosage of the precipitate over time might be a solution, to avoid spots in the batch vessel with high precipitant concentrations and assure homogenous mixing. The increased viscosity of the precipitate suspension decrease mixing efficiency in batch vessels. To overcome these issues, continuous precipitant addition and continuous incubation in tubular reactors was selected, as its effectiveness was already shown by others [34-36].

2 Objectives

The objective of this work is to develop a fully continuous integrated operation of a recombinant antibody purification process for primary recovery and product capture. Primary recovery of a Chinese hamster ovary cell based production process should be performed by continuous cell flocculation and continuous flock separation. Furthermore, a fully continuous product capture step by antibody precipitation and a fully continuous solid-liquid separation of the antibody precipitate should be developed. Feasible operation conditions for precipitation and precipitate re-solubilization have to be found. A focus should be on an uninterrupted continuous product in- and continuous product outflow of the unit operations, which enables easy integration into any continuous purification scheme. A simple and stable process is required, as it reduces the necessity of complex process control strategies.

The main research question: Is steady state a requirement for continuous precipitation?

3 Discussion and additional results

A continuous cell flocculation process for primary recovery and a continuous antibody precipitation process for product capture was developed. In both cases, the feed stream with flocculant/precipitant were combined and mixing and incubation were achieved by simple tubular reactors with built-in static mixers. Membrane filtration was used for continuous flocculation as well as for continuous precipitation to separate the solid fraction from the liquid fraction.

3.1 Continuous cell flocculation

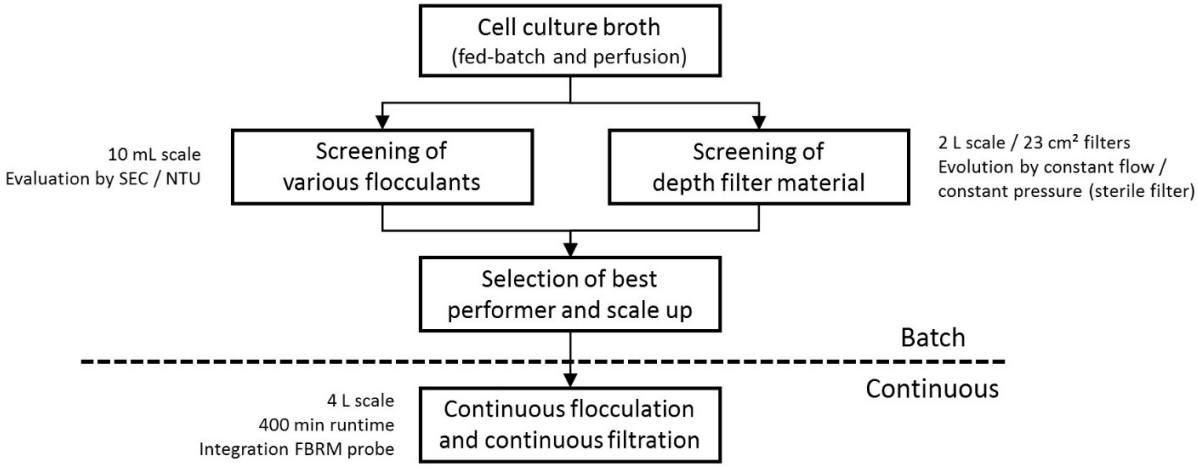

Figure 5: Workflow of the development of the continuous cell flocculation.

The first publication is about the development of a continuous operated cell flocculation and continuous cell removal by parallelization of depth filtration. The workflow, how continuous operation was achieved, is depicted in Figure 5. The flocculation efficiency of various flocculants, flocculant additives and conditions were screened on the basis of different, recombinant antibody containing cell culture harvests produced by fed-batch and perfusion cell culture, respectively. The clearance efficiency was evaluated by size exclusion chromatography and by the reduction of the Nephelometric Turbidity Units (NTU) before and after flocculant addition. Suitable flocculation conditions were then tested on various depth filter materials. This experiments were done by our project partner Merck. To assess the required filter area, flocculated material was clarified and the filterability was determined by constant flow and constant pressure method. The constant flow method plots the filter resistance (pressure per LMH (liter per square meter per hour)) over the filtrate volume (liters per square meter filter area). An increase of the filter

resistance indicates filter plugging. The constant pressure method assess the maximum volume, which can be processed by a filter. Therefore, the filtrate of the constant flow test was filtered at constant pressure (0.5 bar) by sterile filters (0.2 µm). The aim is not to characterize the sterile filters, however it gives an indication about the clearance efficiency of the different tested depth filter materials. pDADMAC in combination with Clarisolve® filters from Merck turned out to be the best performer in regard of product yield, necessary filter area and stability of the flocculant to cell concentration ratio.

To convert batch flocculation process into continuous cell flocculation, the flocculant was continuously added to the cell containing cell culture feed. The two streams were simply combined and the mixture was incubated for a certain period of time in a tubular reactor. Inbuilt static mixers were introduced into this stream to prevent flocks from settling and to achieve mixing due to active pumping through the tubular reactor. Figure 6 shows that tubular reactors with inbuilt static mixers have narrow residence times compared to tubes without static mixers. The residence time of a tubular reactor is defined by the flow velocity through the tube, the tube diameter and the tube length. Such simple design brings many advantages. Residence time and the scale of the tubular reactor can be varied by adapting the flow velocity, the tube dimension or the tube length. Commercially available standard tubing and fittings were used, hence, the setup is very feasible for single-use.

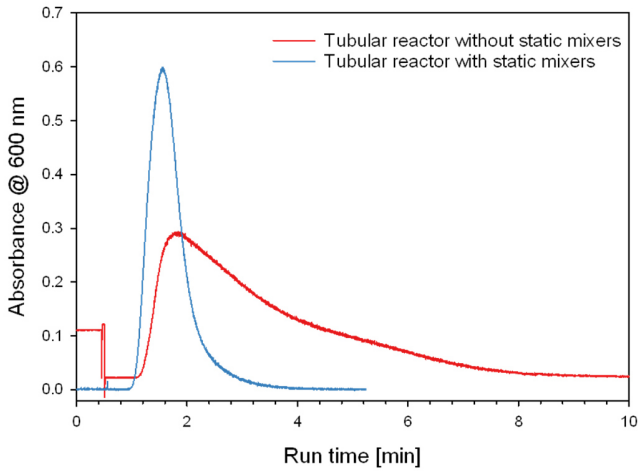

Figure 6: Residence time distribution of an antibody precipitate. (Red) Tubular reactor without static mixers. (Blue) Tubular reactor with inbuilt static mixers.

Continuous solid-liquid separation for flocculated cell culture broth was achieved relatively simple by parallelization of depth filtration. When the feed pressure of the depth filter reached a certain pressure value, hence the filter is supposed to be fully loaded, the filter feed stream was switched to a second

filtration line. Meanwhile, the first filter was exchanged and flushed to be ready for another filter cycle. To avoid manual handling throughout the clarification, more than two filter lines or a bigger filtration area can be installed, alternatively. Additionally, Focused Beam Reflectance Measurement (FBRM) was applied to evaluate flock size and precipitate size. The probe was inserted into a feed stream or stirred vessel to measure the flock size according to the chord length of the reflected laser beam. The signal counts give a hint on the number of flocks in the suspension. It has to be taken into account, that this method does not give an absolute value, but allows to assess the flock characteristics [publication I].

3.2 Continuous operation for product precipitation

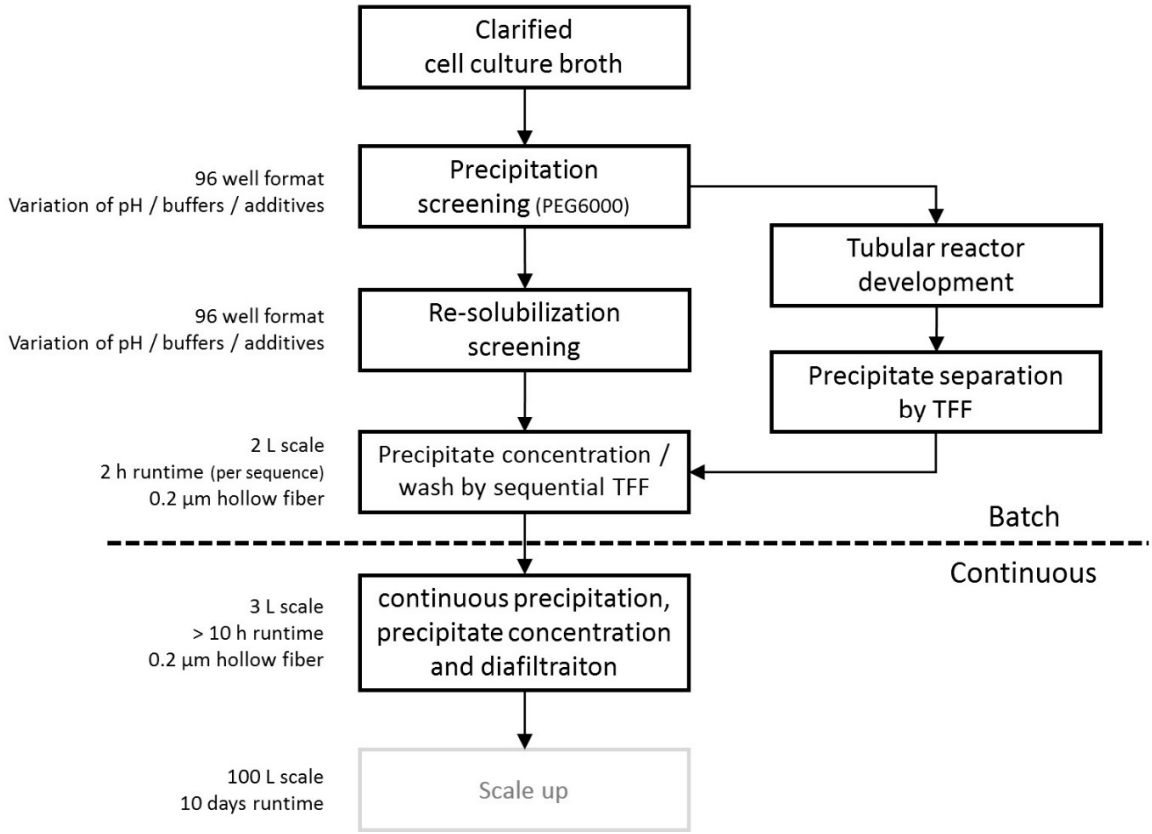

Figure 7: Workflow of the development of the continuous precipitation, precipitate concentration and precipitate diafiltration.

After a primary recovery follows the product capture step, where main impurities such as HCPs and DNA are removed and the product is concentrated. My second publication is about the development of a continuous capture step for antibodies based on protein precipitation by PEG. The workflow of the development of a continuous product capture step by precipitation is depicted in Figure 7.

Various precipitation conditions as well as re-solubilization conditions were screened. To find the best performing conditions PEG6000 amount, buffer composition, pH values and salt addition were varied. In parallel a continuous precipitation reactor similar as for cell flocculation and continuous precipitate separation was developed. The challenge of continuous solid/liquid separation in this operation is: the product (antibody) is present in solid state, whereas majority of impurities remain soluble after PEG precipitation. This is in contrast to flocculation where the antibody stays in solution and solid impurities (cells, cell debris, probably DNA) are removed. Avoiding product precipitate compression is mandatory, because a compressed precipitate pellet results in slow re-solubilization kinetics. Centrifugation or dead-end filtration are not feasible, because the precipitate is compressed and is hard to re-solubilize. Preliminary experiments by Hammerschmidt et al. showed fast re-solubilization kinetics, if the precipitate is in lose state [35]. Continuous precipitate capture by depth filters and re-solubilization by backflushing this filters with solubilization buffer was tested. Unsatisfying yields of 55% were gained obviously because of the very slow solubilization kinetics when the precipitate pellet is compressed on depth filters. This probably goes in line with channeling of the solubilization buffer through the antibody pellet forming filter cake. Hence, the solubilization buffer do not sufficiently get in contact to the precipitate pellet for re-solubilization. Tangential flow filtration (TFF) using microfiltration hollow fiber membranes was selected for precipitate capture because this method guarantees that the precipitate is in lose state and re-solubilize instantaneously. The operation shown by Hammerschmidt et al. only allowed for batch wise concentration and wash of precipitate [35]. For a fully continuous operation I used a tubular reactor and two TFF-units in series.

For continuous precipitation, The antibody containing feed stream of clarified cell culture broth was combined with a feed stream of PEG6000 plus low amounts of zinc. The antibody precipitate was incubated and mixed in a tubular reactor containing inbuilt static mixers. In the first TFF stage, the antibody precipitate was concentrated by circulating the precipitate via a hollow fiber microfiltration membrane. The concentration factor can be defined by the ratio precipitate inflow/permeate outflow of the first stage. After an initial startup phase a concentrated precipitate bleed was withdrawn into the second stage. In the second stage wash buffer was continuously added to the concentrated precipitate and the washed precipitate was circulated again by hollow fiber microfiltration membrane. This continuous diafiltration ensures a reduction of soluble HCPs. The concentrated and washed precipitate was continuously harvested by a low bleed out of the second TFF stage (Figure 8). The whole process was exemplified by product capture of a recombinant antibody out of a cell culture broth. The system runs stable over 10 h and is capable to deal with product titer fluctuations – which may result from continuous

upstream processing – without interventions into the process. To predict a startup phase and when steady state is reached, a numerical based model dependent on in- and outflows of the system was set up. This predicted data of the model nicely fit to the measured data of the continuous run which gives a helpful tool to predict start-up phase and when steady state is reached [publication II].

Figure 8: Set-up of the continuous precipitation, continuous precipitate concentration and continuous diafiltraiton setup. A self-assembled tubular reactor with tow Äkta flux systems in series were used. The TFF systems were connected to 0.2 µm microfiltration hollow fiber modules.

Continuous cell flocculation and continuous precipitation and precipitate harvest use tubular reactors to guarantee a certain residence time, achieve mixing and prevent cell flocks and product precipitate from settling. In contrast to continuous product capture by PCCC with an interrupted product mass flow (continuous product inflow and discontinuous product outflow; Figure 2), this continuous methods ensure a continuous product mass flow for primary recovery and for capture. It avoids surge tanks and complicated automation or monitoring. Just minor adjustments of flow rates based on gravimetrically monitored tank levels have to be automated.

3.3 Process robustness of continuous precipitation

To test process robustness of the developed precipitation, a variety of different concentration conditions like temperature and pH were tested. It was shown that the precipitation kinetics of various precipitation conditions (13.2% (w/v) PEG6000, pH 7.5 and pH 8.4, temperature: 10°C, 20°C and 30°C) tested in a stirred vessel are not constant. Precipitation is induced instantaneously and after the precipitant addition and an initial spike in particle numbers detected by the FBRM probe, the signals of all fraction sizes from 0 up to 1000 µm decreased over time. This observation was true for all tested conditions. It probably represents the aging of the precipitate over time, where the precipitate gets denser and is ruptured by the agitation of the stirrer. Antibody precipitation at pH 8.4 – the isoelectric point of this antibody – showed the strongest response in the FBRM signal over time (Figure 9.A) compared to precipitation at neutral pH, where the signal decrease over time was not as steep. This confirms the theory, as at the pI of a protein, its overall charge and therefore its solubility is the lowest. Steady state of precipitate size distribution and counts was reached after two hours of runtime or longer. Similar results were achieved for CHO cell flocculation with 0.0375% pDADMAC in a stirred vessel. The cell flock size changes over time. Steady-state is reached after around two hours (Figure 9.B). As no steady state in batch experiments was achieved within a feasible time, the question was raised: Is reaching this steady state required for continuous precipitation?

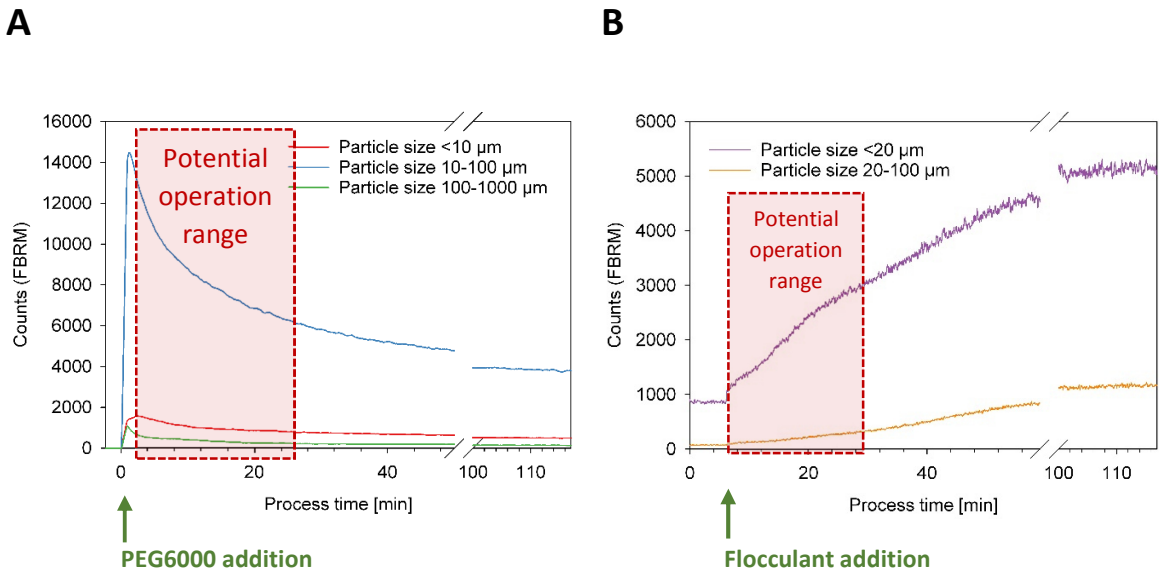

Figure 9: (A) Precipitation kinetics in batch; 13.2% (w/w) PEG6000; pH 8.4 = pI; 300 rpm agitation speed. (B) CHO cell flocculation kinetics in batch; Flocculation kinetics: 0.0375% pDADMAC; 276 rpm agitation speed.

To answer this, the influence of residence time of the continuous precipitation was evaluated. Various reactor lengths were chosen to obtain residence times from 2.5 min up to 25 min. This represents a reasonable operation window as depicted in Figure 9 and will answer the question if we should operate closer to the steady state conditions, or if it is not necessary to reach steady state. The precipitate was monitored over time by Focused Beam Reflectance Measurement (FBRM) in combination with a self-assembled flow cell. The flow profile inside the flow cell is directed that it impacts the probe in a 45 degree angle. It was observe, that dead volumes inside the flow cell as well as how deep the probe penetrate the feed stream has severe influence on the number of counts. This explains, why the number of counts at an antibody concentration of 3.0 g L^{-1} and at a residence time of 25 min (Figure 10.C) are lower (below 3000) than at the other conditions (Figure 10.A/B/D). In preliminary experiments probe fouling was observed starting after approximately two hours of runtime. For this investigation, an operational time of two hours is sufficient, but for operation in a production process, a redesign of the FBRM flow cell is required to gain stable and reproducible results. Anyhow, the size distribution of the precipitate was stable over the tested period independent of the residence time (RT 2.5 min Figure 10.A/B and 25 min Figure 10.C/D).

In parallel to evaluation of the influence of the residence time, the cell culture broth (initial titer 3.3 g L^{-1}) was spiked with protein A purified antibody to a final concentration of 8.0 g L^{-1}, to see, if the antibody titer has an influence on the continuous precipitation. Within the tested conditions, the size distribution of the precipitate was independent of antibody concentration (3.0 g L^{-1} Figure 10.A/C and 8.0 g L^{-1} Figure 10.B/D).

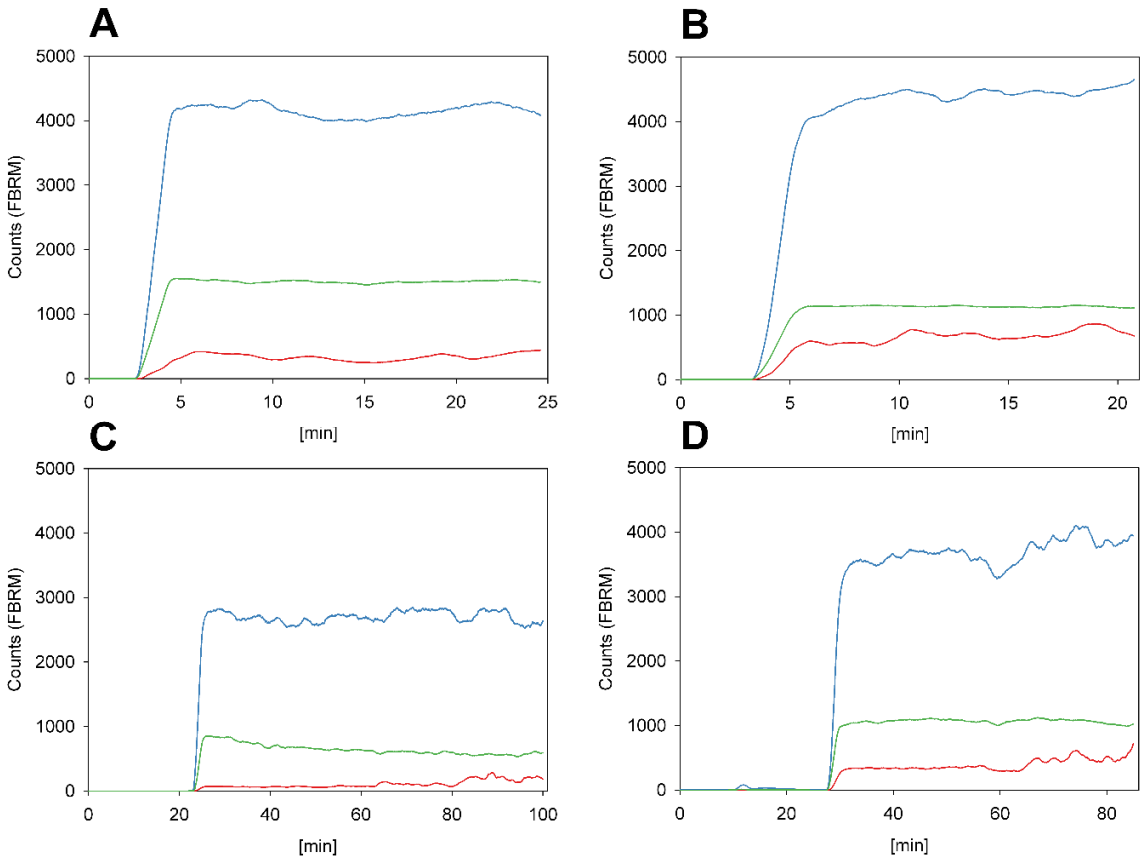

Figure 10: Process robustness of the size distribution of precipitates in a tubular reactor; (A) RT 2.5 min / 3.3 g/L antibody; (B) RT 2.5 min / 8.0 g/L antibody; (C) RT 25 min / 3.3 g/L antibody; (D) RT 25 min / 8.0 g/L antibody. In red: precipitate in a size range below 10 µm; In blue: precipitate in a size range of 10-100 µm; in green: precipitate n a size range of 100-1000 µm.

The results above shows independence of precipitate size in regard of residence time in the tubular reactor as well as in regard of the initial antibody concentration of the clarified cell culture broth. Additionally to the precipitate size characterization by FBRM, the antibody yield, antibody monomer purity and the high molecular weight impurities were determined by size exclusion chromatography to see if residence time and initial antibody concentration have an influence.

As in Figure 11.A depicted, similar yields of 80 to 85% were achieved independent on RT (2.5 min and 25 min) and antibody concentration (3.3 g L^{-1} and 8.0 g L^{-1}). It has to be noted, that this experiments were performed with non-optimized conditions. A step yield of 80 to 85% in an antibody production process would not be acceptable for an antibody capture step.

The monomer purity of the cell culture broth, spiked with protein A purified antibody, was around 30% higher, because the impurities of the CCB were diluted by the addition of pure antibody to gain an

antibody concentration of 8 g L^{-1}. As depicted in Figure 11.B, antibody purity is independent of RT. Anyhow, due to this dilution of the impurities by pure antibody, precipitate of the spiked CCB showed an approximately 10% higher purity than the non-spiked CCB (Figure 11.B). With a similar impurity profile, no dependence on monomer purity is expected.

High molecular weight impurities are 4% higher for CCB compared to spiked CBB. This is again, because the CCB was spiked with purified antibody with an initial HMWI content of 2%. After the precipitation, the HMWI content was reduced by a factor of 2 to 4. It was assumed that precipitated aggregates do not completely re-solubilize by the addition of solubilization buffer and therefore remain solid and are removed before analytics. The option that aggregates do not precipitate was excluded, because antibody aggregates were not enriched or could not be found in the supernatant after antibody precipitation. Moreover, there is no hint of generating new aggregates by precipitation. Within the tested range, the HMWI profile is similar. Hence, precipitation is independent either from residence time or from the antibody concentration within the tested range (Figure 11.C).

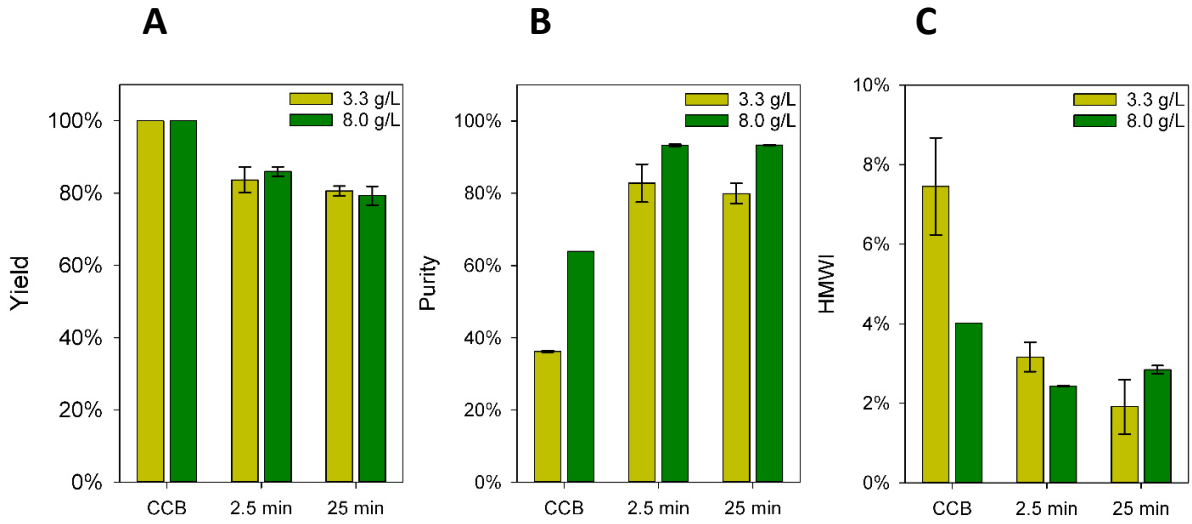

Figure 11: Process robustness of continuous precipitation in tubular reactors; Yield, purity and HMWI at residence times of 2.5 min and 25 min compared to initial cell culture broth (CCB). Olive bars spiked with protein A purified antibody. Standard deviation calculated over experiment period (up to 160 min) and repletion.

Continuous precipitation by tubular reactors is a potential alternative to conventional chromatography based product capture steps. It is a fully continuous unit operation with continuous product inflow and continuous product outflow. It was shown that precipitation is a robust method. It can handle titer

fluctuations or changes in the media matrix, which may result from preliminary interconnected perfusion cell culture. Such a stable process does not require complicated online or real-time measurements, such as UV detectors, fluorescence detectors, multi angel light scattering detectors in combination with refractive index detectors or Raman detectors. It enables simple control strategies without the need of online antibody concentration or HCP profile determination, as it is required for capacity and cycle time assessment in PCCC. A simple and predictable process does not require high capital investment in regards to monitoring and control equipment. Finally, it makes the process more efficient in regard of economical and product safety issues. An ideal production system would be a self-regulating system without the need of any active exertion of influence manually or by controllers. As we deal with biologic systems, various influencing factors contribute to the process. As it was proved, working in steady-state is not compulsory, as long as the process is run in a stable region. Therefore, we have to gain process understanding and find simple and stable solutions to end up in such stable and well-regulated processes.

3.4 Scale up of continuous precipitation

Scale up is a substantial issue for process development. Linear scalability is rarely possible, as handling of large volumes run into several issues in regard of heat transfer [79], mixing efficiency [79, 80], pressure limitations [81] and longer process durations for procedures such as pumping. Moreover, a scale up changes physical impact of various factors such as shear stress, liquid/air interface, etc. which can lead to structural changes of the product and cause product related impurities [82, 83]. Therefore, a simple scale up strategy is desirable, as it will result in safer products, lower production costs and more flexible processes and facilities. In parallel, scale down strategies in continuous production are relevant as well. Continuous processes require lots of material for process development compared to batch processes. Hence, simple (down)scalable processes are desirable reducing time and costs for process development [11].

For continuous processes using tubular reactors, scale up is rather simple. The volumetric throughput of a tubular reactor is either defined by the dimension of the tube or the flow velocity through the reactor. The residence time additionally depends on the length of the tubular reactor. The scale up of a tubular reactor system is therefore quite simple as you can ether increase the flow rate and/or tube diameter at constant residence time. The installation of inbuilt static mixers guarantee sufficient mixing. This prevent cell flocks and antibody precipitate from settling, uniformly distributes the flocculant/precipitant and avoids spots with highly concentrated flocculant/precipitant. High flow velocity results in higher shear rates, which will affect the size of the flocks or precipitate as it break or erode cell flocks and precipitate.

Flow rates up to 10 mL min^{-1} were tested, which corresponds to a linear velocity of approximately 75 cm min^{-1}. No issues were observed. Tubular reactors can be easily realized in single-use design, as tubing, static mixers and connectors are commercially available in various sizes and materials.

Cell flocculation before depth filtration reduced the necessary filtration area by a factor of four. Scale up of the depth filtration is simply done by increasing the filtration area. The parallelization of filtration guaranteed continuous product in- and out flow. To show the feasibility of parallelization of filtration, two filters were connected in parallel. In periodic cycles or if the inlet pressure reaches a critical value, which indicates a fully loaded filter, the filter line was switched manually without interruption of product flow. Scale up is simply achieved by an increase of the filter dimensions. Modular filter modules (e.g. Merck Clarisolve® or Millistak+® filters or Sartorius Sartoclear® filters) make the scale-up of the filter area very simple. An upscale parameter for filtration is the volume processed per membrane area per time unit (LMH or L/m²/h). If filter material, pore size, flow geometry et cetera of a filter module are kept identical, process parameters such as the filter flux and transmembrane pressure can be determined in lab scale and directly brought to process scale filtration by keeping the LMH constant [72].

	Run time 10 h		Run time 10 days	
Product harvest	81%	9.4 g	95%	279 g
Product trapped in system / flush out	14%	1.7 g	<1%	1.7 g
Product loss	3%	0.34 g	3%	8.2 g

Run time 10 days	
95%	2.8 kg
<1%	17 g
3%	82 g

Figure 12: Sketch of a scale up of the continuous precipitation, continuous precipitate concentration and continuous diafiltraiton with a yield and product yield assumption.

Continuous precipitation, continuous precipitate concentration and continuous diafiltraiton was demonstrated for 10 h. After 10 hours, 81 % of antibody had left the system, and a further 14% of precipitated antibody was flushed from the membranes and tubes after shut down. After recovering this product from membranes and tubes, a yield of 95% was gained. In a short experiment of 10 h, what is left in the device (filters and tubes) is very significant, but such a system is expected to be run for 10 days or

more continuously in a production setup. If the system is run for ten days, it can be expected that around 280 g of antibody can be captured. Moreover, the fraction trapped in the system gets negligible (less than 1%) if the process runs for ten days or longer. For such a system, a flush-out of the antibody in the system is not necessary anymore, reducing necessary buffer volumes, handling and avoiding dilution of the antibody at the end of the process. The system is linearly scalable using the aforementioned LMH concept. A scale up by a factor of ten will be capable to capture around 2.8 kg of antibody within ten days. Such amount of antibody is already in the range of the material required for clinical phase, as well as the in the range of the annual demand of some commercially available biopharmaceutical antibodies [84]. A scale up by a factor of ten gains ten times more product but increases the required footprint just a little. The tubular reactor will require a wider tube (9.5 mm ID instead of 4.8 mm ID) and the hollow fiber membrane with ten times the filter area is just twice as long (Figure 12). All in all just a little increase of footprint is needed to capture ten times of the amount of product compared to the lab experiment highlighting the small footprint needed for pilot and process scale continuous manufacturing.

4 Conclusion and Outlook

The ideal goal for a fully continuous platform process for immunoglobulins is to be run in fully continuous and integrated mode with non-interrupted product mass flow from buffer preparation and inoculation until formulation and packaging. One conceivable concept is the integration of several unit operations combining the benefits of constant product in- and outflow, low process monitoring effort and simple automation strategies. A feasible combination would be upstream processing by perfusion cell culture followed by cell retention by TFF or ATF. An economically more beneficial alternative is a hybrid process, where the upstream process is done by fed-batch fermentation, which still results in higher product titers compared to perfusion cell culture [41]. Cell culture harvest requires primary recovery to get rid of cells and cell debris. To boost this primary recovery, cell flocculation was employed. I showed in my first publication that cell flocculation results in better sedimentation and filterability and reduces the required floor space. I developed two options for flocculation, one being the addition of flocculant into the fermentation reactor, the second being the continuous cell flocculation, which guarantees a constant flocculant to cell culture broth contact time. In my second publication, I developed a continuous product capture step by continuous product precipitation, continuous concentration and continuous precipitate wash. The best re-solubilization conditions were shown at pH 3.5, which are a perfect fit for subsequent low pH viral inactivation. This low pH inactivation can be done in a continuous mode, either in a tubular reactor or a bead reactor followed by flow through chromatography for polishing. At the end of the process a continuous viral filtration and continuous ultrafiltration and diafiltration (UF/DF) for formulation has to be integrated (Figure 13).

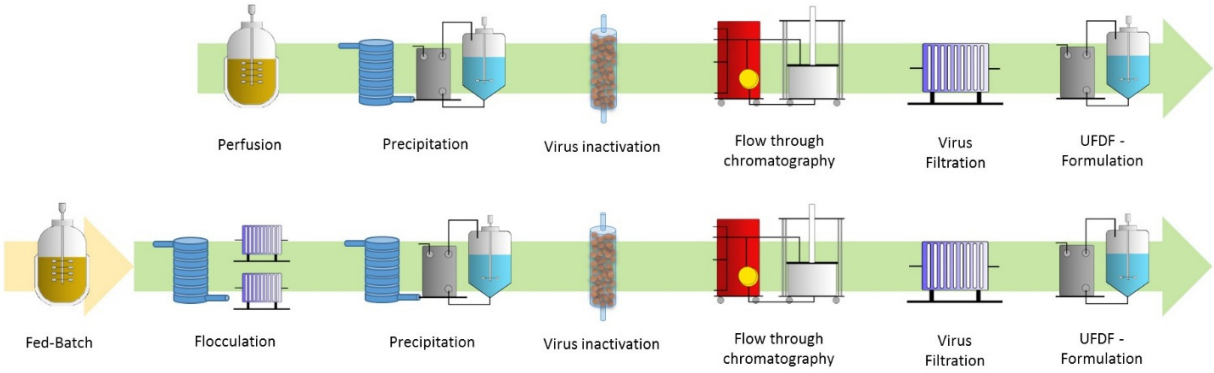

Figure 13: Process scheme of a fully continuous production process and a hybrid process with upstream processing by fed-batch fermentation and continuous flocculation as a primary recovery step.

All of these goes in accordance with regulatory issues. The idea is to pack the whole up- and downstream into compact units with simple control strategies. This requires less space. Capital costs are switched to consumables, which guarantees more flexibility, less validation effort and less set-up time. To pack such a production facility in an intermodal container makes such mobile production units even more flexible (Figure 14) and biopharmaceuticals can be produced on-site where needed.

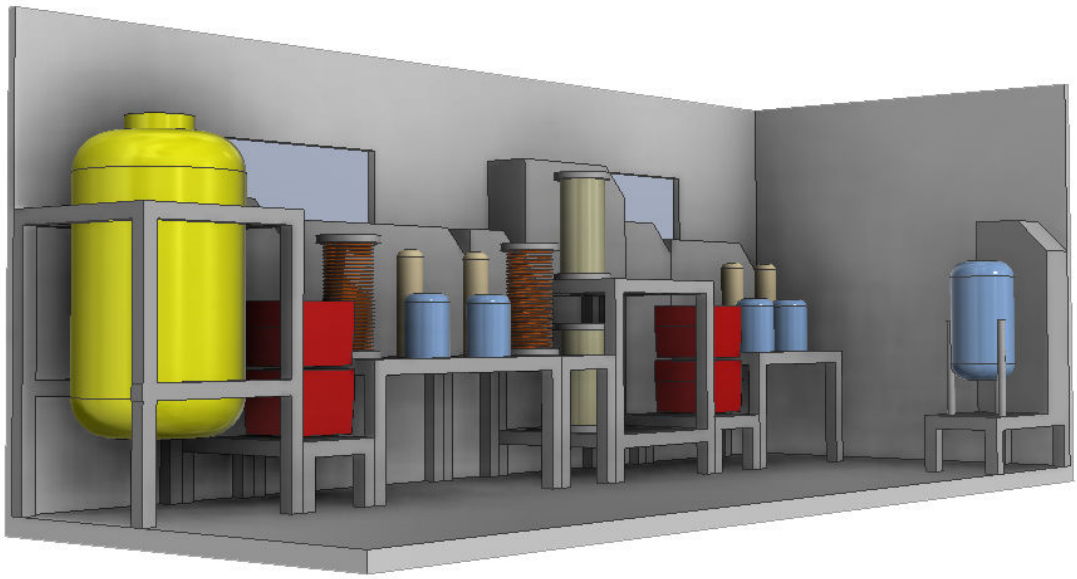

Figure 14 Concept of a compact and flexible continuous production facility in an intermodal container. Image © Peter Satzer

In summary, the objectives of this doctoral thesis were achieved.

- Development of a fully continuous operation for primary recovery by flocculation
- Development of a fully continuous operation for product capture by product precipitation
- Fully continuous scalable solid-liquid separation of product precipitate with beneficial re-solubilization kinetics
- Exemplification of these approaches shown by recombinant antibodies

In this work I showed that, if process parameters such as residence time, product titer and impurity profile are in a certain range, a stable system such as continuous flocculation and continuous precipitation is capable to deal with process fluctuations without the need for process adjustment. Such changes in

concentration, feed flow rate and therefore residence time, and impurity pattern are to be expected from upstream production scenarios like perfusion culture, where the culture ages, and perfusion rates have to be adjusted accordingly. We can therefore answer the research hypothesis if a steady state is necessary for a robust continuous process with a clear no, as I developed a highly robust continuous precipitation based capture step where a steady state of the upstream unit operation is not required. Moreover, I presented two unit operations with continuous, uninterrupted product mass inflow into the system as well as uninterrupted product mass outflow of the system. Both unit operations need minimal control systems in order to run stable and robustly. The systems developed within this thesis, namely the continuous flocculation and the continuous precipitation are an important step to a fully integrated continuous production process of a biopharmaceutical which will result in more flexible, more efficient and safe products for the wellbeing of patients.

5 Publications

5.1 First author publications

Publication I

Continuous cell flocculation for recombinant antibody harvesting

Burgstaller, D., Krepper, W., Haas, J., Maszelin, M., Mohoric, J., Pajnic, K., Satzer, P. (2018). [Article]. Journal of Chemical Technology and Biotechnology, 93(7), 1881-1890. doi: 10.1002/jctb.5500

In this work, I screened the flocculation behavior of various antibody expressing CHO cell harvests, produced in fed-batch and perfusion cell culture. Based on the best performing combination of flocculant and filter, I developed a continuous flocculation process in a tubular reactor and parallelization of filtration.

Publication II

Continuous integrated antibody precipitation with two-stage tangential flow microfiltration enables constant mass flow.

Burgstaller, D., Jungbauer, A., Satzer, P. (2019). [Article]. Biotechnology and Bioengineering, Biotechnol Bioeng 116, 1053-1065. doi: 10.1002/bit.26922

In this work, I screened various precipitation and re-solubilization conditions in small-scale experiments. I converted batch precipitation into continuous precipitation using a tubular reactor and interconnected it to continuous precipitate separation, concentration and precipitate wash using two TFF microfiltration units in series. Additionally, I set up a numerical based model to predict the startup and the shutdown phase.

5.2 Contributions to other publications

Publication III

Mid-manufacturing storage: Antibody stability after chromatography and precipitation based capture step

Krepper, W., Burgstaller, D., Jungbauer, A., Satzer, P., Submitted and excepted with revisions at Biotechnology Progress 2019

This work compares the stability of an antibody precipitate and an antibody in solution purified by protein A. The antibody intermediates were stored at -20 °C, 4 °C and at room temperature. In frequent intervals, samples were measured with various methods to determine structural changes after storage. My contribution was that I precipitated the antibody by continuous precipitation, determined the antibody monomer purity and the high molecular weight impurities by size exclusion chromatography and I performed the isoform characterization by anion exchange chromatography by pH gradient elution.

Publication IV

Fractal dimension of antibody PEG-precipitate: An engineering parameter for comparison of batch and continuous operation.

Satzer, P., Burgstaller, D., Krepper, W. Jungbauer, A., intended for submission to Engineering in Life Sciences

This work deals with the characterization of the particle structure of an antibody precipitate. A MATLAB script for microscopic image processing was written to determine the fractal dimension of precipitates. This fractal dimension of a precipitate can be used as an engineering parameter for precipitation processes in biotechnological applications. My contribution to this work was that I prepared four different precipitates by batch precipitation and continuous precipitation at low and high shear rates. Moreover, I recorded the stacked microscopic images of the precipitates using a wide-field fluorescence microscope.

6 List of figures

7 References

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Research Article

Received: 20 September 2017 Revised: 31 October 2017 Accepted article published: 9 November 2017 Published online in Wiley Online Library: 8 December 2017

(wileyonlinelibrary.com) DOI 10.1002/jctb.5500

Continuous cell flocculation for recombinant antibody harvesting

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Abstract

BACKGROUND: Integrated continuous production technology is of great interest in biopharmaceutical industry. Efficient, flexible and cost effective methods for continuous cell removal have to be developed, before a fully continuous and integrated product train can be realized. The paper describes the development and testing of such an integrated continuous and disposable set-up for cell separation by flocculation combined with depth filtration.

RESULTS: Screening of multiple flocculation agents, depth filters, and conditions demonstrated that the best performance was obtained with 0.0375% polydiallyldimethylammonium chloride (pDADMAC; a polycationic flocculation agent) in combination with Clarisolve® depth filters. Using this set-up, a 4-fold decrease of filtration area was achieved relative to standard filtration without flocculation, with yields of ≥97% and DNA depletion of up to 99%. Continuous operation was accomplished using a simple tubular reactor design with parallelization of the filtration. The reactor length was selected to allow a 13.2-min residence time, which was sufficient to complete flocculation in batch experiments. Continuous flocculation performance was monitored on-line using focused beam reflectance measurement. Filter switch cycles based on upstream pressure were controlled by in-line pressure sensors, and were stable from one filter to the next.

CONCLUSION: It was demonstrated that stable and efficient continuous flocculation associated with depth filtration can be easily accomplished using tubular reactors and parallelization. Continuous cell separation is essential for the development of fully continuous integrated process trains. This cost-efficient disposable design run in continuous mode significantly reduces facility foot print, process costs and enables great flexbility.

Keywords: biochemical engineering; bioseparations; proteins; filtration; animal cell

INTRODUCTION

Flocculation combined with disposable depth filtration enables clarification of cell culture broth via fully integrated disposable processes. When producing antibodies in Chinese hamster ovary (CHO) cells, the product is expressed into the supernatant, and the induction of flock formation enables easier cell removal without compromising product yield. Flocculation entails the addition of a flocculant with the aim of increasing particle size to improve sedimentation or filtration properties. Since mammalian cell surfaces are negatively charged, flocculation is usually performed using cationic polymers to bridge the particles.[1] Common polymers used for cell flocculation include polyethylenimine (PEI)[2]; polydiallyldimethylammonium chloride (pDADMAC)[3,4]; chitosan[5]; and stimulus-responsive polymers, such as modified polyallylamine (mPAA). The addition of such a stimulus initiates flock formation as well as precipitating residual polymer, which can be removed efficiently from the cell culture broth. mPAA is a cationic polymer which is precipitated by the addition of anionic divalent phosphate.[6]

Cell flocculation can also be induced by surface charge neutralization caused by acidification[7,8] or by co-precipitation caused by calcium phosphate precipitation.[9] Following Stokes law, since sedimentation velocity is proportional to the second power of particle size, the flock size of agglomerated cells and cell debris enhances sedimentation and centrifugation performance.[3,5,6] Agglomeration of cells, cell debris, and cell-related fines reduces pore blockage on conventional filters, thus reducing the required filtration

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area.[3,5,10,11] This effect can be further enhanced by using specifically designed depth filters, such as Clarisolve®.[4,6]

Cell culture advancements enable high cell numbers and greater product titres,[12,13] but these increases reduce the effectiveness of conventional primary recovery unit operations. Longer process times and high cell numbers are associated with more nonviable cells and cell debris, further impairing the performance of centrifugation and depth filtration.[14] When cell culture broths with high cell numbers are processed by disk stack centrifuge, more frequent discharge cycles are required, leading to higher product loss. This product loss has an even greater impact at high product titres. Flocculation can have the additional benefit of depleting host cell proteins (HCPs), strong negatively charged DNA, and high molecular weight impurities (HMWI).

Several reports describe biotechnological applications of flocculation in downstream processing. Tomic *et al.* demonstrated that a combination of pDADMAC-assisted flocculation and depth filtration led to enhanced filter performance and DNA reduction.[4] Singh *et al.* found that preliminary acidification, or addition of either chitosan or a 150-kDa modified polymer, resulted in improved filtration throughput and clarification efficiency.[10] Kang *et al.* performed cell flocculation with a stimuli-responsive polymer called benzylated poly(allylamine), followed by depth filtration, which enabled high antibody yields and efficient removal of HCP, DNA, HMWI and residual polymer. Using material pre-treated by this method with a subsequent Protein A chromatography step, Kang *et al.* reportedly achieved the requirements for drug substances. McNerney *et al.* developed a method for two-polymer flocculation using pDADMAC and polyethylene glycol (PEG), which showed beneficial settling properties and increased filter throughput.[3] In their informative review, Felo *et al.* summarize the flocculation methods reported in antibody production by CHO cells.[15] While these prior publications have investigated some applications of batch flocculation for CHO cells, no study has yet included comprehensive and wide screening, or expansion of the technology for use in continuous production.

Continuous integrated processing is of great interest in the biopharmaceutical industry, as it offers economic benefits and a smaller facility footprint and, consequently, lower capital investment. Moreover, a well-controlled continuous process can provide improvements in product quality and process stability compared with batch processes.[16,17] When combined with disposable technology, a continuous process can offer even greater flexibility, which can further reduce the cost and time for implementation in certain production scenarios. Notably, disposable technology is not available for large-scale production. Commercially available bioreactors are limited to 2000 L reactor volume.[18] In addition, disposable downstream equipment is size limited as well (for instance disposable chromatography columns are only commercially available up to 20 L column volume).[19] A bioreactor harvest of over 2000 L cannot be handled using disposable technology unless continuous operation is employed. Since continuous operation requires a smaller scale, it can be combined with disposable technology to enable full-scale production.[20,21]

The simplest tubular reactor design involves the installation of static inner tubular mixers that force the liquid to change axial flow direction, thereby maintaining a homogenous solution or suspension throughout the reactor. The reactor length can be selected to achieve the necessary residence time. For a wide range of operation conditions, plug flow can be assumed, and it is possible to achieve very narrow residence time distributions compared with other continuous reactors, such as continuous stirred tank reactors (CSTR) and mixed suspension mixed product removal (MSMPR).[16] Compared with other reactors, tubular reactors have advantages with regards to simplicity and scalability. On the other hand, in helical coiled tube arrangements (e.g. the coiled flow inverter), Dean vortices are generated and further mixing is achieved by introduction of 90° bends.[22–25] Such reactors have a very narrow operating range because Dean vortices are generated within only a very narrow window of operating conditions, requiring Reynolds numbers of around 30.[26] CSTR can also be used for continuous reactions, but are not suitable in scenarios requiring a narrow residence time distribution since a full wash-out requires at least five reactor volumes. Our group previously demonstrated the simple concept of antibody precipitation using tubular reactors with helical static mixers for continuous operation.[27,28]

This paper describes the development and testing of a reactor for continuous cell flocculation combined with depth filtration for flocculated cell removal. In this reactor, cell broth and flocculant stream were combined and continuously mixed in a tubular reactor filled with helical static mixers. The reactor length was selected to achieve a specific reaction time for flock formation. The set-up was kept simple to facilitate a disposable design and reduce the facility footprint. We performed small batch experiments to examine the flocculation efficiency and working range of five flocculants, and we tested the clarification efficiency with both standard depth filters and filter materials specially developed for pre-treated cell culture broths. To achieve broader applicability of the flocculation and depth filtration conditions, we screened two different antibodies from five different fermentations. Our objective was to develop a continuous cell removal process without product loss, which would offer improved performance compared with the disc stack centrifuge. The flocculant and operating conditions were also evaluated for their efficiency in reducing HCP, DNA, and HMWI. The system was tested on a laboratory scale with a 132-mL-volume reactor.

EXPERIMENTAL

Cell culture

In CHO cells, two antibodies were produced: antibody A (IgG2 subtype) and antibody B (IgG1 subtype). The material was kindly provided by our project partner, LEK, a Sandoz company (Ljubljana, Slovenia). Cell culture broth was used from fed-batch fermentations of antibody B (subtype IgG1) and antibody A (subtype IgG2), as well as from a perfusion culture producing antibody A. Fed-batch fermentations were harvested 13 to 16 days after inoculation. Cell density of the harvested material varied from 4.9×10^6 and 9.6×10^7 cells per mL. Cell viability ranged from 85–98%.

Batch flocculation screening

The cell culture broth was mixed with various concentrations of five different flocculants [pDADMAC solution 10% (Merck KGaA, Darmstadt, Germany), mPAA solution 10% (Merck), $CaCl_2$ (Sigma-Aldrich, St. Louis, MO, USA), caprylic acid (Sigma-Aldrich), and PEG with an average molecular weight of 6000 g mol^{-1} (PEG6000; Merck KGaA)], followed by a 20 min incubation with gentle mixing. Next, the flocculated mixture was centrifuged for 10 min at 1000 *g* in an Eppendorf centrifuge 5810R with rotor A-4-62 (Hamburg, Germany). We determined the nephelometric turbidity units (NTU) of the supernatant using a portable turbidimeter (2100Q Hach, Loveland, CO, USA).

Journal of Chemical Technology & Biotechnology published by John Wiley & Sons Ltd on behalf of Society of Chemical Industry.

The samples were sterilized by 0.2 µm filtration, and frozen at −80 °C for shipment and further analysis. Size exclusion chromatography was performed to assess yield, purity, and HMWI.

Batch filter screening

Prior to use, the 23-cm^2 µPod® format depth filters (Clarisolve® 20MS, 40MS, and 60HX grades – developed for filtration of pre-treated feeds with particle size distributions of 20 µm, 40 µm and 60 µm; Millistak + ® HC D0HC and F0HC, all from Merck KGaA) were flushed and vented with water for injection. Cell culture broth was mixed with various concentrations of different flocculants, and incubated for 20 min in a stirred reactor vessel. Next, this mixture was pumped through the filter by a Masterflex® L/S® peristaltic pump (Cole-Parmer, Vernon Hills, IL, USA) at a constant flow (120 liters per square meter per hour (LMH)), following the Pmax™ method recommended by the manufacturer.[29] Volume and pressure were monitored over time using a scale and disposable in-line pressure sensors (PendoTECH, Princeton, NJ, USA), with data collection by the DAQ system 2.0 (Merck KGaA). Filtration was stopped upon reaching a defined pressure limit of 2.0 bar, or when the 1 L prepared feed was completely processed. The filtration efficiency of the filtration pool was determined based on NTU measurement with a 2020wi Portable Turbidity Meter (LaMotte, Chestertown, MD, USA). The possible volume feed per m^2 of filtration area was calculated from the collected process volume and pressure data.

The processed feed was then filtrated through a sterile filter (Express® SHC Optiscale®-25 0.5/0.2 µm; Merck KGaA) using constant applied pressure, with calculation of the maximum volume flow through the sterile filter, following the Vmax™ method recommended by the manufacturer.[29] Again, volume was monitored using a scale, and data acquired with the DAQ system 2.0 (Merck KGaA). The necessary sterile filter area was calculated based on the gradual pore-plugging model.

Continuous flocculation and filtration

For continuous flocculation, we designed a self-assembled tubular reactor. Standard lab tubes (Tygon® R-3603, 4.8 mm inner diameter; Saint-Gobain, Courbevoie, France) were filled with static mixers (HT-40-6.30-24-AC; Material Acetal; Stamixco AG, Wollerau, Switzerland). These were combined with polycarbonate Luer fittings (Cole-Parmer) to enable the installation and flushing of one filter, while the other filter was fed with flocculated cell culture broth. Cell culture broth was pumped at 10 mL min^{-1} by a Masterflex® L/S® Peristaltic Pump (Cole-Parmer), and pDADMAC solution 10% (Merck KGaA, Darmstadt, Germany) was added in-line using a Gemini 88 syringe pump (Scientific, Inc., Holliston, MA, USA) at a ratio that produced a final concentration of 0.0375% pDADMAC. The tubular reactor length was selected to achieve a 13.2 min residence time at a flow rate of 10 mL min^{-1} before the feed reached the depth filter (261 LMH). Disposable pressure sensors (PendoTECH) were used to collect pressure curves for each filter individually. In addition, particle characterization was performed using a self-assembled Teflon-based flow cell combined with a Focused Beam Reflectance Measurement probe (Particle Track G400; Mettler Toledo, Columbus, OH, US). Data from the Particle Track were evaluated using iC FBRM 4.4 software (Mettler Toledo, Columbus, OH, US). Flock removal was performed using 23 cm^2 Clarisolve® 40MS depth filters in µPod® format (Merck KGaA), and 50 mL fractions were manually collected for assessment of yield, purity, and HMWI.

Size exclusion chromatography

Size exclusion chromatography was used to assess antibody yield, purity, and the amount of HMWI. High-performance liquid chromatography was carried out by isocratic elution on a Dionex UltiMate 3000 HPLC system equipped with a diode array detector (Thermo Scientific, Waltham, MA, USA). The running buffer was 50 mmol L^{-1} sodium phosphate buffer with 150 mmol L^{-1} NaCl (Sigma-Aldrich) at a pH of 7.0, prepared with 0.22 µm filtration (GSWP04700, Merck KGaA). 10 µL of a 0.2 µm vacuum-filtered sample (0.2 µm GHP AcroPrep™ 96 filter plate; Pall Life Sciences, Ann Arbor, MI, USA) was applied to a TSKgel® G3000SWXL HPLC Column (5 µm, 7.8 × 300 mm) with a TSKgel SWXL Guard Column (7 µm, 6.0 × 40 mm; Tosoh, Tokyo, Japan). Chromeleon™ 7 software (Thermo Scientific) was used to monitor the signals at 215 nm (for HMWI) and 280 nm (for purity and yield). Product purity was defined by the ratio of product peak area (monomer) to sum of all peak areas. Product yield was calculated dividing the product peak area (monomer) of the flocculated material by the product peak area (monomer) of the cell culture broth.

Isoform characterization

To determine the acidic and basic antibody isoforms, a CIEX-HPLC method was used with a linear pH gradient, based on the method developed by Lingg *et al.*[30] Measurements were performed on an Agilent 1100 HPLC system with a ProPac™ WCX-10G Guard Column (10 µm, 4 × 50 mm) and a ProPac™ WCX-10 column (10 µm, 4 × 250 mm) (Thermo Scientific). Samples were diluted to 1 g L^{-1} and the injection volume was 100 µL. Mobile phase A was 5.5 mmol L^{-1} HEPES, 4.2 mmol L^{-1} Bicine, 9.5 mmol L^{-1} CAPSO, 0.8 mmol L^{-1} CAPS, and 6.3 mmol L^{-1} NaCl (pH 8.0), and mobile phase B was 10.5 mmol L^{-1} Bicine, 2.5 mmol L^{-1} CAPSO, 7.0 mmol L^{-1} CAPS, pH 10.5. With a flow rate of 1 mL min^{-1}, the method set-up comprised a 1 CV equilibration step at 0% B, a linear gradient step from 0–55% B in 1 CV, a linear gradient step from 55–75% B in 10 CV, a linear gradient step from 75–100% B in 1 CV, and a wash step at 100% B for 1 CV. The outlet was monitored at 280 nm.

DNA assay

Double-stranded DNA content was determined using a fluorescent nucleic acid stain Quant-iT™ PicoGreen® dsDNA assay kit (Life technologies, Waltham, MA, USA) following the manufacturer's instructions. All experiments were performed in 96-well plates. Measurements were taken using a Genius Pro plate reader (Tecan, Männedorf, Switzerland) at a 480-nm excitation wavelength and a 520-nm emission wavelength.

RESULTS AND DISCUSSION

Screening of flocculants and filters for primary separation

Five different flocculants [PEG6000, pDADMAC, 15-kDa modified polyallylamine (mPAA), caprylic acid, and calcium chloride] were first screened for their ability to efficiently flocculate cells and clear host cell impurities, such as DNA, proteins, and product-related impurities (e.g. HMWI). To identify optimum conditions suitable for broader applications, screening was performed using cell culture broths from two fed-batch cultures of antibody B, two fed-batch cultures of antibody A, as well as cell culture broth from a perfusion culture producing antibody A. Clearance efficiency was determined by the decrease of turbidity after centrifugation – a centrifugal force that will only sediment larger particles and can thus be used as a rough indication of flocculation efficiency.

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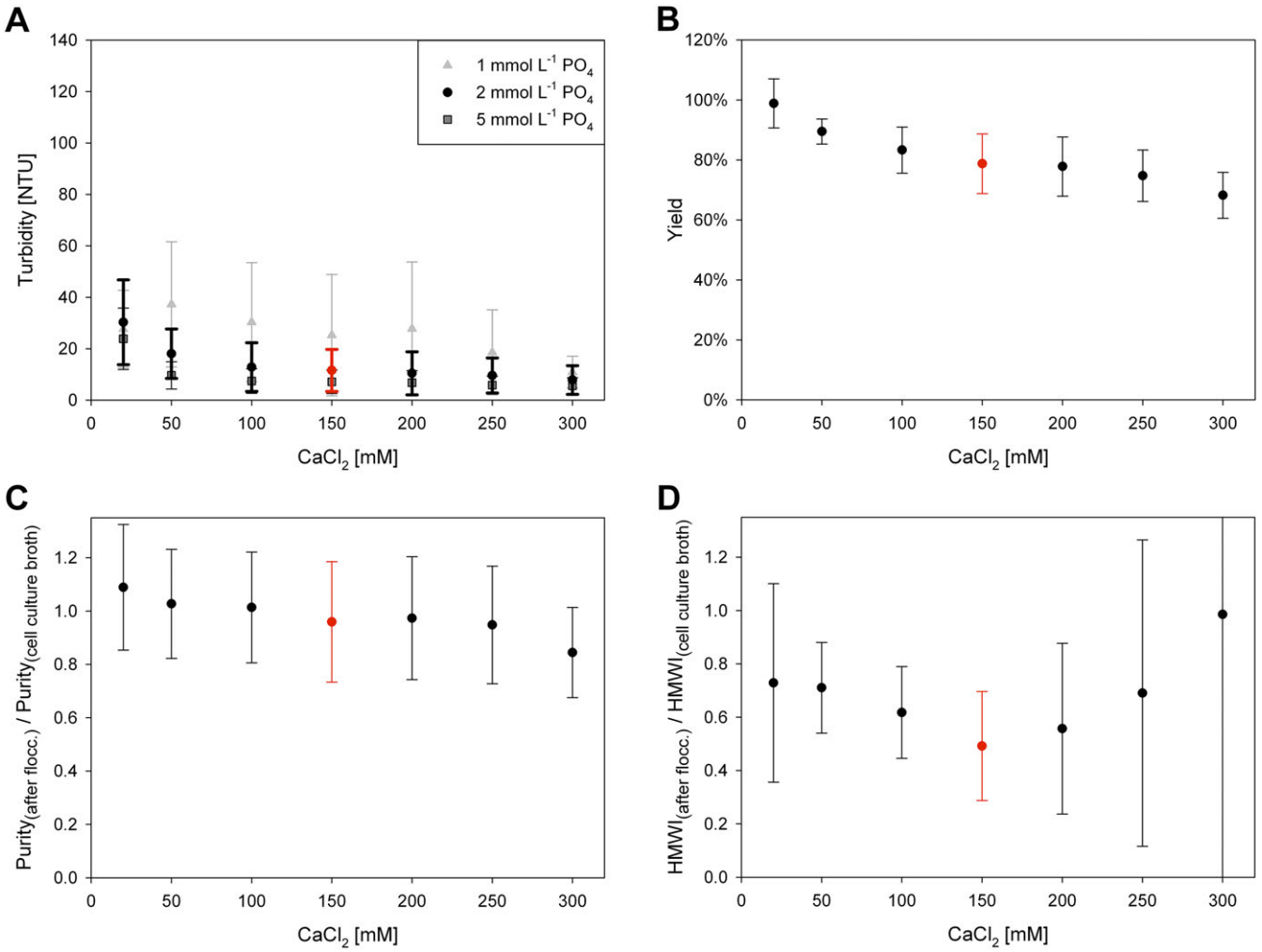

Figure 1. Performance of $CaCl_2$ flocculation (flocc.) in terms of turbidity after flocculation (A), yield (B), purity (C), and high molecular weight impurities (HMWI) (D). Turbidity is shown for addition of phosphate at three different concentrations. All other data are shown only for addition of 2 mmol L^{-1} phosphate. The standard deviations shown represent the data from five different fermentations. Selected conditions for filter screening are highlighted in red. NTU, nephelometric turbidity units.

In the first round of experiments, the five flocculants were added to the supernatant as follows: PEG6000 in a range from 0 to 8%, pDADMAC from 0.025% to 0.1%, mPAA from 0.025% to 0.8% with additional phosphate from 0 to 100 mmol L^{-1}, caprylic acid from 0.25% to 2%, and calcium chloride from 20 to 200 mmol L^{-1} with additional phosphate from 0 to 10 mmol L^{-1}. Caprylic acid did not reduce turbidity at all, and resulted in a supernatant that was almost impossible to filtrate through a 0.2 µm sterile filter for sample preparation. Flocculation with as little as 4% PEG6000 resulted in ~40% antibody loss, probably due to low antibody solubility with addition of PEG6000. A process that results in this degree of product loss, especially at a step as early as the primary separation of cells, is not an economically viable option. Thus, PEG6000 and caprylic acid were excluded from further experiments.

The next round of screening experiments included three flocculants: calcium chloride, pDADMAC, and mPAA. Five different cell culture harvests (two fermentations producing IgG1, and three fermentations producing IgG2) were screened to identify the optimal concentration for flocculation, and to determine the deviation across different fermentations. This enabled identification of the most stable conditions for a flocculation-based platform process. The cell culture broths showed different antibody concentrations and levels of HMWI; therefore, the purity and HMWI levels were normalized after flocculation to the levels of the respective supernatants to improve comparability. Turbidity was not normalized, so as to reflect the ability of the flocculation method to reduce fermentations of different initial turbidities to the same robust turbidity after flocculation.

Screening of calcium chloride

Flocculation with calcium chloride was screened at concentrations ranging from 20–300 mmol L^{-1} with addition of 1 mmol L^{-1}, 2 mmol L^{-1}, and 5 mmol L^{-1} phosphate. $CaCl_2$ showed moderate clearance efficiency over the tested range (Fig. 1(A)). Among all tested batches, the best clearance and most stable performance was with the addition of 2 and 5 mmol L^{-1} phosphate. With addition of 1 mmol L^{-1} phosphate, the clearance efficiency showed high batch-to-batch variation. Clearance improved with increasing $CaCl_2$ concentration, but at the expense of up to 30% yield loss (Fig. 1(B)) and with no increase of purity (Fig. 1(C)). The reduction of HMWI (which predominantly included antibody aggregates) was highly variable among all screened production batches,

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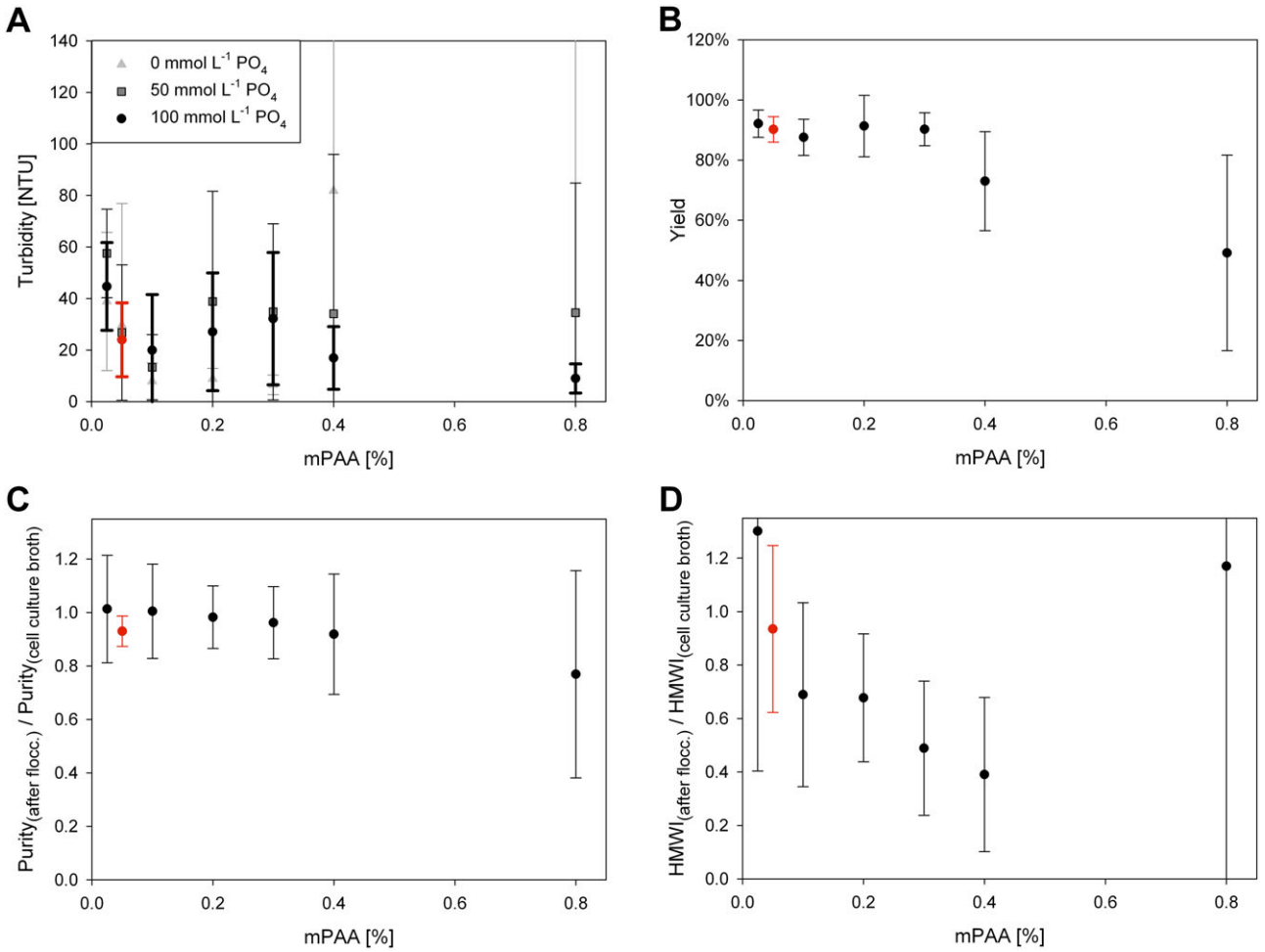

Figure 2. Performance of mPAA flocculation (flocc.) in terms of turbidity after flocculation (A), yield (B), purity (C), and high molecular weight impurities (HMWI) (D). Turbidity is shown for three different phosphate concentrations. All other data are shown only for addition of 100 mmol L^{-1} phosphate. The standard deviations shown represent the data from five different fermentations. Selected conditions for filter screening are highlighted in red. NTU, nephelometric turbidity units.

with an optimum reduction at an intermediate $CaCl_2$ concentration (Fig. 1(D)). Overall, a broad and stable operation range was observed with 50 mmol L^{-1} to 300 mmol L^{-1} of $CaCl_2$ in the presence of 2 mmol L^{-1} phosphate. Within this range, ~80–95% DNA removal (data not shown) was detected. However, product loss at the primary separation stage is economically unacceptable. Therefore, the yield loss with increasing $CaCl_2$ concentration narrows the operation window to low-to-intermediate $CaCl_2$ concentrations. For filter screenings, 150 mmol L^{-1} $CaCl_2$ with 2 mmol L^{-1} phosphate was chosen.

Screening of mPAA

According to the manufacturer, mPAA is a smart polymer. Addition of the stimulant phosphate induces flock formation. mPAA was screened at concentrations ranging from 0.025–0.8% with the addition of 50 mmol L^{-1} and 100 mmol L^{-1} phosphate and without additional phosphate. Clearance efficiency showed a narrow operation range at around 0.1% mPAA with high batch-to-batch variations, especially without the addition of phosphate as a stimulant (Fig. 2(A)). The best and most stable operating conditions were observed with addition of 100 mmol L^{-1} phosphate. Minor yield losses (below 10%) were detected at low-to-intermediate mPAA concentrations (Fig. 2(B)), and higher yield losses with high mPAA concentrations. Purity was not increased with mPAA (Fig. 2(C)). Use of mPAA reduced HMWI by up to 50% (Fig. 2(D)), at the expense of an overall high batch-to-batch variation and high yield losses. For further filter screening experiments, 0.05% mPAA was used with 100 mmol L^{-1} phosphate as the danger of yield loss was considered to be more significant than the benefit of HMWI-removal. If a specific process benefits more from the HMWI-removal than from higher yields, we would recommend to move to the other side of the operation window (at around 0.1 to 0.2% mPAA).

Screening of pDADMAC

pDADMAC was tested at concentrations ranging from 0.005% to 0.15%. With pDADMAC concentrations within the range of 0.01% to 0.05%, the clearance efficiency was very broad (Fig. 3(A)), as previously reported.[4] In accordance with classical flocculation behaviour, inefficient flocculation was observed at very high and very low concentrations, and good performance with intermediate concentrations. At low concentration, the polymer addition is not sufficient to build large flock structures, while an excess of polymer covers the complete cell surface and effectively shields cells and prevents them from bridging and further flock formation. Apart from minor batch-to-batch variations, significant loss of yield (Fig. 3(B)) or influence on product purity (Fig. 3(C))

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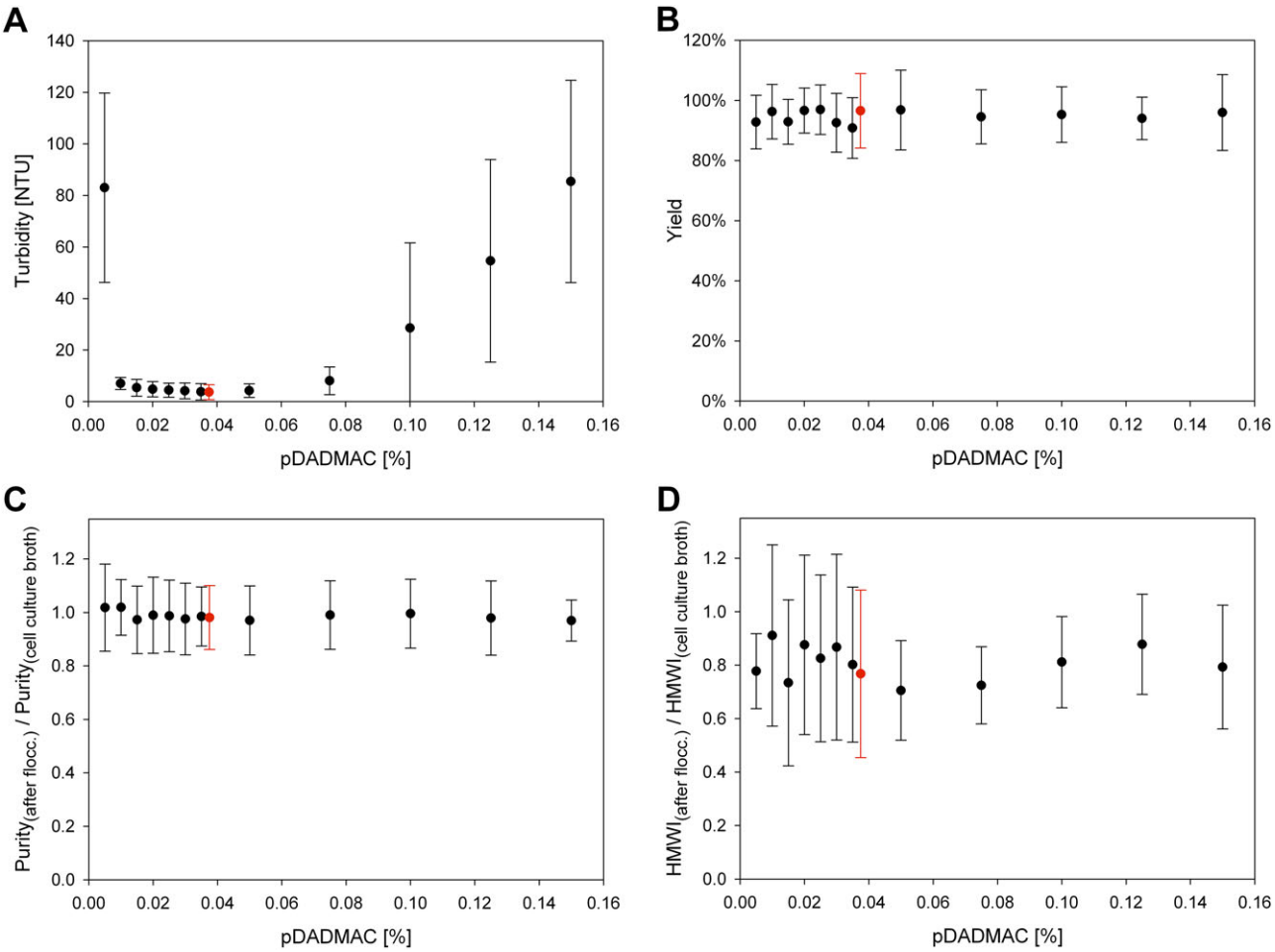

Figure 3. Performance of pDADMAC flocculation (flocc.) in terms of turbidity after flocculation (A), yield (B), purity (C), and HMWI (D). The standard deviations shown represent the data from five different fermentations. Selected conditions for filter screening are highlighted in red. NTU, nephelometric turbidity units.

was not observed. HMWI levels were not reduced and showed high variability, with relatively high variations across all screened fermentation batches (Fig. 3(D)). With all utilized pDADMAC concentrations, a DNA reduction of around 97–99% was achieved compared with the cell culture broth. A pDADMAC concentration of 0.0375% was selected for further filtration screenings, because it is right in the middle of the operation window (0.01–0.075%) and results in good process robustness.

Figure 4 compiles the selected conditions for filter screening (150 mmol L^{-1} $CaCl_2$ with 2 mmol L^{-1} phosphate; 0.05% mPAA with 100 mmol L^{-1} phosphate and 0.0375% pDADMAC). pDADMAC as flocculant shows the best result in terms of yield with an average yield of 97% followed by mPAA with an average yield of 90% and $CaCl_2$ with an average yield of 79% (Fig. 4(A)). Product purity after flocculation compared with product purity of the cell culture broth was not increased. For pDADMAC a normalized purity of 0.98 was achieved, for $CaCl_2$ a purity 0.96 and for mPAA a purity of 0.93 (Fig. 4(B)). $CaCl_2$ is capable to reduce HMWI by a factor of roughly 2, but at the risk of losing yield. The average HMWI content achieved after flocculation compared with HMWI content of the cell culture broth was 0.49 for $CaCl_2$, 0.77 for pDADMAC and 0.94 for mPAA (Fig. 4(C)). These conditions were carried over to filtration screening experiments.

Filter screening

To further screen the flocculation options, the filterability of suspensions obtained with each flocculant was tested using different filter set-ups. The best flocculant concentrations were applied, determined by earlier screening (0.0375% pDADMAC, 0.05% mPAA with 100 mmol L^{-1} phosphate, and 150 mmol L^{-1} $CaCl_2$ with 2 mmol L^{-1} phosphate), and used untreated cell culture broth as a control. The filters tested included conventional positively charged cellulose-based depth filters containing diatomaceous earths as a filtration aid (Millistak + ® HC D0HC and F0HC grades from Merck Millipore) and Clarisolve® depth filters that were specifically developed for flock removal after flocculation of cell culture broth. Clarisolve® depth filters comprise both polypropylene layers and positively charged cellulose layers containing diatomaceous earth. These layered filter materials have different pore sizes, and an increased headspace to accommodate the filtrated cell mass.

After flocculant addition and incubation, the filter capacity was assessed by applying a constant flow to the filter and recording the resulting pressure curves. To compare the flocculation agents and untreated material in combination with Clarisolve® filters, a control filter train was run using untreated cell culture broth as well as flocculated cell culture broth with a Millistak + ® HC D0HC and F0HC filter series. As the last primary separation step, depth filtration was followed by sterile filtration using polyether

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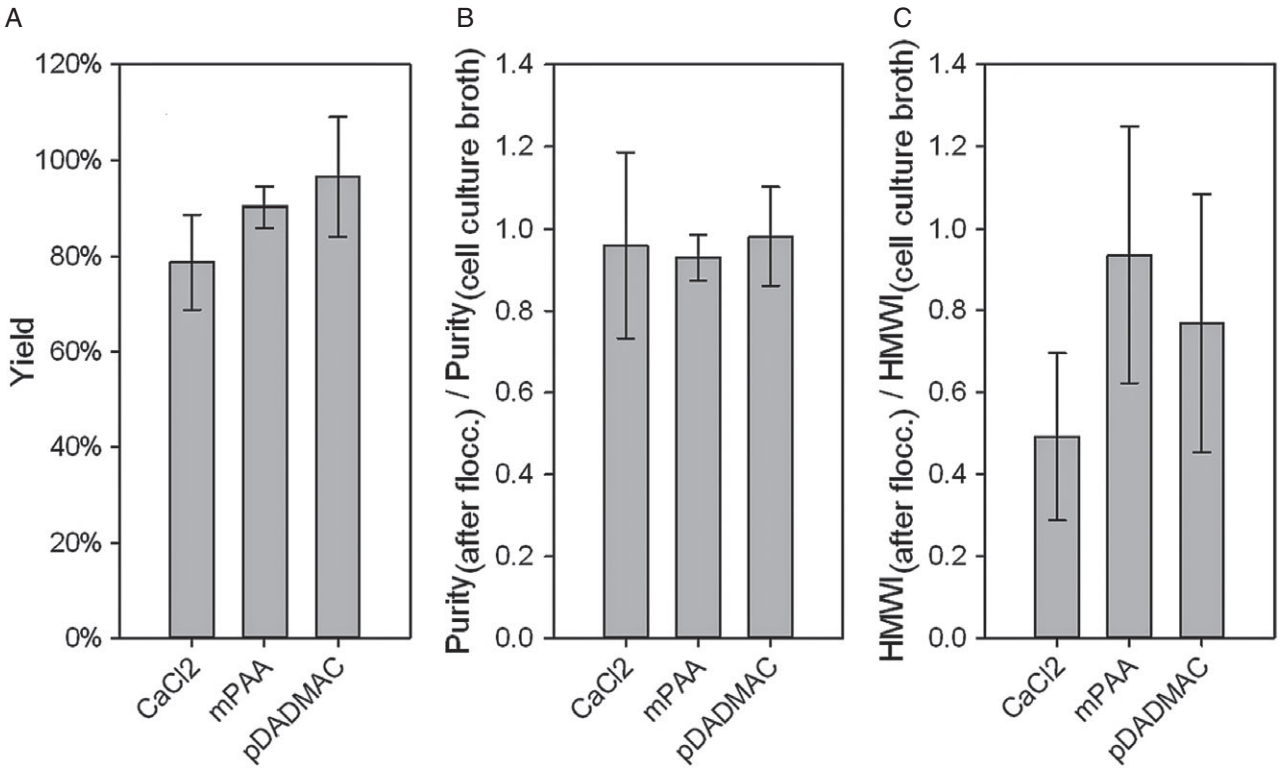

Figure 4. Summary of the selected flocculation conditions (150 mmol L^{-1} $CaCl_2$ with 2 mmol L^{-1} phosphate; 0.05% mPAA with 100 mmol L^{-1} phosphate and 0.0375% pDADMAC) for further filter screenings in terms of yield (A), purity (B) and HMWI (C). The standard deviations shown represent the data from five different fermentations.

sulfone-based bi-layer membrane disks. The filtrates from the depth filtration experiments were applied to the sterile filter using constant pressure, and the flow-through was monitored. Using the data from depth filtration and sterile filtration, the filtration area necessary to process 100 L of harvest in 2 hours was calculated. Assessment of the required filter area for sterile filtration also serves as quality control of the depth filtration, since any fine material getting past the depth filtration step will quickly clog the subsequent sterilization filter.

All of the filtration screening results were compiled from the different fermentation batches to identify the best-performing option (based on the lowest required filter area in m^2), and the most stable option (based on the lowest standard deviation from batch to batch). Figure 5 shows the required filter areas for sterile filtration and depth filtration, together with the standard deviations for each filter indicated by red error bars. Filtration of the flock suspension generated using mPAA showed the largest variation in the required sterile filter area, in the range of $\pm 1.8\ m^2$. Half of the experiments showed significantly worse filtrate quality after depth filtration, with an average value of $3.34\ m^2$. In contrast, the other half of the experiments showed good performance, with an average value of $0.16\ m^2$. Especially when using mPAA, the cell culture broth (and hence the fermentation) seemed to exert a crucial influence, and must be considered. Variation in cell viability and substances added upstream such as detergents or trace elements (such as iron, copper or other metal ions) that are not tracked during up- or downstream might have a strong influence on the stimulus responsive polymer mPAA. With flocculated material, the depth filtration area was reduced by a factor of ~4 compared with the standard filtration train, which is consistent with reports by Tomic *et al.*[4] The best flocculant was pDADMAC, as it

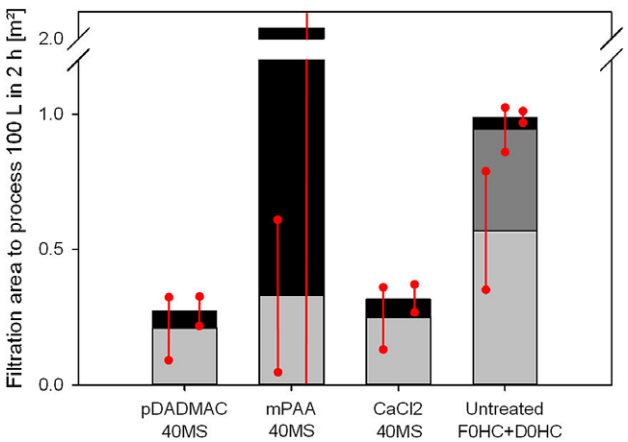

Figure 5. Required filter area (in m^2) for the filtration of 100 L of harvest in 2 hours. The grey bars indicate the filter area required for depth filtration. The F0HC + D0HC filter train represents a benchmark filter train, comprising two depth filtrations in series. The black bars show the necessary sterile filter area, which indicates the filtrate quality after depth filtration. The standard deviations shown represent the data from different fermentations, including nine different fermentations using pDADMAC as flocculant, four using mPAA, five using $CaCl_2$, and seven using the benchmark filter train. The best performers for each pre-treatment option are shown.

required the lowest filter area and showed consistent performance across all experiments, closely followed by $CaCl_2$. $CaCl_2$ has the disadvantage of lower yield in comparison with pDADMAC (79% vs 97%). Clarisolve® filters were not suitable for the filtration of untreated cell culture broth. A turbid filtrate indicates an inefficient clearance performance probably because cells are not retained

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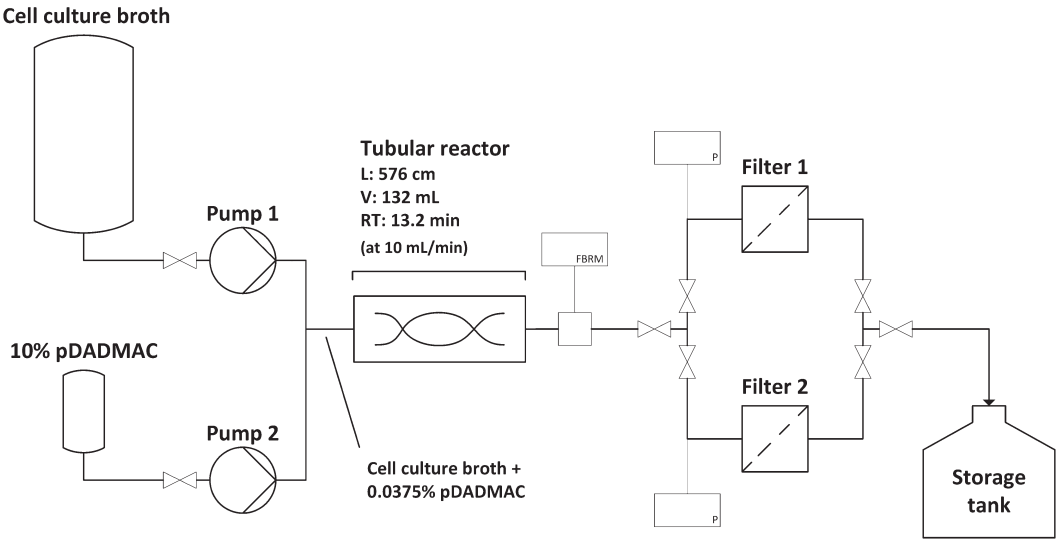

Figure 6. Flow scheme of the continuous flocculation set-up. Harvested cell culture broth and flocculant are combined in a flow rate-dependent manner to a final concentration of 0.0375% pDADMAC. Residence time in the tubular reactor is 13.2 min at a flow rate of 10 mL min^{-1}. Mixing is achieved by static mixer elements. Flock size is monitored using a FBRM probe. Pre-filter pressure is monitored by in-line pressure sensors. Continuous operation is guaranteed by parallelization of filtration.

due to the bigger pore sizes of Clarisolve® filters. In contrast, filtration of flocculated material with a Millistak + ® HC D0HC and F0HC filter series results in low filter capacity (data not shown). Since pDADMAC showed the lowest necessary filtration area in this experiment, along with high yield, stable purity and HMWI, a wide operation range, and additional DNA removal of up to 95%, this condition was selected for the establishment of continuous flocculation (Fig. 3).

Continuous flocculation

After identifying pDADMAC at 0.0375% in combination with Clarisolve® 40MS grade depth filter as the best performing option, continuous operation was designed using this set-up. For continuous flocculation, a simple tubular reactor was assembled using Luer fittings and standard laboratory tubing filled with static helical mixers (Fig. 6). The fittings and tubing were mounted on an acrylic glass baseplate. The residence time could be adjusted by changing the tubular diameter, flow velocity, and reactor length. To ensure permanent mixing, helical static mixers were used that guaranteed macromixing within seconds.[31,32] The total reactor length was 576 cm, with a tube void volume of 185 mL. To ensure the correct residence time, the reactor's volume was determined experimentally after filling it with static mixers. Gravimetrical determination revealed that the built-ins reduced the reactor volume by a factor of approximately 1.4, down to 132 mL. The theoretical residence time was 13.2 min at a flow rate of 10 mL min^{-1}. The Reynolds number was 24.8, indicating a laminar flow. The calculations were based on in-house CHO cell culture broth with a dynamic viscosity of 1.2 mPas and a density of 1010 kg m^{-3}. Pressure was measured over time. The flock size was determined by focused beam reflectance measurement using a custom-made Teflon flow cell. Yield, purity, and HMWI were assessed off-line by size exclusion chromatography. Continuous filtration was achieved by using two parallel depth filtration units. While one filter was filtering the process stream, the other filter was exchanged, vented, and flushed in preparation for the next cycle.

To demonstrate the possibility of fully continuous filtration, three filter switches were used and five continuous runs performed. Engineering runs were first performed with limited analytics (without the FBRM sensor) for development, demonstrating the feasibility of filter switching, and different filter set-ups, flow rates, and reactor lengths. From this preliminary screening of set-ups, Clarisolve 40MS filters were selected as the best option, and fully continuous operation of three filter switches with full analytics (pressure curves, yield, purity, and HMWI) and with an in-line FBRM sensor was demonstrated (scheme shown in Fig. 6, data shown in Fig. 7). We opted to switch filters before reaching the manufacturer recommended pressure rating (maximum delta pressure, 2.0 bar), to enable the testing of three filter switches within a reasonable working day: the first two filters were switched at 0.7 bar, and the third at 1.4 bar. Superimposition of all three pressure curves showed consistent pressure behaviour for all three filters (Fig. 7(B)). A constant average yield of 97% was achieved over the whole 400 min run time. Product purity was also stable at ~18%, indicating no formation of additional by-products via cell lysis or any other processes. HMWI coming out of the reactor steadily increased from 3% to 5%, with 3% achieved after switching to a new filter, and increasing to 5% when reaching the filter's pressure limit (Fig. 7(C)). The cell culture broth contained 4.1% HMWI. Thus, it was assumed that HMWI bound to the fresh filter material to a certain extent at low pressure, and were released either due to increasing pressure or due to their replacement by some HCP that bound more strongly to the filter. Overall, comparison of the average of the harvested fractions with the feed material did not indicate the generation of any additional aggregates. Figure 7(C) shows that each filter switch cycle was accompanied by decreases in yield and HMWI, quickly followed by increases and steady behaviour. This was caused by the dead volume of the filter that was filled with wash solution. After flushing out the wash solution, the yield and HMWI values followed a consistent pattern for all three filter switches.

The FBRM probe signal showed a slight increase in the counted particles over time, followed by a decrease and an unstable signal after reaching a pressure of ~0.4 bar (Fig. 7(A)). The filter switches

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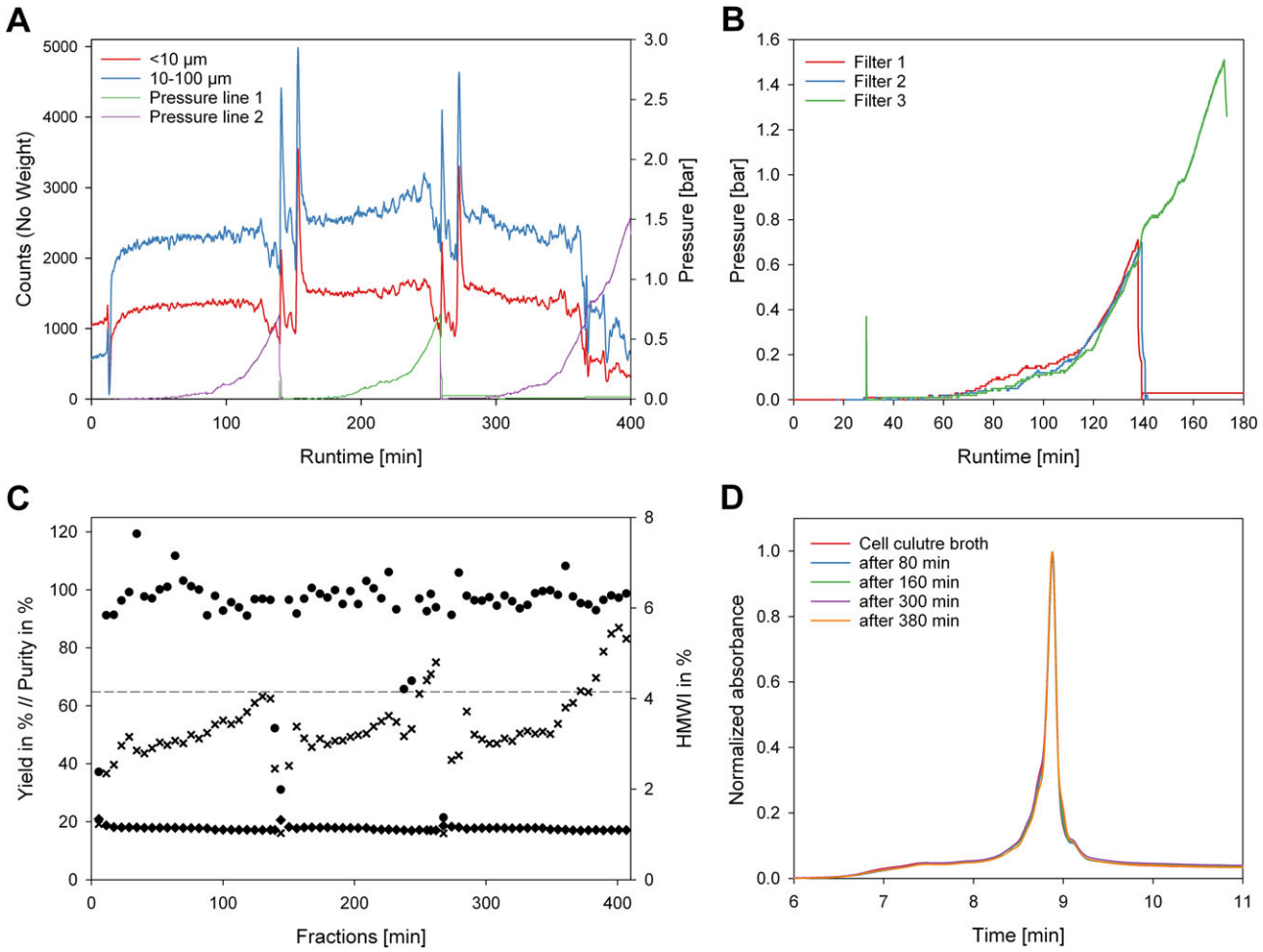

Figure 7. (A) Focused beam reflectance measurement (FBRM) signal and pressure trends over time. (B) Overlay of pressure curves of three filter switch cycles. (C) Yield (dots), purity (diamonds), and high molecular weight impurities (HMWI) (crosses) over time (50 mL fractions). Dashed line indicates the initial HMWI. (D) Overlay of isoform patterns of antibody exposed to pDADMAC at different time points. Separation by cation exchange chromatogram and highly linear pH gradient elution.

were marked by two spikes in the FBRM signal, after which the signal reverted to a steady state. After a 350 min run-time and during the loading of the third filter, the signal became completely unstable. One possible explanation is that the custom-made flow cell was susceptible to changes in pressure above 0.4 bar, which could lead to changes in the flow pattern in the flow cell, resulting in signal changes. Another explanation is probe fouling over time, which is also indicated by a high fouling index reported by the iC FBRM 4.4 software. The flow cell must be optimized to prevent both of these effects. Nevertheless, the developed method is promising for monitoring flock size *in situ* and online.

To ensure product quality during pDADMAC flocculation product aggregates and fragments were monitored via size exclusion chromatography of untreated and flocculated product. No change in antibody peak shape or retention time was observed – just the lower molecular weight pattern that represents HCPs that slightly differ in shape and height, according to SEC measurements. The pattern of charge variants was measured using cation exchange chromatography with a linear pH gradient method, optimized for exceptional linearity.[30] No new isoforms were generated during pDADMAC flocculation or were selectively removed (Fig. 7(D)).

Cytotoxicity was not expected to be an issue. According to the application note, when using 0.0375% pDADMAC for flocculation, the residual pDADMAC was 10 ppm after Protein A chromatography and was below 1 ppm after cation and anion exchange chromatography.[11] No cytotoxic or haemolytic effects are expected with 1 ppm pDADMAC, while only minor toxicity is reported at 10 ppm pDADMAC[11] and 0.01 mg mL^{-1} pDADMAC.[33] McNerney *et al.* report even lower pDADMAC concentrations of 5 ppm after flocculation and depth filtration, and of 0.5 ppm in the subsequent protein A pool.[3] Therefore, it is assumed that pDADMAC will be sufficiently removed from the final product by further processing, which will involve at least two chromatography steps. Overall, the use of pDADMAC requires no additional adjustment of ordinary cell culture broths, has a wide operational range, and is simple to implement.

This pilot trial demonstrated stable continuous operation of antibody flocculation without detrimental effects on product quality, yield, or purity, and with the additional benefits of DNA removal, a small footprint, and a low capital investment.

CONCLUSIONS

The paper describes a novel, stable, and scalable method for continuous flocculation. Various flocculants, conditions, and depth filters were screened in batch operations and the findings applied to

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the development of a continuous operation. By using flocculation, the required depth filtration areas were successfully reduced by a factor of 4 compared with a conventional filtration train. Moreover, the selected flocculant pDADMAC showed no formation of aggregates, fragments, or charge variants, and was associated with minimal antibody losses of 3% and almost complete DNA removal. The continuous run offers great advantages in terms of a small footprint. Specifically, the set-up developed using tubular reactors and peristaltic pumps has the benefits of a cost-efficient disposable design and significantly reduced filter area and facility footprint. Continuous flocculation enables the integrated continuous clarification of cell culture broth, without requiring continuous centrifuges or large filtration units that are necessary for conventional filtration.

ACKNOWLEDGEMENTS

We extend special thanks to Simon Caserman (National Institute of Chemistry, Slovenia) for producing the FBRM flow cell. This work was funded by the European Union's Horizon 2020 program under grant agreement no 635557 (Project: Next-generation biopharmaceutical downstream process). A.J. received also funding from Ministry of Science, Research and Economy (BMWFW), the Federal Ministry of Traffic, Innovation and Technology (bmvit), the Styrian Business Promotion Agency SFG, the Standortagentur Tirol, the Government of Lower Austria and Business Agency Vienna through the COMET-Funding Program managed by the Austrian Research Promotion Agency FFG.

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Received: 13 November 2018 | Revised: 27 December 2018 | Accepted: 9 January 2019

DOI: 10.1002/bit.26922

ARTICLE

WILEY BIOTECHNOLOGY AND BIOENGINEERING

Continuous integrated antibody precipitation with two-stage tangential flow microfiltration enables constant mass flow

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Funding information
H2020 LEIT Biotechnology, Grant/Award Number: 635557; European Union's Horizon 2020 program

Abstract

Continuous precipitation is a new unit operation for the continuous capture of antibodies. The capture step is based on continuous precipitation with PEG6000 and Zn^{++} in a tubular reactor integrated with a two-stage continuous tangential flow filtration unit. The precipitate cannot be separated with centrifugation, because a highly compressed sediment results in poor resolubilization. We developed a new two-stage tangential flow microfiltration method, where part of the concentrated retentate of the first stage was directly fed to the second stage, together with the wash buffer. Thus, the precipitate was concentrated and washed in a continuous process. We obtained 97% antibody purity, a 95% process yield during continuous operation, and a fivefold reduction in pre-existing high-molecular-weight impurities. For other unit operations, surge tanks are often required, due to interruptions in the product mass flow out of the unit operation (e.g., the bind/elute mode in periodic counter-current chromatography). Our setup required no surge tanks; thus, it provided a truly continuous antibody capture operation with uninterrupted product mass flow. Continuous virus inactivation and other flow-through unit operations can be readily integrated downstream of the capture step to create truly continuous, integrated, downstream antibody processing without the need for hold tanks.

KEYWORDS
diafiltration, IgG, membrane, polyethylene glycol, tubular reactor

1 | INTRODUCTION

Continuous and batch precipitation methods were described by our group and others as an economically viable option for the continuous capture of antibodies from clarified culture supernatants (Grosshans, Wang, Fischer, & Hubbuch, 2018; Hammerschmidt, Hintersteiner, Lingg, & Jungbauer, 2015; Hammerschmidt, Hobiger, & Jungbauer, 2016; Hammerschmidt, Tscheliessnig, Sommer, Helk, & Jungbauer, 2014; Kateja, Agarwal, Saraswat, Bhat, & Rathore, 2016; Rathore, Kateja, Agarwal, & Sharma, 2016; Tscheliessnig et al., 2014). This method is quite robust; it readily accommodates varying feed concentrations, and it can be combined with flocculation. Converting the process into a continuous operation is relatively simple with tubular reactors (Burgstaller et al., 2018; Hammerschmidt et al., 2016; Hekmat, Huber, Lohse, von den Eichen, & Weuster-Botz, 2017; Kateja et al., 2016; Raphael & Rohani, 1999). However, one problem that remains unsolved is the continuous solid–liquid separation step, which comes after the actual precipitation, particularly when the solid fraction is the desired product. Centrifugation is an option for continuous solid–liquid separation, but it is difficult to realize for the continuous harvest of polyethylene glycol (PEG)-precipitates. We have observed that extreme compaction of the precipitate is detrimental to the process yield, because the dissolution of dense precipitates requires vigorous stirring and shaking, and the air–liquid interphases generated cause protein aggregation and denaturation. In contrast, the precipitation reaction is a gentle technology that provides high yield; therefore, we must develop new, gentle

continuous solid–liquid separation and dissolution methods to establish a continuous antibody precipitation process with high yield.

One way to avoid compaction of the precipitate is membrane filtration, which avoids harsh resolubilization conditions altogether. Continuous membrane filtration can be realized by running at least two staggered filtration processes. However, that strategy interrupts the fully continuous operation, and the antibody must be harvested in cycles. Another possibility is single-pass tangential flow filtration (TFF), which allows the solid to be harvested in a single pass through a cascade of membrane filters (Casey, Gallos, Alekseev, Ayturk, & Pearl, 2011; Rucker-Pezzini et al., 2018). The third choice is counter-current TFF. This method is used for extremely large-scale production in other industry sectors, like oil processing. Counter-current TFF can also be readily connected to continuous diafiltration, which can be used for washing and/or dissolution of the precipitate (Jungbauer, 2013; Nambiar, Li, & Zydney, 2018).

In the present study, we focused on antibody precipitation with PEG as the capture step. Previous studies have shown that PEG with a molecular mass of 6,000, at a concentration in the range of 14%, provided a good tradeoff between yield and viscosity in process solutions (Grosshans et al., 2018; Hammerschmidt et al., 2015; Hammerschmidt et al., 2016; Sommer et al., 2014, 2015). Although the amount of PEG can be reduced by increasing the molecular mass, this causes an increase in the viscosity. High viscosity can be a problem in certain unit operations, particularly when membrane filtration is used. The upper limit of the transmembrane pressure governs the maximum achievable flux. Accordingly, the cross-flow velocity of highly viscous fluids is low, and consequently, the mass transfer rate through the membrane is slow. These engineering relationships for optimizing membrane filtration have been well established and dictate that lower viscosity is beneficial for increasing the flow rate. Therefore, reducing the viscosity is a critical goal for an efficient process.

It has been shown that the addition of a bivalent cation, such as zinc, might substantially reduce the PEG concentration needed for complete precipitation of the product (Przybycien,). Indeed, Zn^{++} bridges the protein molecules in a flocculent-like manner, therefore, it can reduce the necessary PEG concentration (Iyer & Przybycien, 1995; Yang et al., 2000). We observed that even at neutral pH, where the net charge of our antibody is positive, this method works, presumably because of bridging effects in between negative batches of the antibody. A combination of Zn^{++} and PEG could greatly reduce the viscosity of the PEG solution, which would be very beneficial for membrane filtration. PEG precipitation can be used as a platform process, but to obtain high yields, the exact precipitation and resolubilization conditions must be fine-tuned for each antibody to optimize the process for maximum productivity. Currently, this optimization can be accomplished with high throughput screening methods in microtiter plates (Grosshans et al., 2018).

Precipitation is directly scalable to any size. The necessary equipment is readily available for all manufacturing scales, and the method is highly stable and largely independent of the concentration of product in the feed stream. When the protein concentration is within a broadly constant range, there is no need to adjust the precipitant concentration, and the unit can be run without including on-line measurements of product concentrations, which simplifies the method and avoids expensive equipment and measurements. In the presence of host cell proteins (HCP), product concentration determinations are very tricky; even the best current technologies are limited in this application (Brestrich, Rüdt, Büchler, & Hubbuch, 2018; Jiang et al., 2017). In the present study, we optimized precipitation and dissolution conditions to mitigate problems caused by high viscosity due to PEG concentrations. We also developed a novel method for connecting and running multiple TFF unit operations to facilitate continuous harvesting, concentrating, and washing procedures for a given precipitate. This method can be directly integrated into any production facility and continuous process sequence.

2 | EXPERIMENTAL

2.1 | Cell culture

Immunoglobulin G1 was produced in Chinese hamster ovary (CHO) cells in a 1000 L fed-batch fermentation. For the primary separation, one fraction of the fed-batch (cell density $7.5*10^6$ cells/ml, cell viability 91.7%) was filtered (Millistak+® D0HC/F0HC+ Millipore Express® SHC in-line; Merck KGaA, Darmstadt, Germany) on Day 1. The second fraction of the fed-batch (cell density $7.3*10^6$ cells/ml, cell viability 89.3%) was flocculated with 0.0375% of the polycationic polymer, polydiallyldimethylammonium chloride (pDADMAC; Merck KGaA) and filtered (Clarisolve® 40MS+ Millipore Express SHC in-line; Merck KGaA) on Day 2. The cell culture broths were kindly provided by LEK, a Sandoz company (Ljubljana, Slovenia)

2.2 | Precipitant optimization

For buffer and pH optimization, 50 mM phosphate buffer (Merck KGaA) in a pH range of pH 6.0 to 8.0 and 50 mM tris(hydroxymethyl) aminomethane buffer (Merck KGaA) in a pH range of pH 7.0 to 9.0 were tested in the presence of 8 to 12% PEG (PEG6000, average molecular weight, 6000 g mol^{-1}; Merck KGaA). Before testing, the cell culture broth (with the desired antibodies) was adjusted to the appropriate pH value with either hydrochloric acid (25% HCl) or sodium hydroxide (10 M NaOH). In a second round, we optimized the addition of zinc chloride ($ZnCl_2$), in the concentration range of 1 to 10 mM, and sodium chloride, in the concentration range of 50 mM to 150 mM, in 50 mM Tris buffer with pH 7.0. No pH adjustment of the cell culture broth was required. Finally, solubility curves were created over a range of 0 to 15% PEG6000, with and without the addition of 2 mM $ZnCl_2$. All optimization experiments were performed in 96 deep-well plates. After 20 min of incubation at room temperature on the end-over-end shaker (Stuart rotator SB3; Cole-Parmer, Vernon Hills, IL), the plate was centrifuged at 4,000rcf for 10 min (Centrifuge Heraeus Multifuge X3, Rotor HIGHPlateTM6000;

Thermo Fisher Scientific, Waltham, MA). The supernatant was withdrawn and analyzed with protein A affinity chromatography (described in section 2.9). All PEG6000 concentrations are defined in terms of weight per volume (w/v) unless stated otherwise.

2.3 | Resolubilization optimization

The antibody was precipitated for 20 min in a 100-ml stirred vessel by adding 15 ml of 40% PEG6000 in 50 mM Tris buffer, pH 7.0, 34.8 ml 50 mM Tris buffer, pH 7.0 and 200 µl 1 M $ZnCl_2$ solution to 50 ml of cell culture broth to gain a final concentration of 6% PEG6000 and 2 mM $ZnCl_2$ in 50 mM Tris buffer, pH 7.0. The precipitate was washed twice. Next, the precipitate was centrifuged at 2,000rcf for 5 min. The supernatant was withdrawn, and an equal volume of 6% PEG6000 solution with 2 mM $ZnCl_2$ in 50 mM Tris buffer, pH 7.0 was added. The pellet was resuspended and adjusted to the particular pH (pH 3.5–pH 8.0) by titration with 25% HCl. For the buffer and pH optimizations, the pH-adjusted, resuspended precipitate was added, according to its pH value, to either 50 mM sodium acid buffer (pH 3.5–pH 6.0) or 50 mM phosphate buffer (pH 6.0–pH 8.0) at dilution ratios of 1:2, 1:3, and 1:5. In the second step, we evaluated samples to determine the influence of adding 0 to 150 mM ammonium phosphate and sodium chloride. All experiments were performed in 96 deep-well plates. After incubating for 20 min at room temperature on the end-over-end shaker, the samples were analyzed with protein A affinity chromatography (described in section 2.9).

2.4 | Sequential TFF–proof of concept

The cell culture broth was combined with 40% (w/w) PEG6000 in 100 mM Tris buffer, pH 7.5 to achieve a final 13.2% (w/w) PEG6000 (Grosshans et al., 2018). The precipitate suspension was incubated for 20 min in a stirred vessel. Next, it was continuously pumped via a transfer pump at a flow rate of 15 ml min^{-1} into the stirred tank vessel of an Äkta flux S system (GE Healthcare, Uppsala, Sweden). In the first stage, the precipitate was concentrated by passing it through a 50 cm^2 hollow fiber membrane (pore size, 0.2 µm; GE Healthcare) at a constant feed rate of 124 ml min^{-1} and a constant permeate flow of 13.8 ml min^{-1}. A constant bleed was withdrawn at a flow rate of 1.2 ml min^{-1}. After 185 min of run-time, the transfer of the precipitate suspension was terminated. The precipitate was further concentrated for 35 min until a feed pressure of 170,000 Pa was achieved. In the second stage, the withdrawn precipitate was combined with the precipitate that remained in the Äkat flux S system (310 ml) and washed with 13.2% (w/w) PEG6000 in 100 mM Tris buffer, pH 7.5 (2690 ml). In this washing stage, we applied the same conditions as those used in the first stage. The precipitate feed was terminated after 190 min of run-time and further concentrated for 10 min until a feed pressure of 170,000 Pa was achieved. Samples were prepared and diluted for size exclusion chromatography analysis.

2.5 | Influence of residence time in continuous precipitation with tubular reactors

Continuous precipitation was achieved with a self-assembled tubular reactor setup (described in section 2.7). The cell culture broth was combined with 40% (w/w) PEG6000 in 100 mM Tris buffer, pH 7.5, with a peristaltic pump (Ismatec ISM597D; Cole-Parmer) to achieve a final precipitation condition of 13.2% (w/w) PEG6000 (Grosshans et al., 2018). The hold time (residence time) in the tubular reactor was varied within a range of 2.5 to 22.5 min, by adapting the length of the tubular setup. The run-time of the continuous precipitation was varied up to 160 min. Samples were taken at various time points and analyzed with size exclusion chromatography. We calculated the standard deviations of yields, antibody purities, and high molecular weight impurities (HMWI), based on experimental repetitions and sampling at various time points.

2.6 | Influence of membrane pore size

The cell culture broth was combined with 40% PEG6000 plus 11.43 mM $ZnCl_2$ in 50 mM Tris buffer, pH 7.0 to achieve a final precipitation condition of 7% PEG6000 with 2 mM $ZnCl_2$ in a volume of 500 ml. The stirred tank of the Äkta flux S system (GE Healthcare) was used as a reactor vessel. The stirrer speed was set to 250 rpm. After 30 min of hold time, the precipitate was concentrated on a 50-cm^2 hollow fiber module fit with a filter membrane (either 0.1 or 0.45 µm pore size; GE Healthcare), until a feed pressure of around 100,000 Pa was achieved. The feed flow rate was set to 200 ml min^{-1}, and the permeate flow rate was set to 7.2 ml min^{-1}.

2.7 | Continuous precipitation

Continuous precipitation was achieved with a self-assembled tubular reactor setup. Spirally-arranged standard lab tubes (Tygon® R-3603, 4.8-mm inner diameter; Saint-Gobain, Courbevoie, France) filled with static mixers (HT-40-6.30-24-AC; Material Acetal; Stamixco AG, Wollerau, Switzerland) were vertically stacked. The setup was connected with polycarbonate Luer fittings (Cole-Parmer). The cell culture broth was continuously pumped at a flow rate of 6.6 ml min^{-1} (transfer pump stage one) and combined with a feed stream of 1.4 ml min^{-1} (permeate pump stage one) of 40% PEG6000 plus 11.43 mM $ZnCl_2$ in 50 mM Tris buffer, pH 7.0. This ratio resulted in precipitation conditions of 7% PEG6000 with 2 mM $ZnCl_2$. The hold time in the tubular reactor was 22.5 min at this flow rate. For continuous operation, two Äkta flux S (GE Healthcare) setups were connected in series. The precipitate was continuously fed into stage one and concentrated in the 0.2-µm hollow fiber module (GE Healthcare) with a filter area of 420 cm^2. In stage two, the precipitate was washed with the continuous addition of 7% PEG6000 with 2 mM $ZnCl_2$ in 50 mM Tris buffer, pH 7.0 and concentrated in the 0.2-µm hollow fiber module (GE Healthcare) with a filter area of 110 cm^2. Both hollow fiber modules were run at a 400 ml min^{-1} feed rate. The permeate flow rate was 7.2 ml min^{-1}. An external peristaltic pump

(Ismatec ISM 597; Cole-Parmer) with a flow rate of 0.8 ml min^{-1} was used to pump the concentrated precipitate continuously, from stage one to stage two, and to withdraw the washed precipitate continuously out of stage two. A second external peristaltic pump (Ismatec IPC; Cole-Parmer), with a flow rate of 7.2 ml min^{-1}, was used at the permeate site in stage one. For preliminary experiments, we used hollow fiber modules of various dimensions (e.g., 50 cm^2 with 0.1-μm, 0.2-μm, and 0.45-μm filters; GE Healthcare).

2.8 | Sample preparation for analytics

The precipitate was centrifuged at 2,000rcf for 1 min (Eppendorf centrifuge 5415R; Eppendorf, Hamburg, Germany). Then, the supernatant was withdrawn, and an equal volume of resolubilization buffer was added (gravimetrically determined). After resuspension and dilution, the samples were filtered through 0.2-μm filters before injection onto a high-performance liquid chromatography (HPLC) column.

2.9 | Protein A affinity chromatography

Protein A affinity chromatography was used to determine the antibody concentration. For HPLC, we used a Dionex UltiMate 3000 HPLC system equipped with a diode array detector (Thermo Fisher Scientific). Mobile phase A was a 50 mM phosphate buffer with 150 mM NaCl, pH 7.0. Mobile phase B was a 100 mM glycine buffer, pH 2.5. All buffers were filtered through 0.22-μm filters (GSWP04700; Merck KGaA) and degassed. The system was run at a flow rate of 2.5 ml min^{-1}. We loaded 20 μl of the sample, filtered through 0.2-μm filters (0.2-μm GHP AcroPrepTM 96 filter plate; Pall Life Sciences, Ann Arbor, MI), on a POROS A 20 μm Column (2.1 × 30 mm, 0.1 ml; Thermo Scientific). The column was washed with 10 column volumes of mobile phase A, eluted with 20 column volumes of 100% mobile phase B, and re-equilibrated with 30 column volumes of mobile phase A. The absorbance at 280 nm was measured. We used a similar protein A-purified IgG1 as the calibration standard. The calibration range was 0.1 to 3 mg mL^{-1}. We evaluated and quantified the results with the Chromeleon™ 7 software (Thermo Fisher Scientific).

2.10 | Size exclusion chromatography

We used size exclusion chromatography to determine the soluble HMWI and to estimate the product purity. For HPCL analytics, we used a Dionex UltiMate 3000 HPLC system equipped with a diode array detector (Thermo Fisher Scientific). The running buffer was a 50 mM sodium phosphate buffer with 150 mM NaCl, pH 7.0 (Merck KGaA). The buffer was filtered through 0.22-μm filters (GSWP04700; Merck KGaA) and degassed. After filtering the sample through 0.2-μm filters (0.2-μm GHP AcroPrepTM 96 filter plate; Pall Life Sciences), we applied 10 μl of the sample to a TSKgel® G3000SWXL HPLC Column (5 μm, 7.8 × 300 mm) in combination with a TSKgel SWXL Guard Column (7 μm, 6.0 × 40 mm; Tosoh, Tokyo, Japan). The absorbances at 215 nm (for HMWI determinations) and 280 nm (for purity estimations) were recorded, and the results were evaluated with the Chromeleon™ 7 software (Thermo Fisher Scientific). The antibody purity was calculated as the ratio of the monomer peak area to the sum of all peak areas, based on the 280 nm signal. The HMWI content was calculated as the ratio of the HMWI peak area to the sum of the monomer peak area plus the HMWI peak area, based on the 215 nm signal. This method just determines the soluble HMWI. Insoluble aggregates will be filtered out and not be detected. When $ZnCl_2$ was present in the sample, a wash step was performed to remove $ZnCl_2$, because $ZnCl_2$ seemed to interact with the size exclusion chromatography column media. Briefly, 350 μl of sample was pipetted into Amicon® spin tubes with a molecular weight cut-off of 50 kDa (Merck KGaA). The spin tubes were placed in 1.5 ml reaction tubes and centrifuged at 16,100rcf for 5 min (Eppendorf centrifuge 5415R; Eppendorf). Then, 350 μl of 50 mM sodium acid buffer, pH 3.5 (resolubilization buffer), was added to the sample, and the sample was centrifuged again. This buffer exchange procedure was repeated five times.

3 | RESULTS AND DISCUSSION

3.1 | Optimization of precipitation conditions

To optimize the precipitation protocol for this specific antibody, we fine-tuned the precipitation conditions in microtiter plates. In a previous study, we found that the optimal PEG size for antibody precipitation was PEG6000 (Sommer et al., 2014). Therefore, here, we tested different PEG6000 concentrations. We also tested the effects of adding $ZnCl_2$ to the feed material and the effects of flocculation before precipitation. Flocculation was described in a previous study (Burgstaller et al., 2018). In short, pDADMAC was used for cell flocculation, and cells were removed with depth filtration. In an initial test, we determined the optimal concentration of $ZnCl_2$ to add to the clarified culture supernatant (Figure 1a). We found that 7% PEG combined with 2 mM $ZnCl_2$ precipitated more than 99% of the antibody. However, higher $ZnCl_2$ concentrations seemed to give diminishing returns. In a second test, we assessed the influence of prior flocculation on antibody precipitation; for precipitation, we used 2 mM $ZnCl_2$ and different PEG6000 concentrations. Interestingly, preflocculated material required a higher PEG concentration than unflocculated material to precipitate the same amount of antibody. When pDADMAC was used in flocculation, host DNA coflocculated with the cell debris (Burgstaller et al., 2018; Tomic et al., 2015). We previously showed that an antibody and DNA coprecipitated during PEG precipitation (Hammerschmidt et al., 2016; Sommer et al., 2015). Accordingly, we speculated that the coprecipitation of DNA with an antibody could explain why a lower PEG concentration was required for unflocculated material. In theory, the solubility of the antibody should be independent of the feed solution; however, due to this coprecipitation, we will subsequently refer to the solubility as an "apparent solubility." For both flocculated and unflocculated materials, the addition of $ZnCl_2$ greatly

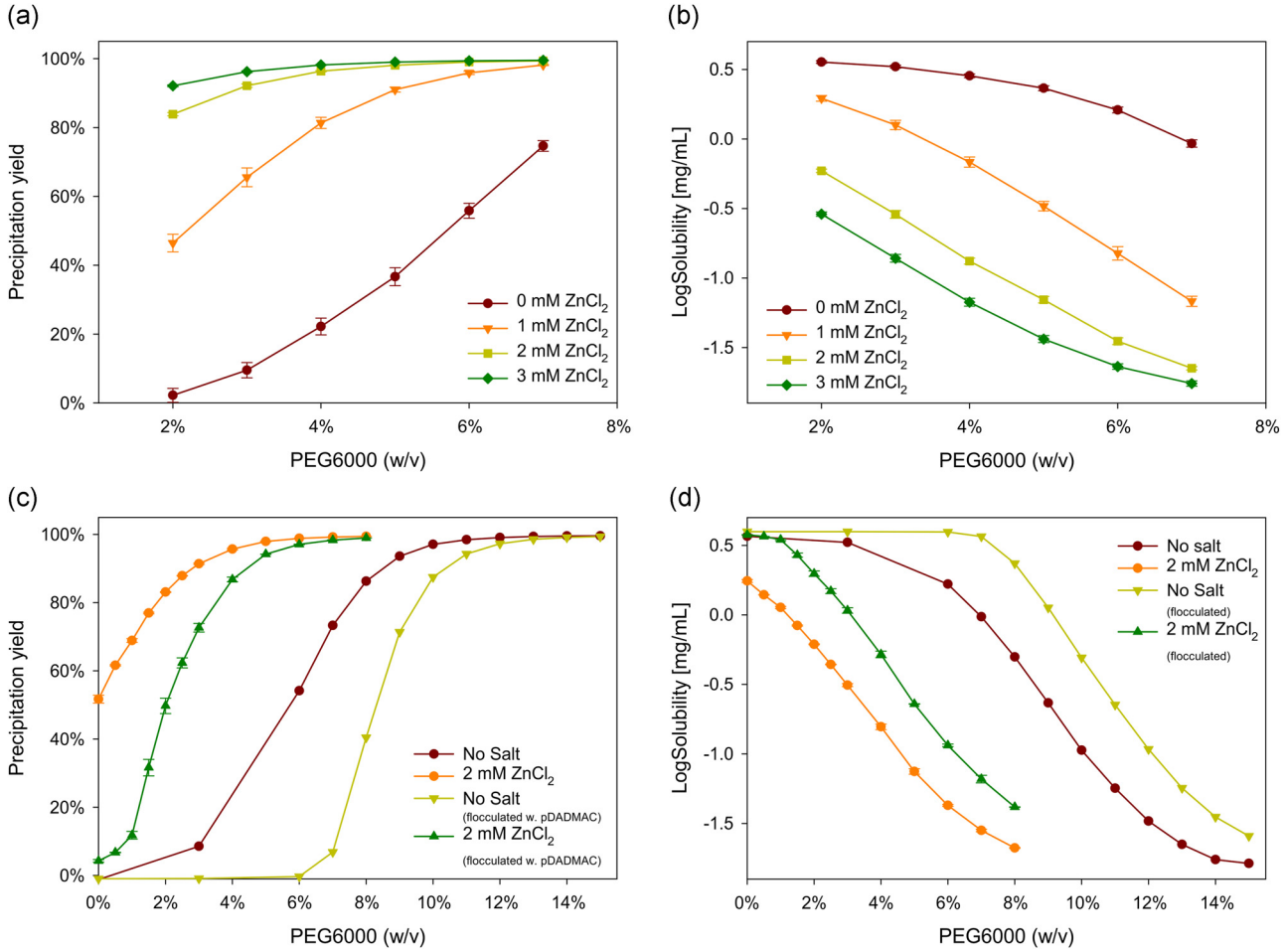

FIGURE 1 Optimization of antibody precipitation. (a) Precipitation yield with increasing PEG and $ZnCl_2$ concentrations; (b) solubility curve for increasing PEG and zinc concentrations; (c) influence of flocculation and added salt on precipitation yield; (d) resulting apparent solubility. The experiments were performed at pH 7.0. PEG: polyethylene glycol [Color figure can be viewed at wileyonlinelibrary.com]

reduced the apparent solubility; 7% PEG was sufficient for precipitating unflocculated material, and 12% PEG was required for precipitating flocculated material. Viscosities of 7 to 12% PEG6000 solutions are amenable to pharmaceutical downstream processes. The addition of $ZnCl_2$ is a very common practice in different unit operations for bioprocessing. Similar zinc concentrations are found in other settings. For example, in-house CHO cell media contains 0.7 to 1.6 mM Zn^{++}, and standard cell culture media, such as Dulbecco's modified Eagle's medium /Ham's F-12 CHO media, contains 0.432 mM Zn^{++} (Wong, Ho, & Yap, 2004).

The key to a successful precipitation process is an efficient resolubilization buffer. A common method for resolubilization is to dilute the precipitate in various buffers. However, to maintain low process volumes, we tested new resolubilization buffers to optimize the dilution ratio. In an initial test, we evaluated a 50 mM sodium acetate buffer at various pH levels (3.5, 5.0, and 6.0) and a 50 mM phosphate buffer at pH 6.0, pH 7.0, and pH 8.0. These buffers were tested at different dilution ratios of 1:2, 1:3, and 1:5. Then, the best conditions for each buffer system were used for the second round of optimization. For the acetate buffer, the best condition was pH 3.5 at a dilution ratio of 1:2; it provided a 91% resolubilization yield. For the phosphate buffer, the best condition was pH 7.0, at a dilution ratio of 1:5; it provided a 94% resolubilization yield. For both buffer systems, we attempted further optimization of the resolubilization yield by adding ammonium phosphate and sodium chloride in different concentrations (Figure 2). Based on these results, we determined that optimal resolubilization was achieved with 50 mM sodium acetate buffer at pH 3.5 without the addition of salt. The phosphate buffer achieved a slightly higher resolubilization yield than the acetate buffer, but the phosphate system was based on a 1:5 dilution, compared with the 1:2 dilution required for the acetate system. Because our objective was to concentrate the antibody solution as much as possible, we selected the 1:2 dilution with the acetate system. In addition, the acetate buffer required as little salt addition as possible. This feature provided an advantage for subsequent polishing steps, such as ion exchange chromatography. Moreover, a pH of 3.5 would be advantageous for a subsequent viral inactivation at low pH, which is commonly performed after the capture step in antibody purification. Thus, the low pH, minimal dilution, and minimal salt addition conditions that provided efficient resolubilization were expected to fit well in any commonly used antibody purification scheme, without further modification.

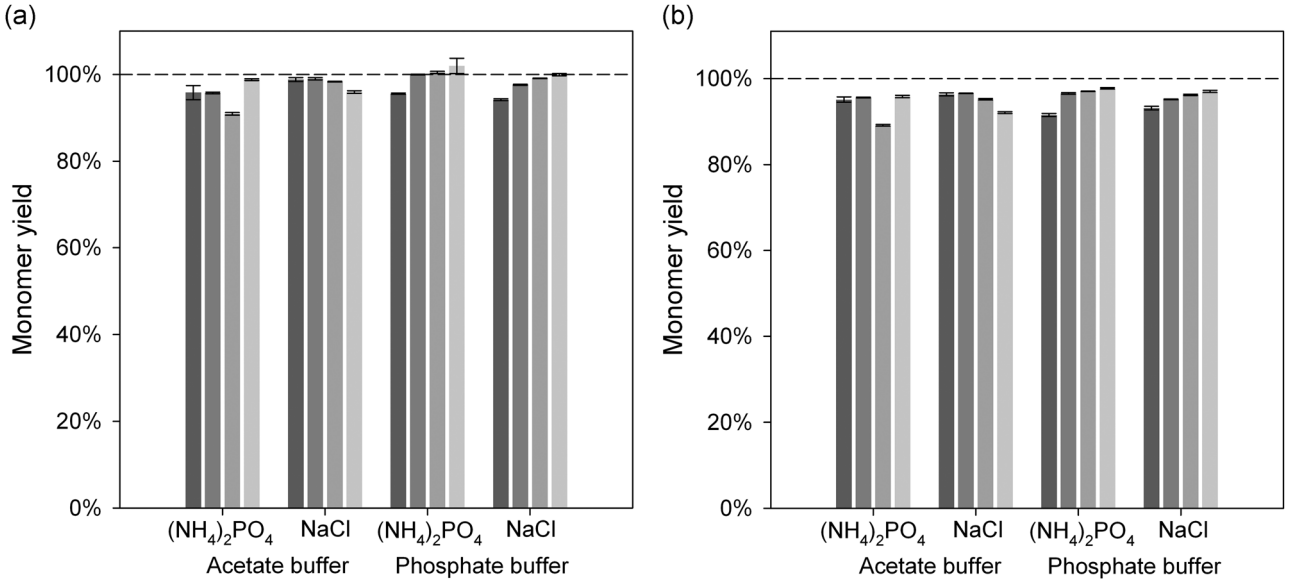

FIGURE 2 Effects of added salts on antibody monomer yields in optimized antibody precipitation and resolubilization conditions. Resolubilization was performed with 50 mM acetate buffer, pH 3.5, at a dilution of 1:2 or with 50 mM phosphate buffer, pH 7.0, at a dilution of 1:5, with or without added $(NH_4)_2PO_4$ or NaCl. Increasing salt concentrations are indicated with decreasing gray shading (dark gray: 0 mM to light gray: 150 mM; salt added) (a) Antibody monomer yield from nonflocculated cell culture broth under various resolubilization conditions. (b) Antibody monomer yield from flocculated cell culture broth under various resolubilization conditions

3.2 | Sequential TFF

We used TFF for harvesting, concentrating, and washing the precipitate, because TFF does not compress the precipitate, compared with centrifugation or dead-end filtration. Compressed precipitates have very slow resolubilization kinetics because the resolubilization buffer cannot penetrate deeply into the compressed structure of the precipitate. Avoiding compaction of the precipitate facilitates resolubilization significantly and reduces the process time. A viable, integrated capture step for continuous operations requires volume reduction, contaminant removal, and a mode of operation transfer from batch TFF.

Initially, we tested the feasibility of the intended continuous integrated TFF method in sequential operations (Figure 3a). In each stage, the precipitate was fed with a constant flow rate from a tank into the retentate vessel of the TFF unit. A controlled bleed flow from the retentate vessel was collected, and the material that permeated the membrane went to waste. The feed and bleed flow rates remained constant. The permeate flow rate was adjusted to maintain a constant volume in the retentate vessel. The bleed stream contained the concentrated antibody product. This product was then diluted with wash buffer and used to feed the next stage. With this approach, we conducted several sequential stages of concentrating and washing the precipitate with a single bench-top instrument.

For the three sequential stages (one concentration stage and two wash stages), we used the same 0.2-μm hollow fiber membrane with a tangential flow of 675 L $m^{-1}h^{-1}$ (LMH; Hammerschmidt et al., 2016) for concentration. The permeate flow was regulated to 75 LMH (Hammerschmidt et al., 2016) with a pump. At the retentate vessel, the ratio of bleed flow:feed flow was 1:12.5, which resulted in a theoretical concentration of 12.5-fold, once the unit reached a steady state. After each stage, the collected, concentrated antibody precipitate slurry was diluted (1:10) with wash buffer, and subsequently, it was concentrated again in the next unit. This procedure mimicked a continuous process with a step-wise diafiltration process to remove nonprecipitated contaminants. After each such stage, the precipitate was dissolved and measured with size exclusion chromatography for purity (Figure 3b). We observed that, after the second stage, the purity did not increase further; therefore, we selected a two-stage process for the continuous operation. Moreover, the size exclusion chromatography results showed that no additional aggregation occurred, compared to that observed in the cell culture broth.

During the run-time, we also determined the concentration of antibody in the retentate vessel. We found that the concentration varied over the run-time of 200 min, and it did not reach a steady state within this period. To estimate the time to steady state for the concentration and wash steps, we setup a simple mass balance model. The feed concentration of our precipitated antibody and all flow rates of the system were known. We summed the accumulated antibody in the permeate vessel and in the bleed stream at each time point of the process. Then, the accumulated mass of precipitated antibody (m) at any given time point (t) could be modeled with for both steps separately, as follows:

$$m_{\text{RETENTATE VESSEL}}(t)=\frac{dm_{\text{FEED}}}{dt}-\frac{dm_{\text{BLEED}}}{dt}-\frac{dm_{\text{PERMEATE}}}{dt}. \quad (1)$$

The antibody precipitate feed (m_{FEED}) into the system was constant over time. We assumed that the antibody was completely precipitated; hence, the loss of antibody through the permeate

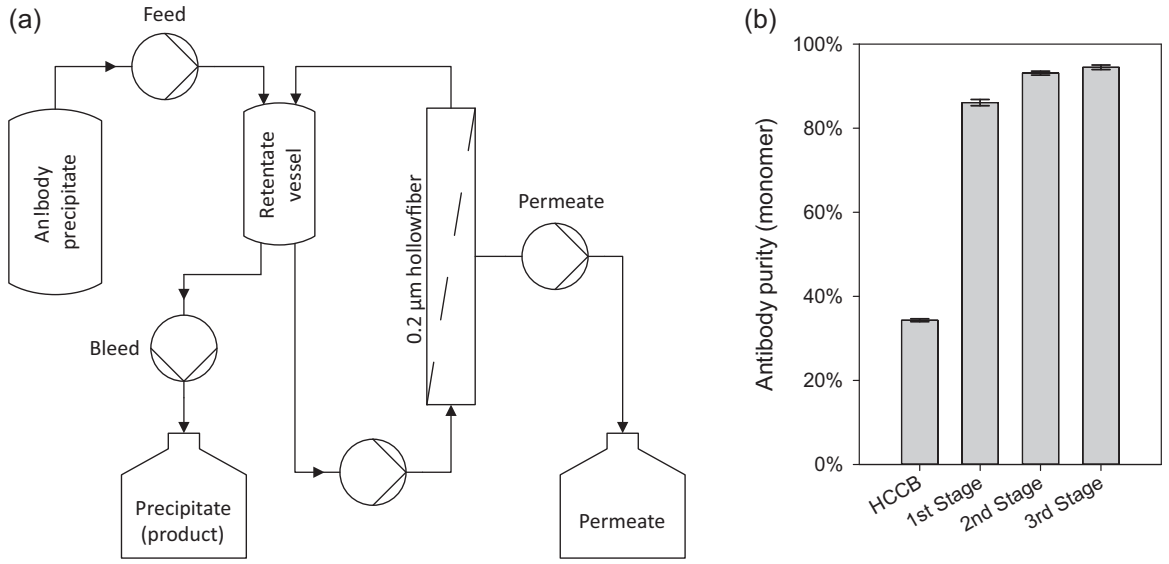

FIGURE 3 Sequential tangential flow filtration (TFF)/discontinuous diafiltration. (a) Flow diagram of the sequential TFF. (b) The purity of the antibody monomer: before TFF, in the host cell culture broth (HCCB); after the antibody precipitate concentration (1st Stage); after the first antibody precipitate wash step with continuous diafiltration (2nd Stage); and after the second antibody precipitate wash step (3rd Stage)

($m_{PERMEATE}$) was zero. This assumption was also confirmed later, during the integration of both steps into a single process. Steady-state occurred when the mass flow of the feed was equal to the mass flow of the bleed, and $m_{RETENTATE\ VESSEL}$ remained constant. Knowing the initial antibody concentration, we solved the equation numerically and matched the data with experimentally determined antibody concentrations from the first stage (Figure 4a). The concentration was normalized to the cell culture supernatant concentration; thus, the curve started below 1, because the cell culture supernatant had to be diluted with the PEG6000 stock solution for precipitation. Therefore, the reported concentration factor represented the concentration relative to the culture supernatant. When we assumed 100% yield for the numerical model, the results fit very well to the measured antibody concentrations, until a run-time of 120 min. After that, we lost antibody, and the final yield was 81%, most likely due to high antibody concentrations and formation of aggregates that could not resolubilize. The experiments to test the feasibility of sequential TFF were performed with nonoptimized buffer conditions (13.2% [w/w] PEG6000, pH 7.5). As 81% yield in antibody

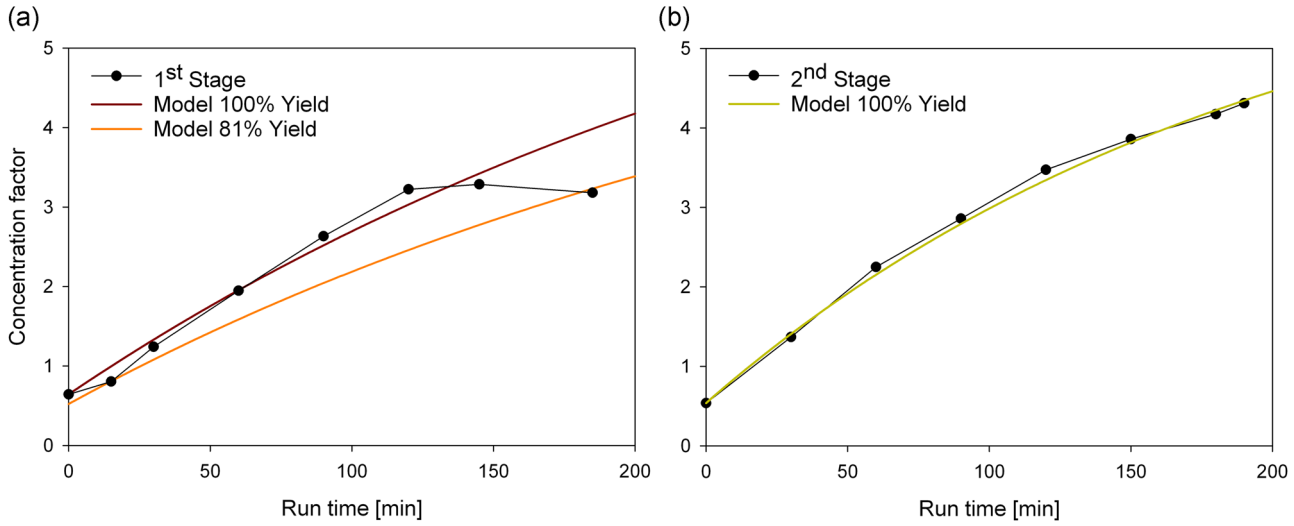

FIGURE 4 Change in the concentration factor during antibody precipitate concentration with discontinuous diafiltration. The concentration factor was the concentration of antibody relative to the concentration of antibody in the cell culture supernatant. (a) Changes in antibody precipitate concentration factors during the 1st stage. Solid lines: the expected concentration factor changes, at a yield of 100% (red) or 81% (orange). (b) Changes in antibody precipitate concentration factors during the 2nd stage, when the antibody precipitate obtained from the 1st stage was further concentrated. The first stage product was washed at a 1:10 dilution in wash buffer. Green line: the expected concentration factor change, at a yield of 100% [Color figure can be viewed at wileyonlinelibrary.com]

production is not acceptable in an antibody capture step, the integrated process was performed with optimized buffer conditions and zinc addition.

Figure 4b shows the antibody concentration over time during the second stage, where the antibody precipitate was washed with discontinuous diafiltration. As in the first stage (Figure 4a), we numerically solved the mass balance model (Equation (1)). The model fit the measured antibody concentrations very well at a theoretical yield of 100% (Figure 4b). No antibody was lost in this wash step and product loss due to high concentrations of antibody did not appear to be an issue in this stage. The higher purity during the second step compared to the first step indicated that contaminants, like HCP, DNA, and media components, were less likely to interfere with the precipitate. Once the majority of these contaminants is removed, high antibody concentrations can be achieved without losing antibody. We achieved an overall yield of 81% in both steps combined, with this sequential antibody precipitate concentration and wash procedure. We increased this yield by integrating the two units.

3.3 | Variation of residence time in continuous precipitation

A tubular reactor is necessary for a transition from batch precipitation to continuous precipitation. We have used tubular reactors in previous studies (Burgstaller et al., 2018; Hammerschmidt et al., 2016) for precipitation, but we did not quantify the influence of the tubular reactor length. Here, we assessed the impact of the reactor length (corresponding to the residence time in the reactor) during continuous antibody precipitation in the tubular reactor. We measured the impact of residence time on HMWI content, antibody yield, and antibody purity (Figure 5). The residence time in our setup was defined by the tube length and diameter and the flow rate of material through the tube. The diameter of the tube and the flow rate were constant (8 ml min^{-1}); we changed the reactor length to determine whether longer or shorter reactors might lead to different process performances. Thus, we tested different residence times, ranging between 2.5 and 22.5 min, by varying the tubular length from 1.2 to 10.8 m. Figure 5 shows the effects of the various tested residence times, in terms of yield (Figure 5a), purity of the antibody monomer (Figure 5b), and HMWI content (Figure 5c). All three parameters showed stable performance, independent of the residence time. We achieved an 82.4 ± 3.5% precipitation yield and an antibody monomer purity of 80.0 ± 5.0% with nonoptimized buffer conditions in this experiment (using 13.2% [w/w] PEG6000 and pH 7.5). As 80% yield is typically not acceptable for antibody purification processes, all further experiments were carried out with the optimized buffer conditions including $ZnCl_2$. Interestingly, the HMWI content was reduced significantly by the precipitation and resolubilization steps (from about 7% to about 2%), for all reactor lengths, with slight variations. We speculated that this efficiency occurred because the HMWIs precipitated easily but were difficult to resolubilize; thus, these particles remained precipitated upon resolubilization, and thus, they were lost. Because the reactor length did not seem to have a significant impact on the performance of the precipitation, we decided to use a residence time of 22.5 min, because this closely matched the incubation time of 20 min we used in all batch experiments in the present and previous studies.

3.4 | Continuous diafiltration

Before starting the continuous operation, we tested the influence of the pore size of the hollow fiber membrane. We did not observe different results with 0.1 and 0.45-μm pores; therefore, we selected a 0.2-μm pore size (see Supporting Information Figure 1).

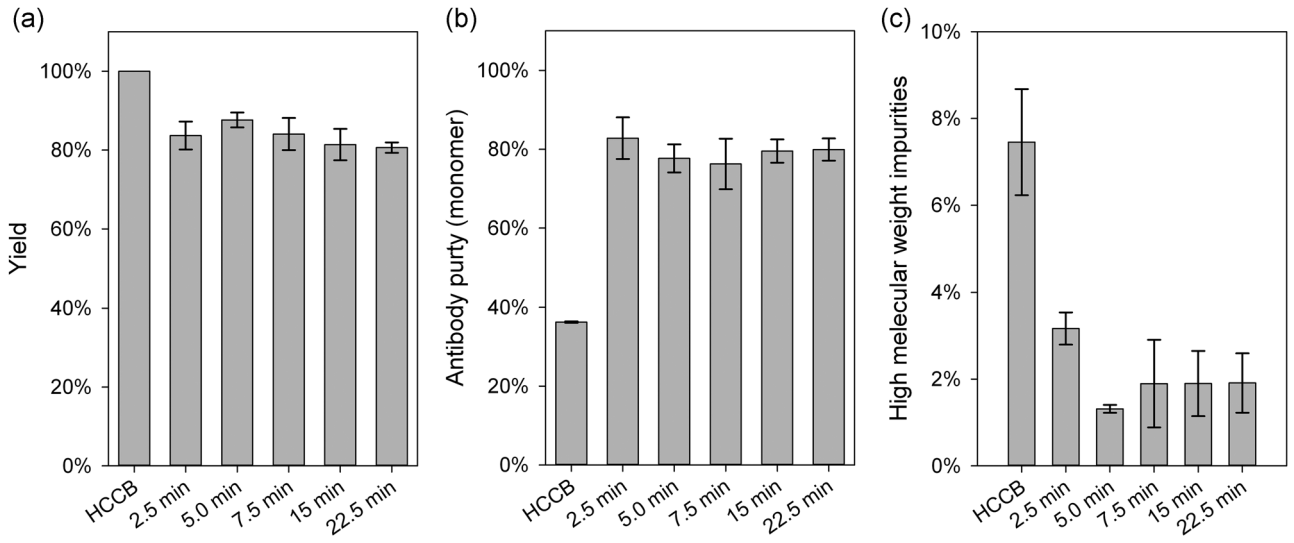

FIGURE 5 Influence of the residence time during continuous precipitation in a tubular reactor. (a) Antibody yield; (b) purity of the antibody monomer; and (c) high molecular weight impurities were measured after different residence times (2.5–22.5 min). The host cell culture broth (HCCB) served as the reference. The whiskers indicate standard deviations, which included variations in the measurements taken at different time points over the experiment period (20–160 min) and variations between experimental repetitions

Next, we integrated all the components that we had optimized or determined into one continuous precipitation step with a TFF system (Figure 6). The optimal precipitation conditions determined in section 3.1 were used in the in-line feed into the tubular reactor and in the feed to the retentate vessel of the first unit. A constant bleed was pumped directly into the retentate vessel of the second unit, without any holding tanks between the units, and both units were run as described in section 3.2. The bleed from the second unit was collected, and this was the concentrated purified precipitated antibody.

First, we combined the precipitant stream (PEG6000 with $ZnCl_2$) with the cell culture stream in a specific ratio by adjusting the flow rates of the peristaltic pumps to achieve final precipitation conditions of 7% PEG6000 and 2 mM $ZnCl_2$. The precipitation was conducted in a self-assembled tubular reactor, with helical static mixers. These static mixers ensured a narrow residence time distribution, which prevented precipitate from settling. The length and diameter of the tubular reactor and the flow rate through the tubular reactor defined the precipitate residence time. In our case, the residence time was 22.5 min, as described above (section 3.3). After the tubular reactor, the precipitate was pumped into the first TFF unit. In a first stage, the precipitate was concentrated with tangential microfiltration (pore size 0.2 µm; membrane area 420 cm^2). The precipitate stream over the membrane was 571 LMH. We selected a concentration factor of 10 (ratio of the feed flow to the bleed flow), because the concentration factor selected for previous experiments (i.e., 12.5) resulted in product loss at high precipitate concentrations. In the first 60 min of the process, the bleed into the second unit was closed off to accelerate equilibration to a steady state. After 60 min, the bleed was continuously fed into the retentate tank of stage two, and a wash buffer containing 7% PEG600 and 2 mM $ZnCl_2$ was continuously added to achieve a 1:10 dilution of the bleed from the first unit.

In the second unit, again, we selected feed and bleed flow rates to achieve a concentration factor of 10, and the retentate tank level was maintained at a constant volume. Again, in this unit, we closed off the bleed to allow equilibration for a startup phase of 60 min. After 60 min (120 min after process start), the bleed out of stage-two was started. The membrane of the second unit (pore size 0.2 µm; membrane area 110 cm^2) was smaller than that of the first unit, because, in the sequential simulation process (section 3.2), we observed reduced fouling in the second stage. The precipitate stream over this second membrane was 2,182 LMH. At 180 min after the process started, we continuously harvested the washed precipitate, which was bled at a low flow rate out of the stage-two unit.

The changes in antibody concentration during stage one runtime are depicted in Figure 7a. The antibody concentration continuously increased up to 25.0 mg ml^{-1} during a 540-min runtime. In comparison, the maximum antibody concentration predicted with the numerically solved mass balance model, assuming a yield of 100%, was 25.6 mg ml^{-1}. Thus, the numerically solved model fit the measured antibody concentration very well. Both the model and the measured data showed that steady state was reached after around 400 min, when the antibody concentration leveled off. The concentration factor was 8.1 compared with the initial antibody concentration in the cell culture broth before stage one. The concentration changes in the antibody and the harvested antibody during stage two are depicted in Figure 7b. Stage two started when the precipitate bleed in unit one was started after 120 min. The flow rate of the added wash buffer was the same as the flow rate of permeate removal from this unit, to maintain a constant feed inflow and product outflow in the second stage. Therefore, no additional precipitate concentration was achieved in this second stage. Indeed,

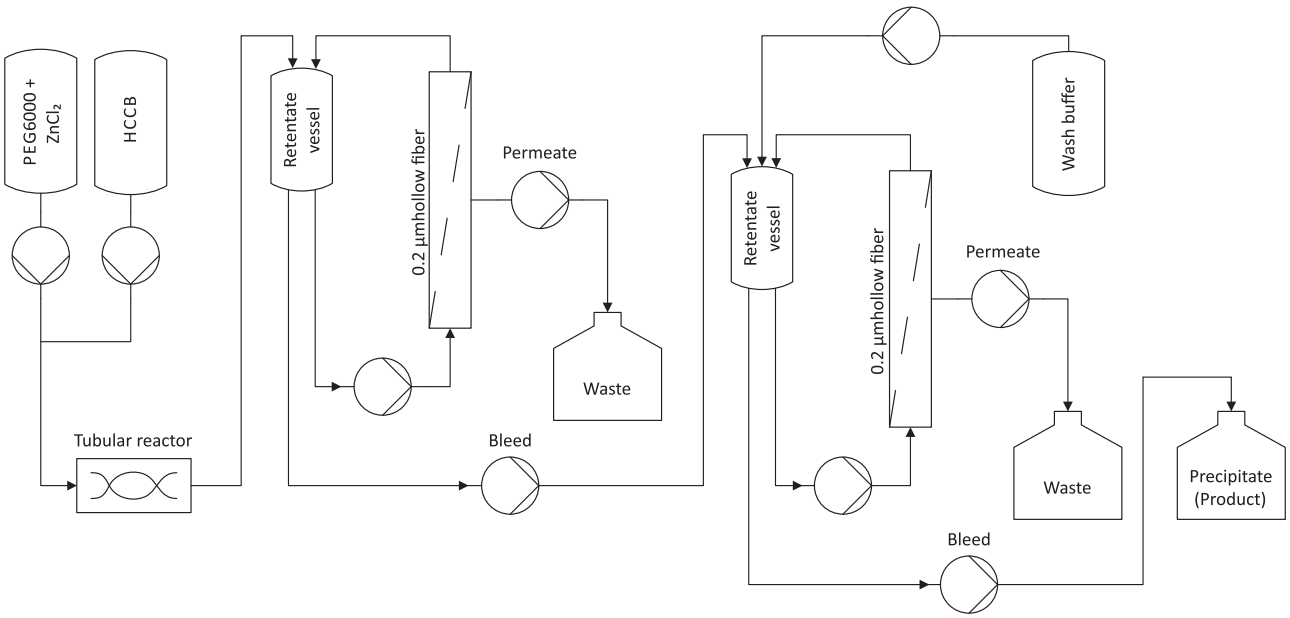

FIGURE 6 Flow diagram of continuous antibody precipitation in a tubular reactor setup. With tangential flow filtration (0.2 µm hollow fiber), the precipitate is separated (first bleed) and then washed (second bleed) in a continuous process. HCCB: host cell culture broth; PEG: polyethylene glycol

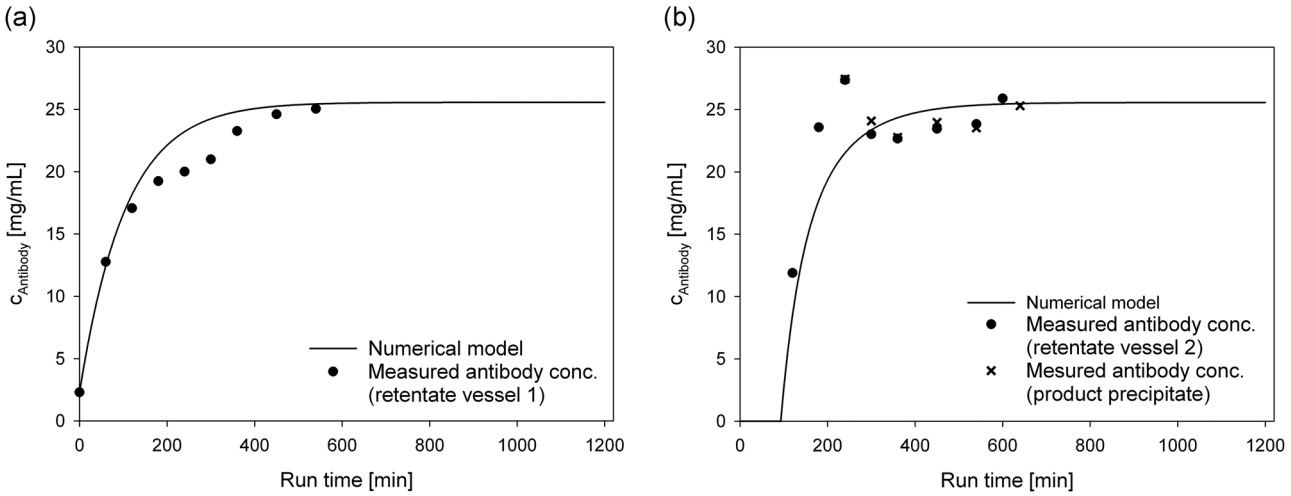

FIGURE 7 Continuous antibody precipitation with two-stage tangential flow filtration. Plots compare the measured antibody concentrations (symbols) and the numerically solved mass balance model data (solid lines) during (a) the first stage, and (b) the second stage; filled circles: measured antibody in retentate vessel; crosses: the harvested antibody precipitate

this stage was dedicated as a washing step to remove residual soluble HCP and other contaminants. The continuous harvest of antibody precipitate was initiated after 180 min. After an initial spike of antibody concentration to 27.4 mg ml^{-1}, the antibody concentration leveled off to roughly 25 mg ml^{-1}, as expected and predicted by the model. The antibody concentration in the harvest was, as expected, analogous to the antibody concentration in the retentate tank of stage two. The model predicted that the steady state would be achieved after 400 min of run-time. This prediction matched the experimental data. Because we started stage two with an inflow of precipitate that was more highly concentrated than the inflow of stage one, the startup phase for stage two (Figure 7b) was much shorter than that of stage one (Figure 7a). Thus, the two-stage tangential flow microfiltration setup produced an overall concentration factor of 8.2 for the continuous harvest or antibody precipitate. The monomer yield is defined as the recovered amount of antibody over the amount of antibody, which has been fed into the system. The monomer yield of the process was 95%, after recovering precipitate from the instruments. We lost 2% of antibody monomer in the permeate of stage one, which reduced our yield; however, we did not lose a significant amount of antibody in the permeate of stage two (below 1%). We harvested 81% of the antibody when we shut it down after 600 min. After flushing the system with buffer, we recovered an additional 5% of the antibody that had remained in the tubular reactor of the continuous precipitation; another 6% that had remained in the membrane and tubes of stage one; and another 3% that had remained in the membrane and tubes of stage two. Overall, the antibody monomer recovery of the entire process was 97%. A longer process duration would most likely produce a higher yield and a more favorable relationship between the precipitate harvested at the outlet and the precipitate that was harvested by flushing the system.

To determine product quality and purity, we measured monomer purity and the HMWI removal with size exclusion chromatography. Unfortunately, we could not measure samples that contained $ZnCl_2$ with our standard size exclusion chromatography analytics, because we observed atypical tailing. Moreover, the stationary phase of the column seemed to be affected by running $ZnCl_2$-treated samples. Even long elution steps or regeneration steps could not reverse this effect. We assumed that the stationary phase of the size exclusion chromatography column (5-μm silica particles with diol phase groups, 250 Å pores) was not fully inert to Zn ions. Thus, the Zn ions could have mediated weak ionic interactions with proteins, and thereby constrained the elution. Although this effect might be of additional scientific interest, we did not investigate it further in the present study. To avoid this tailing, we had to wash the resolubilized samples several times to remove $ZnCl_2$ before size exclusion chromatography analysis. Figure 8a shows the HMWI of various samples from the continuous run. The HMWI content was reduced from 7.3% in the initial host cell culture broth (HCCB) to 3.5% after resolubilization, at the beginning of precipitation. After 300 min of run-time, the HMWI content was further reduced to 2.4%. After 300 min of precipitate wash in stage two, the HMWI content was reduced to 1.4%. This resulted in a fivefold reduction of HMWI compared with the initial HMWI content of the HCCB. We speculate that we might not have actually removed HMWI during the precipitation step. Instead, these impurities might have formed aggregates that were carried through the system, and they were unable to resolubilize at the end. Therefore, the HMWI were actually removed when precipitate was discarded from the resolubilized antibody. HMWI can cause severe immunological issues. Subsequent polishing steps such as (flow-through) ion exchange chromatography, as usual in an antibody downstream process, will further reduce remaining HMWI.

The antibody monomer purity (Figure 8b) increased from an initial 34 to 91%, at the beginning of the precipitation; then, it

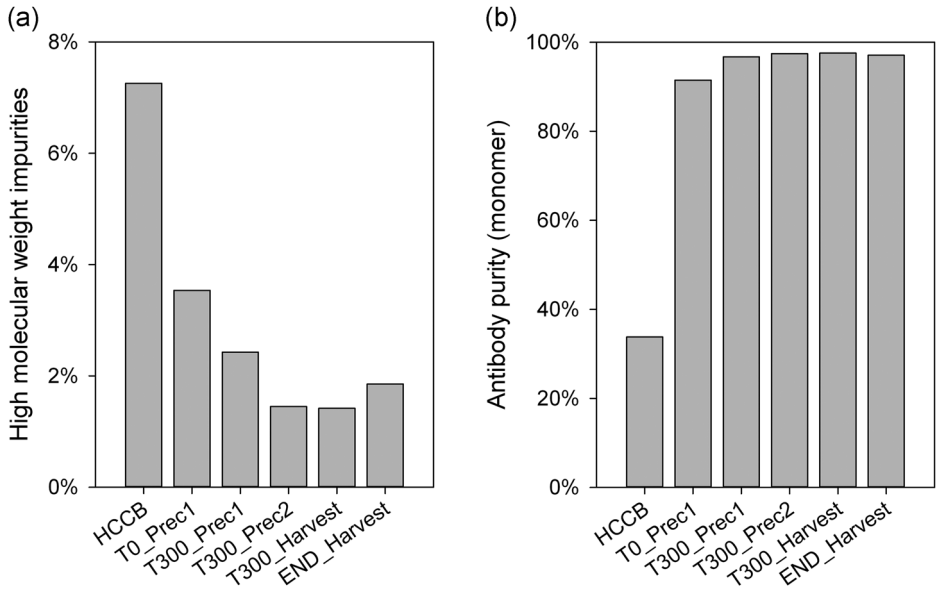

FIGURE 8 Removal of high molecular weight impurities and antibody purity measurements. (a) High molecular weight impurities in the host cell culture broth (HCCB), reduced HMWI in the resolubilized pellet in stage one (T0_Prec1) and again at 300 min (T300_Prec1). In stage two, reduced HMWI after 300 min (T300_Prec2). T300_Harvest: the precipitate harvested after 300 min; END_Harvest: pool of the precipitate harvested at the end of the process. (b) Antibody purity of the same fractions shown in (a). HMWI: high molecular weight impurities

increased again to 97% after 300 min of run-time. This purity is comparable with that achieved in elutes from protein A chromatography; moreover, in addition, we reduced the HMWI. We expect also that the specific purification performance in regard to DNA, HCP, as well as nature of HCP, can match the performance of protein A chromatography, as was already shown in other publications (Hammerschmidt et al., 2016; Sommer et al., 2015). To achieve continuous protein A affinity chromatography, the process must be cycled or performed with counter-current loading. However, that method interrupts the continuous mass flow, and in turn, it significantly increases the residence time distribution of the entire process. In contrast, our process provided continuous, uninterrupted mass flow, and a fully continuous process could be maintained. Furthermore, the outflow of the resolubilization reactor could be fed directly into a continuous virus inactivation solution, (Klutz, Lobedann, Bramsiepe, & Schembecker, 2016) and subsequent flow-through chromatography (Ichihara, Ito, Kurisu, Galipeau, & Gillespie, 2018) would not interrupt the mass flow.

The process described here had several advantages over other methods. First, it incorporates a simple, robust setup of readily available instrumentation, because TFF is a standard unit operation currently used in the pharmaceutical industry for buffer exchanges, perfusion systems, and other operations (Rucker-Pezzini et al., 2018). Second, precipitation is, by nature, a concentration-independent process; the addition of precipitant only depends on the volume that must be processed, not on the specific antibody concentration in the feed stream. Thus, a very simple control loop is required to maintain steady-state precipitation, and it is not necessary to perform complex, expensive on-line measurements of antibodies in a complex feedstock. This feature greatly simplifies the purification process, particularly in the context of perfusion cultures with changing antibody concentrations. Third, our TFF-based precipitation method provided a continuous steady outflow of product. This steady stream could be directly connected to the subsequent unit operations without intervening surge tanks.

4 | CONCLUSION

In this study, we developed a truly continuous antibody capture process. The antibody was continuously precipitated from clarified culture supernatant, and the antibody precipitate was continuously concentrated and washed with yield and purity similar to those achieved with protein A affinity chromatography. This process was robust because fluctuations in the feed stream were readily handled. There was no need for on-line protein concentration monitoring. The setup could be readily realized as a disposable unit, because the necessary equipment, such as tubing, fittings, static mixers, and hollow fiber modules are commercially available. This process is truly continuous compared with other, quasi/semicontinuous chromatography processes, which require a cyclic operation. Our process did not require surge tanks. This process could be used to integrate capture, virus inactivation, and even first intermediate purifications.

ACKNOWLEDGMENTS

This study was funded by the European Union's Horizon 2020 program under grant agreement number 635557 (Project:

Next-generation biopharmaceutical downstream process). A.J. was supported by the Federal Ministry for Digital and Economic Affairs (bmdw), the Federal Ministry for Transport, Innovation, and Technology (bmvit), the Styrian Business Promotion Agency SFG, the Standortagentur Tirol, Government of Lower Austria and ZIT-Technology Agency of the City of Vienna through the COMET-Funding Program managed by the Austrian Research Promotion Agency FFG. The funding agencies had no influence on the conduct of this study. We extend special thanks to Christian Leisner and GE Healthcare for loaning us a second ÄKTA flux S system.

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

How to cite this article: BurgstallerD, JungbauerA, SatzerP. Continuous integrated antibody precipitation with two-stage tangential flow microfiltration enables constant mass flow. *Biotechnology and Bioengineering*. 2019;1–13. https://doi.org/10.1002/bit.26922