

Continuous cell flocculation for recombinant antibody harvesting

Daniel Burgstaller,^a Walpurga Krepper,^a Josselyn Haas,^b Marine Maszlin,^b Jure Mohoric,^c Katja Pajnic,^c Alois Jungbauer^{a,d*} and Peter Satzer^a

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 $\geq 97\%$ 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 flexibility.

© 2017 The Authors. *Journal of Chemical Technology & Biotechnology* published by John Wiley & Sons Ltd on behalf of Society of Chemical Industry.

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}; chitosan⁵; 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

* Correspondence to: A Jungbauer, Department of Biotechnology, University of Natural Resources and Life Sciences, Vienna (BOKU), Muthgasse 18, 1190 Vienna, Austria. E-mail: alois.jungbauer@boku.ac.at

a Department of Biotechnology, University of Natural Resources and Life Sciences, Vienna (BOKU), Vienna, Austria

b Merck, 67120 Molsheim, France

c LEK, a Sandoz company, 1000 Ljubljana, Slovenia

d Austrian Centre of Industrial Biotechnology (ACIB), Vienna, Austria

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).

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 HMW.

Batch filter screening

Prior to use, the 23-cm² 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 m² 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 cm² Clarisolve[®] 40MS depth filters in µPod[®] format (Merck KGaA), and 50 mL fractions were manually collected for assessment of yield, purity, and HMW.

Size exclusion chromatography

Size exclusion chromatography was used to assess antibody yield, purity, and the amount of HMW. High-performance liquid chromatography was carried out by isocratic elution on a Dionex Ulti-Mate 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 HMW) 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 CIEH-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. HMW). 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.

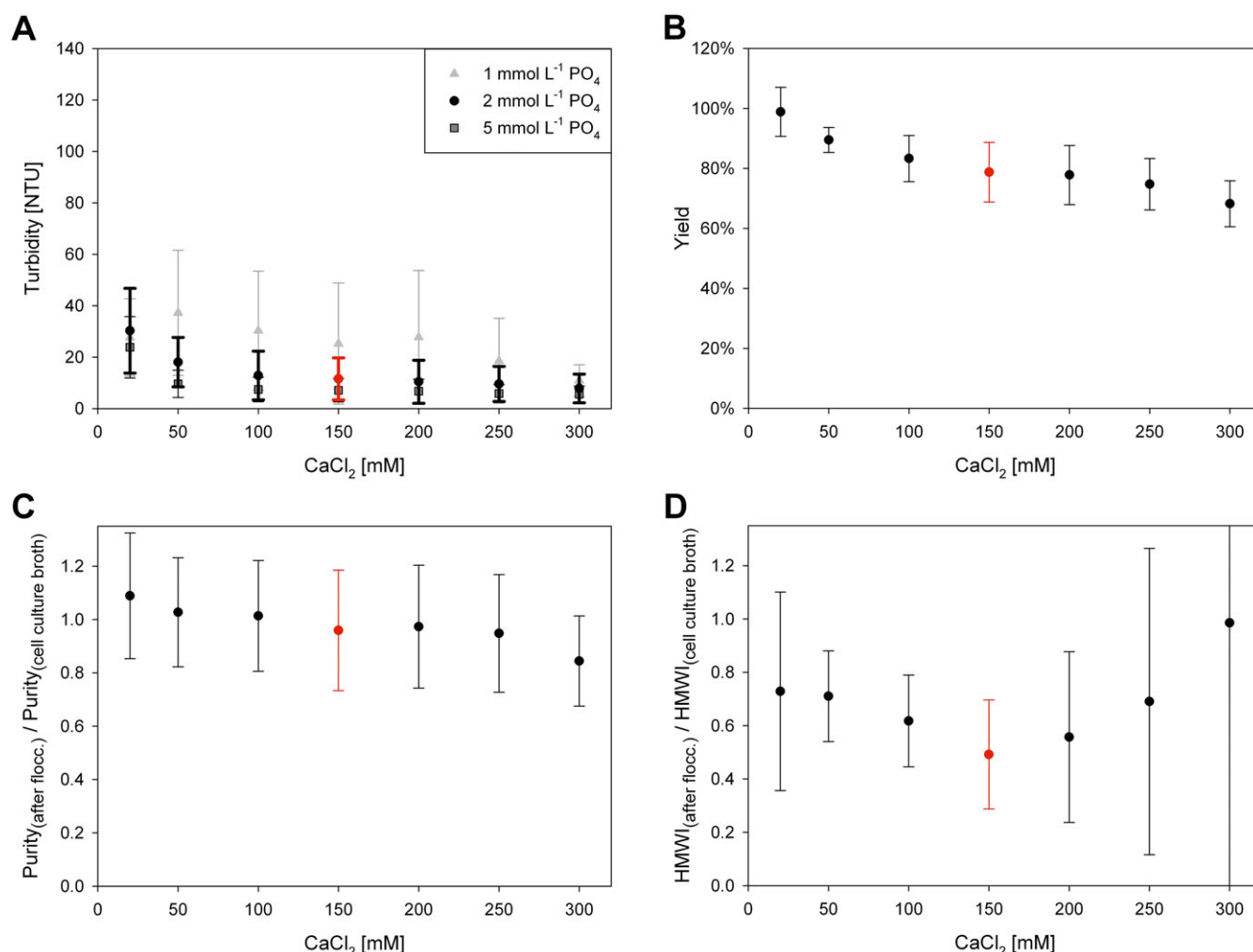


Figure 1. Performance of CaCl₂ flocculation (floc.) in terms of turbidity after flocculation (A), yield (B), purity (C), and high molecular weight impurities (HMWl) (D). Turbidity is shown for addition of phosphate at three different concentrations. All other data are shown only for addition of 2 mmol 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 HMWl; therefore, the purity and HMWl 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. CaCl₂ 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 CaCl₂ 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 HMWl (which predominantly included antibody aggregates) was highly variable among all screened production batches,

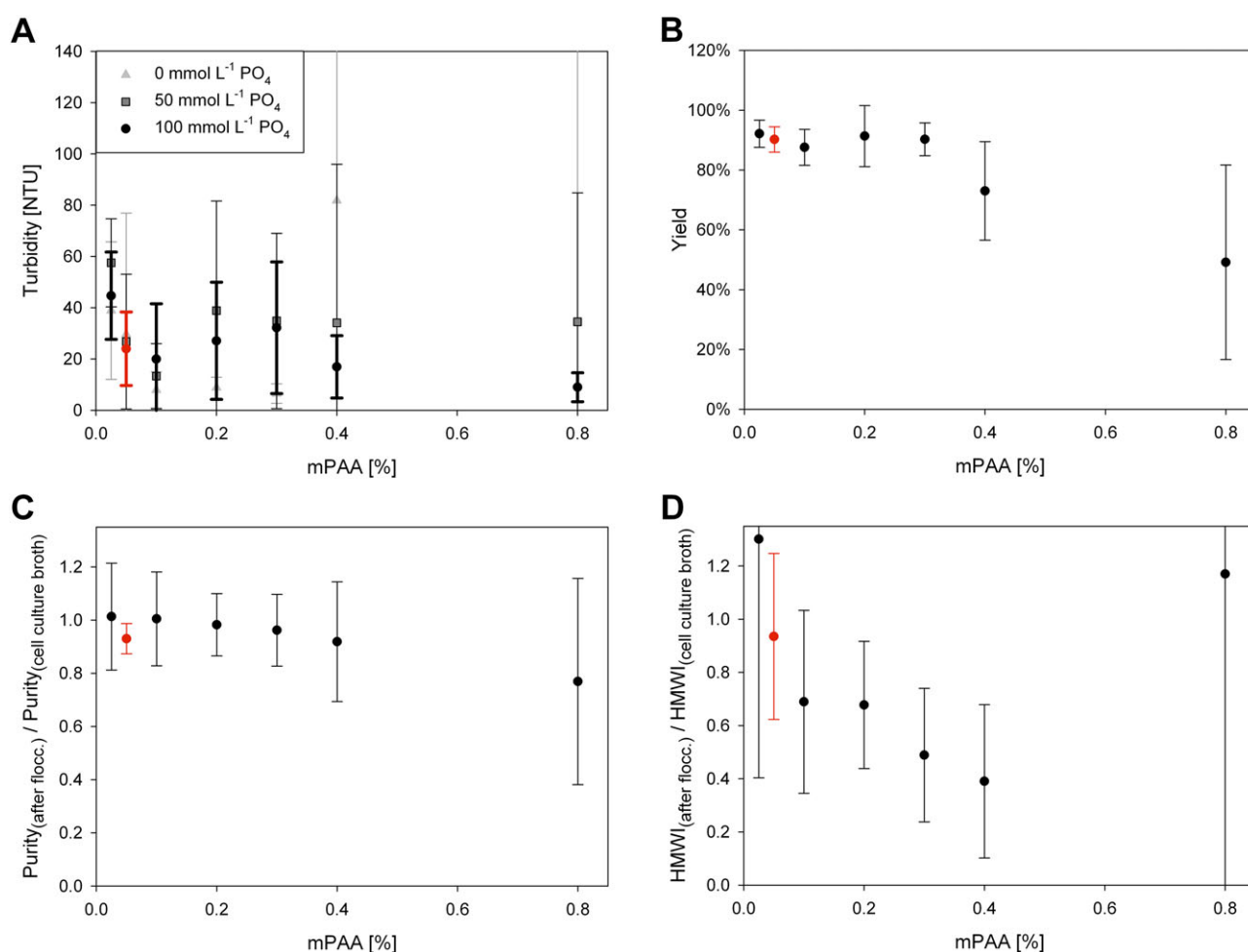


Figure 2. Performance of mPAA flocculation (flocc.) in terms of turbidity after flocculation (A), yield (B), purity (C), and high molecular weight impurities (HMWI) (D). Turbidity is shown for three different phosphate concentrations. All other data are shown only for addition of 100 mmol L⁻¹ phosphate. 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 CaCl₂ concentration (Fig. 1(D)). Overall, a broad and stable operation range was observed with 50 mmol L⁻¹ to 300 mmol L⁻¹ of CaCl₂ 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 CaCl₂ concentration narrows the operation window to low-to-intermediate CaCl₂ concentrations. For filter screenings, 150 mmol L⁻¹ CaCl₂ 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))

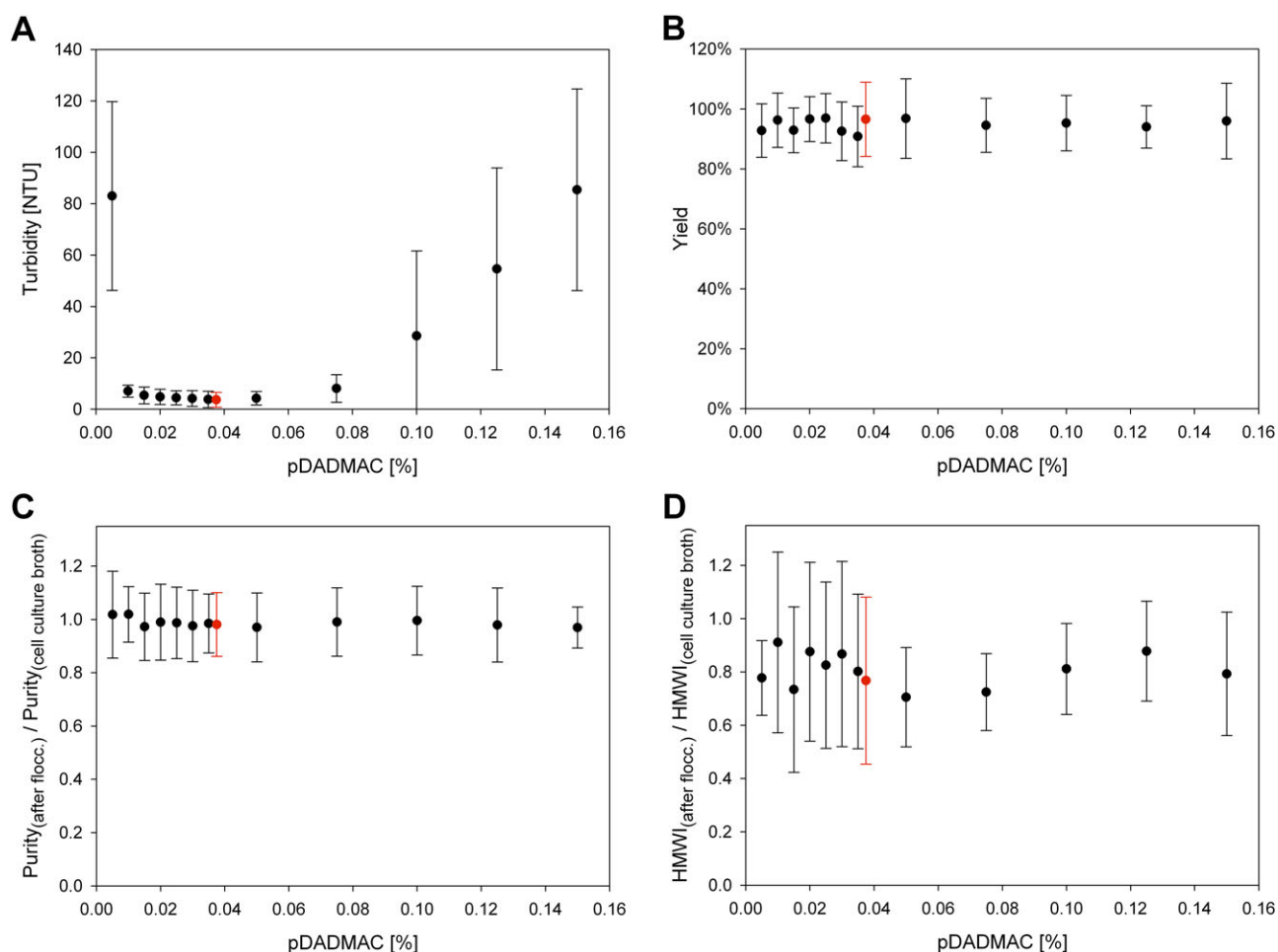


Figure 3. Performance of pDADMAC flocculation (floc.) in terms of turbidity after flocculation (A), yield (B), purity (C), and HMWl (D). The standard deviations shown represent the data from five different fermentations. Selected conditions for filter screening are highlighted in red. NTU, nephelometric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

was not observed. HMWl 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 CaCl₂ 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 CaCl₂ 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 CaCl₂ a purity 0.96 and for mPAA a purity of 0.93 (Fig. 4(B)). CaCl₂ is capable to reduce HMWl by a factor of roughly 2, but at the risk of losing yield. The average HMWl content achieved after flocculation compared with HMWl content of the cell culture broth was 0.49 for CaCl₂, 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 CaCl₂ 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

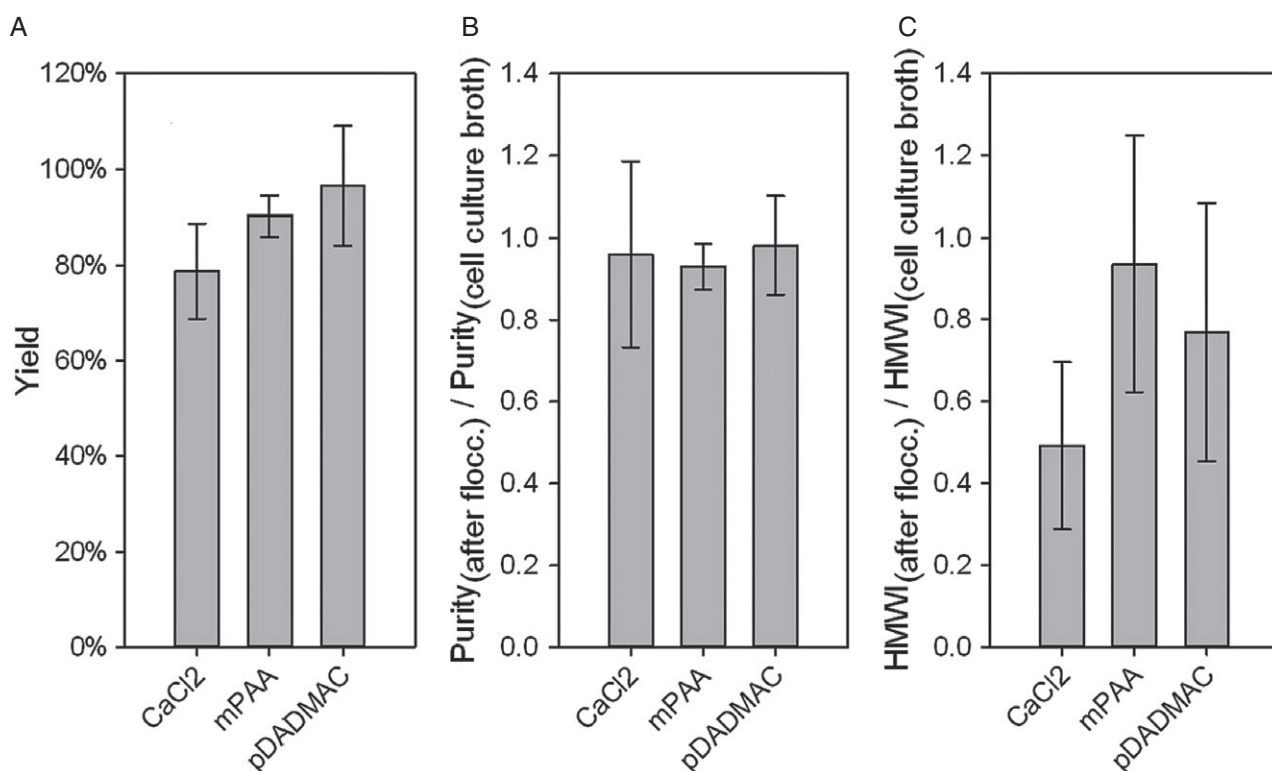


Figure 4. Summary of the selected flocculation conditions (150 mmol L⁻¹ CaCl₂ with 2 mmol L⁻¹ phosphate; 0.05% mPAA with 100 mmol L⁻¹ phosphate and 0.0375% pDADMAC) for further filter screenings in terms of yield (A), purity (B) and HMWl (C). The standard deviations shown represent the data from five 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 m²), 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 ± 1.8 m². Half of the experiments showed significantly worse filtrate quality after depth filtration, with an average value of 3.34 m². In contrast, the other half of the experiments showed good performance, with an average value of 0.16 m². 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

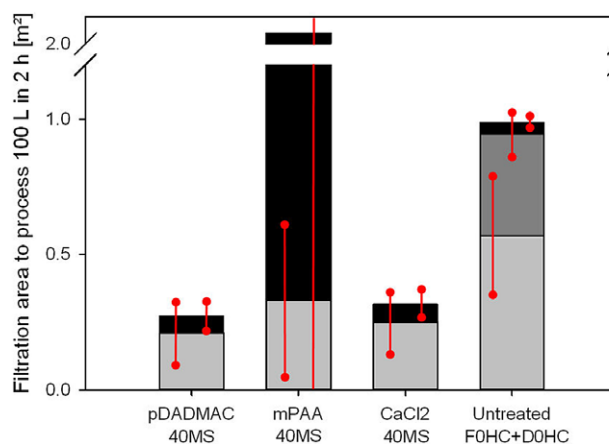


Figure 5. Required filter area (in m²) for the filtration of 100 L of harvest in 2 hours. The grey bars indicate the filter area required for depth filtration. The F0HC + D0HC filter train represents a benchmark filter train, comprising two depth filtrations in series. The black bars show the necessary 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 CaCl₂, 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 CaCl₂. CaCl₂ 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

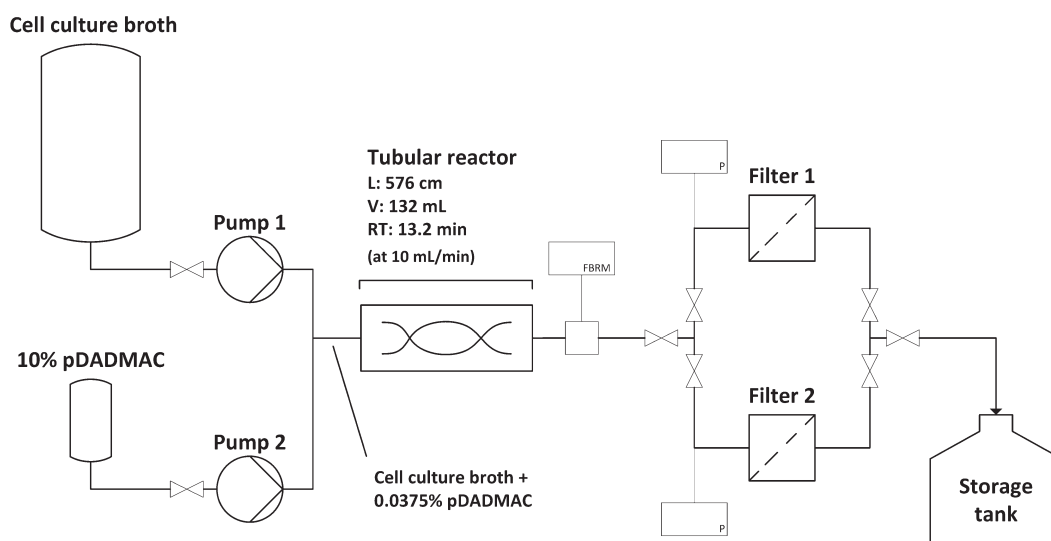


Figure 6. Flow scheme of the continuous flocculation set-up. Harvested cell culture broth and flocculant are combined in a flow rate-dependent manner to a final concentration of 0.0375% pDADMAC. Residence time in the tubular reactor is 13.2 min at a flow rate of 10 mL min^{-1} . Mixing is achieved by 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. Gravimetric 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

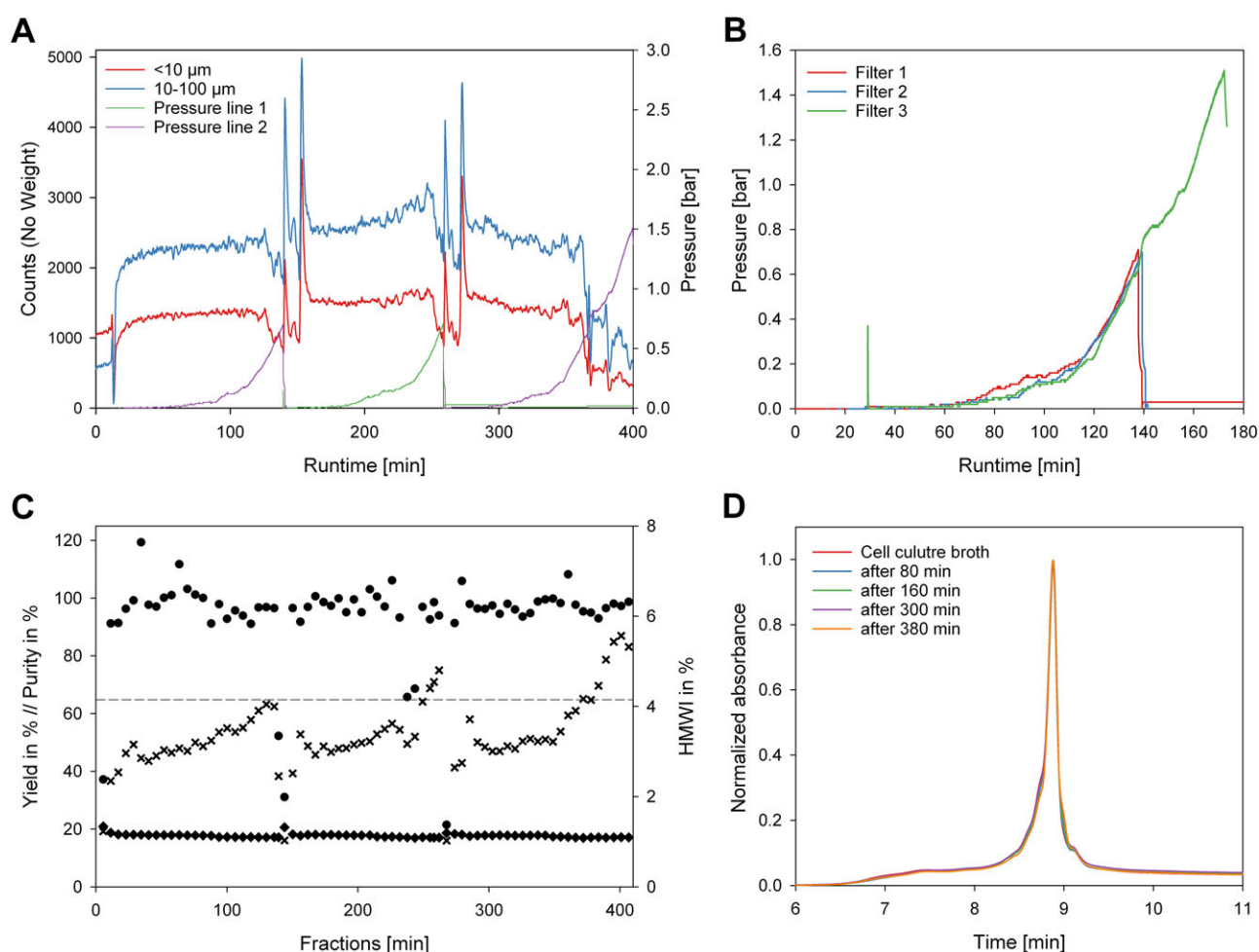


Figure 7. (A) Focused beam reflectance measurement (FBRM) signal and pressure trends over time. (B) Overlay of pressure curves of three filter switch cycles. (C) Yield (dots), purity (diamonds), and high molecular weight impurities (HMWI) (crosses) over time (50 mL fractions). Dashed line indicates the 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 pDADMAC¹¹ and 0.01 mg mL⁻¹ pDADMAC.³³ McEnerney *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

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.

REFERENCES

- Gregory J and Barany S, Adsorption and flocculation by polymers and polymer mixtures. *Adv Colloid Interface Sci* **169**:1–12 (2011).
- Salt DE, Hay S, Thomas ORT, Hoare M and Dunnill P, Selective flocculation of cellular contaminants from soluble-proteins using polyethyleneimine: a study of several organisms and polymer molecular-weights. *Enzyme Microbial Technol* **17**:107–113 (1995).
- McNerney T, Thomas A, Senczuk A, Petty K, Zhao X, Piper R et al., PDADMAC flocculation of Chinese hamster ovary cells: enabling a centrifuge-less harvest process for monoclonal antibodies. *mAbs* **7**: 413–428 (2015).
- Tomic S, Besnard L, Furst B, Reithmeier R, Wichmann R, Schelling P and Hakemeyer C, Complete clarification solution for processing high density cell culture harvests. *Sep Purif Technol* **141**:269–275 (2015).
- Riske F, Schroeder J, Belliveau J, Kang X, Kutzko J and Menon MK, The use of chitosan as a flocculant in mammalian cell culture dramatically improves clarification throughput without adversely impacting monoclonal antibody recovery. *J Biotechnol* **128**:813–823 (2007).
- Kang YK, Hamzik J, Felo M, Qi B, Lee J, Ng S et al., Development of a novel and efficient cell culture flocculation process using a stimulus responsive polymer to streamline antibody purification processes. *Biotechnol Bioeng* **110**:2928–2937 (2013).
- Westoby M, Chrostowski J, de Villemorin P, Smelko JP and Romero JK, Effects of solution environment on mammalian cell fermentation broth properties: enhanced impurity removal and clarification performance. *Biotechnol Bioeng* **108**:50–58 (2011).
- Lydersen BK, Brehm-Gibson T and Murel A, Acid precipitation of mammalian cell fermentation broth. *Ann NY Acad Sci* **745**:222–231 (1994).
- Pham L, Ye H, Cosset FL, Russell SJ and Peng KW, Concentration of viral vectors by co-precipitation with calcium phosphate. *J Gene Med* **3**:188–194 (2001).
- Singh N, Pizzelli K, Romero JK, Chrostowski J, Evangelist G, Hamzik J et al., Clarification of recombinant proteins from high cell density mammalian cell culture systems using new improved depth filters. *Biotechnol Bioeng* **110**:1964–1972 (2013).
- Merck Millipore, Application Note (AN33330000): pDADMAC Flocculant Reagent for Use with Clarisolve® Depth Filters Merck Millipore (2015). [Online]. Available: www.merckmillipore.com/INTERSHOP/web/WFS/Merck-AT-Site/de_DE/-/EUR/ShowDocument-Pronet?id=201306.13788
- Huang YM, Hu W, Rustandi E, Chang K, Yusuf-Makagiansar H and Ryll T, Maximizing productivity of CHO cell-based fed-batch culture using chemically defined media conditions and typical manufacturing equipment. *Biotechnol Prog* **26**:1400–1410 (2010).
- Clincke MF, Molleryd C, Samani PK, Lindsog E, Faldt E, Walsh K and Chotteau V, Very high density of Chinese hamster ovary cells in perfusion by alternating tangential flow or tangential flow filtration in WAVE Bioreactor-part II: applications for antibody production and cryopreservation. *Biotechnol Prog* **29**:768–777 (2013).
- Iammarino M, Nti-Gyabaah J, Chandler M, Roush D and Göklen K, Impact of cell density and viability on primary clarification of mammalian cell broth. *BioProcess Int* **5**:38–50 (2007).
- Felo M, Kang YK, Hamzik J, Balderes P and Ludwig DL, Industrial application of impurity flocculation to streamline antibody purification processes. *Pharmaceut Bioprocess* **3**:115–125 (2015).
- Jungbauer A, Continuous downstream processing of biopharmaceuticals. *Trends Biotechnol* **31**:479–492 (2013).
- Zydney AL, Continuous downstream processing for high value biological products: a review. *Biotechnol Bioeng* **113**:465–475 (2016).
- Langer ES and Rader RA, Single-use technologies in biopharmaceutical manufacturing: a 10-year review of trends and the future. *Eng Life Sci* **14**:238–243 (2014).
- Brod H, Vester A and Kauling J, Möglichkeiten und Grenzen von Disposable-Technologien in biopharmazeutischen Verfahren. *Chemie Ingenieur Technik* **84**:633–645 (2012).
- Brower M, Hou Y and Pollard D, Monoclonal antibody continuous processing enabled by single use, in *Continuous Processing in Pharmaceutical Manufacturing*. Wiley-VCH Verlag GmbH & Co. KGaA, 255–296 (2014).
- Klutcz S, Magnus J, Lobedann M, Schwan P, Maier B, Niklas J et al., Developing the biofacility of the future based on continuous processing and single-use technology. *J Biotechnol* **213**:120–130 (2015).
- Ruthven DM, The residence time distribution for ideal laminar flow in helical tube. *Chem Eng Sci* **26**:1113–1121 (1971).
- Saxena AK and Nigam KDP, Coiled configuration for flow inversion and its effect on residence time distribution. *AIChE J* **30**:363–368 (1984).
- Vashisth S and Nigam KDP, Liquid-phase residence time distribution for two-phase flow in coiled flow inverter. *Ind Eng Chem Res* **47**:3630–3638 (2008).
- Kateja N, Agarwal H, Saraswat A, Bhat M and Rathore AS, Continuous precipitation of process related impurities from clarified cell culture supernatant using a novel coiled flow inversion reactor (CFIR). *Biotechnol J* **11**:1320–1331 (2016).
- Klutcz S, Kurt SK, Lobedann M and Kockmann N, Narrow residence time distribution in tubular reactor concept for Reynolds number range of 10–100. *Chem Eng Res Des* **95**:22–33 (2015).
- Hammerschmidt N, Hobiger S and Jungbauer A, Continuous polyethylene glycol precipitation of recombinant antibodies: sequential precipitation and resolubilization. *Process Biochem* **51**:325–332 (2016).
- Hammerschmidt N, Hintersteiner B, Lingg N and Jungbauer A, Continuous precipitation of IgG from CHO cell culture supernatant in a tubular reactor. *Biotechnol J* **10**:1196–1205 (2015).
- Merck Millipore, Application Note (AN1512EN00): Filter Sizing Methods. Merck Millipore (2000). [Online]. Available: <https://www.scribd.com/document/352405072/Filter-Sizing-Methods-Application-Note>
- Lingg N, Tan E, Hintersteiner B, Bardor M and Jungbauer A, Highly linear pH gradients for analyzing monoclonal antibody charge heterogeneity in the alkaline range. *J Chromatogr A* **1319**:65–71 (2013).
- Fang JZ and Lee DJ, Micromixing efficiency in static mixer. *Chem Eng Sci* **56**:3797–3802 (2001).
- Hessel V, Lowe H and Schonfeld F, Micromixers - a review on passive and active mixing principles. *Chem Eng Sci* **60**:2479–2501 (2005).
- Fischer D, Li Y, Ahlemeyer B, Kriegelstein J and Kissel T, In vitro cytotoxicity testing of polycations: influence of polymer structure on cell viability and hemolysis. *Biomaterials* **24**:1121–1131 (2003).