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An automated, low volume, and high-throughput analytical platform for aggregate quantitation from cell culture media

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ABSTRACT

High throughput screening methods have driven a paradigm shift in biopharmaceutical development by reducing the costs of good manufactured (COGM) and accelerate the launch to market of novel drug products. Scale-down cell culture systems such as shaken 24- and 96-deep-well plates (DWPs) are used for initial screening of hundreds of recombinant mammalian clonal cell lines to quickly and efficiently select the best producing strains expressing product quality attributes that fit to industry platform. A common modification monitored from early-stage product development is protein aggregation due to its impact on safety and efficacy. This study aims to integrate high-throughput analysis of aggregation-prone therapeutic proteins with 96-deep well plate screening to rank clones based on the aggregation levels of the expressed proteins. Here we present an automated, small-scale analytical platform workflow combining the purification and subsequent aggregation analysis of protein biopharmaceuticals expressed in 96-DWP cell cultures. Product purification was achieved by small-scale solid-phase extraction using dual flow chromatography (DFC) automated on a robotic liquid handler for the parallel processing of up to 96 samples at a time. At-line coupling of size-exclusion chromatography (SEC) using a 2.1 mm ID column enabled the detection of aggregates with sub-2 µg sensitivity and a 3.5 min run time. The entire workflow was designed as an application to aggregation-prone mAbs and "mAb-like" next generation biopharmaceuticals, such as bispecific antibodies (BsAbs). Application of the high-throughput analytical workflow to a shake plate overgrow (SPOG) screen, enabled the screening of 384 different clonal cell lines in 32 h, requiring $< 2\ \mu g$ of protein per sample. Aggregation levels expressed by the clones varied between 9 and 76%. This high-throughput analytical workflow allowed for the early elimination of clonal cell lines with high aggregation, demonstrating the advantage of integrating analytical testing for critical quality attributes (CQAs) earlier in product development to drive better decision making.

1. Introduction

Monoclonal antibodies (mAbs) continue to dominate the biopharmaceutical market with more than 50 products currently approved in the EU and USA and revenues projected to reach 300 billion dollars by 2025 [1,2]. Further advancements in protein engineering have led to the development of next generation mAbs such as antibody-drug conjugates (ADC), multi-specific antibodies (msAbs), and antibody fragments including antigen-binding fragments (Fab) and single-chain variable fragments (scFvs) [3]. These novel therapeutics have expanded the horizon for the treatment of cancer, autoimmune and infectious diseases such as Ebola, HIV and COVID-19 [4–6]. Despite their success, the introduction of next generation mAbs poses further industrial challenges mostly due to the increased engineering complexity and the lack of platform technologies [7–9]. In this context, bispecific antibodies (BsAbs), engineered to display two different antigen-binding sites, have proven auspicious for the treatment of cancer, autoimmune diseases and several other conditions due to their multi-target mechanism of action [10]. This class of therapeutics encompasses several different species and a variety of formats

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whose characteristics along with their therapeutic and engineering principles have been extensively reviewed in the literature [2,4,6,11,12]. Currently available on the market include the anti-neoplastic agent Blincyto® (bispecific T-cell engager (BiTE) blinatumomab, Amgen/Micromet) and Hemlibra® (full-length IgG-like BsAb emicizumab-kxwh, Chugai/Genentech, a subsidiary of Roche) used against cancer and haemophilia A, respectively. Despite holding therapeutic promise, the challenges associated with BsAbs developability have hindered their market thriving [5,12,13]. The increased molecular complexity of these products often leads to low expression titres and poor stability resulting in high levels of high-molecular-weight (HMW) impurities [14–21]. These impurities include covalently or non-covalently bound aggregate species such as dimers, trimers, tetramers, etc., often forming because of misfolding events [22]. Aggregation is a highly immunogenic protein modification classified as a critical quality attribute (CQA) of therapeutic proteins [23–26]. Aggregation poses developability issues concerning not only the safety and efficacy of the final product but also the increased time and COGM. Indeed, aggregates are removed during downstream processing, thus a high percentage of aggregation may result in considerable product waste [27]. To overcome these challenges, bioprocess engineering strategies have been focusing on increasing the expression titres and purification yield as well as minimizing protein aggregation to reduce the costs of goods [28–31]. Considerable emphasis is given to analytical testing to identify and quantify aggregation for the selection of the top clones during early-product development.

In this context, next generation manufacturing (NGM) technology focuses on delivering new processing methodologies to enable a paradigm shift in the cost and speed of biopharmaceutical development, with particular attention to important product quality attributes such as aggregation. Under this umbrella, high-throughput process development (HTPD) tools have proven successful to accelerate biopharmaceutical development and process optimization [32,33]. Scale-down cell culture systems, such as shaken 24- and 96-deep well plates (DWPs) have successfully been employed in HTPD activities such as clone selection and optimization of the media and supplements composition during cell line development (CLD) [34,58]. High-throughput analysis is fundamental to efficiently identifying aggregation-prone candidates during both discovery and development [25,35–37]. However, the implementation of analytical testing in such low-volume cell cultures is often hampered by the limited-expression titres and sample volume available for analysis. Moreover, the analysis of aggregates represents analytical challenges due to their large size and inherent structural complexity [27,38]. Thus, the selection of both clones and process conditions during the very early stages of CLD is mostly driven by expression titre, whilst aggregation analysis is introduced at a later stage. Both the increased complexity of next generation therapeutic proteins and the need for COGM reduction and process development acceleration push the research of more efficient analytical tools to drive decision making during biopharmaceutical development.

Over the last decade, a variety of research has demonstrated the suitability of multi-dimensional liquid chromatography (mD-LC) methodologies as high throughput solutions to leverage lead candidate and clone selection, process optimization, and extended characterization [39–46]. 2D IEX-SEC and SEC-IEX methods were developed to bridge size and charge variants analysis to support mAb development studies [44–46] and 2D-LC Protein A-SEC for measuring titre and aggregation of a target mAb from HCCF [25,43]. Although combining purification and product quality analysis in a fully automated fashion, mD-LC methods lack parallelization capability because of the sequential nature of LC system injections. Processing hundreds of samples at a time, which is required for testing 96-DWPs, demands shorter analysis time than 2D-LC methods can often provide, therefore such application requires greater throughput capability.

Microfluidics-based platforms such as LabChip GXII (PerkinElmer) have been applied to the high-throughput analysis of protein size and purity [29,30,47]. However, chip-based techniques use denaturing conditions and lack sufficient resolution for the separation of aggregates which constitutes a bottleneck [30]. Size exclusion chromatography is the industry gold-standard methodology for the analysis of soluble protein aggregates [49]. The resolution achieved with SEC is superior in comparison to electrophoretic and light-scattering-based methods, Taylor dispersion analysis (TDA), analytical ultracentrifugation (AUC) and field-flow fractionation (FFF) [20,49]. Modern ultra-high performance liquid chromatography (UHPLC) methods using 4.6 mm ID x 150 mm SEC columns packed with sub-2 µm particles, provide enhanced analysis throughput in comparison to high-performance liquid chromatography (HPLC) [48,50,51]. Rea et al. developed a capillary SEC method coupled to fluoresce detection with picogram sensitivity [52,53]. However, small-scale SEC methods have not been widely adopted in the biopharma community due to the limited column availability and the need for dedicated low-flow instrumentation with specialised setups to minimize the extra-column dead volume [52].

Another challenge of developing analytical methods to support biopharmaceutical development is assuring their simple operation and flexibility of application to a variety of molecular formats. Because high-throughput tools are often expected to be used for routine testing, they must be simple, robust and easy to perform by non-expert scientists. They should also provide a platform approach for a defined class of molecules, thus reducing the need for additional method development.

This study presents the development of a high-throughput analytical platform integrating the purification and aggregation analysis of mAbs and BsAbs expressed in 96-DWP cell cultures. The aim was to design an analytical workflow suitable to process 300 µL cell cultures to implement as a high-throughput tool for routine testing providing data with minimal sample requirements. The choice of the technology used for both purification and aggregation analysis was driven by ease of operations and the potential for automation. Protein-A resin-filled pipette tips (PhyTip columns from Biotage) were adopted due to their feasibility in processing small volumes and providing purified material for analytical testing at high concentrations [55]. The use of a Tecan EVO200 robotic liquid handler enabled the parallel purification of up to 96 samples at a time and ensured consistent performance. The development of a rapid small-scale high-throughput SEC method (HTSEC) on a narrow bore 2.1 mm ID column allowed for aggregation analysis with sub-2 μg sensitivity and unsupervised processing of a 96-DWP on a UHPLC system. The PhyTip purification/HTSEC workflow was applied to a large shake plate overgrow (SPOG) screen testing 384 different clonal cell lines cultured in both fed-batch and semi-continuous perfusion conditions in 96-DWPs. This demonstrated the ability of the high-throughput analytical workflow to link product quality (PQ) analysis to cell engineering strategies and efficiently drive the CLD process.

2. Experimental section

2.1. Chemicals and materials

Sodium phosphate dibasic, sodium phosphate buffer monobasic, sodium chloride, sodium hydroxide, hydrochloric acid, ammonium acetate, acetic acid, glycine hydrochloride, and L-methionine sulfoximine were purchased from Sigma-Aldrich (Gillingham, UK). CD-CHO medium and Gibco™ 1x DPBS were purchased from

Thermo Fisher Scientific (Gloucester, UK). Production media and feed were proprietary to AstraZeneca.

2.2. Samples

Two IgG1, kappa mAbs (mAb-1 and mAb-2), and two BsAbs (BsAb-1 and BsAb-2) were kindly provided by AstraZeneca (Cambridge, UK) and used for method development. To generate a high% aggregate sample for method development, the mAb-1 and mAb-2 samples were stressed at 70 °C and 300 rpm shaking for 60 min. CLD screen samples were provided from culturing in 96-DWP for 10 days, shaking at 350 rpm and adding a feed solution at regular intervals RED. The semi-continuous perfusion samples were generated using replicate plates which were then centrifuged daily from day 4 of culture, removing 75% of the media volume and replacing it with a media solution containing feed and glucose.

2.3. Equipment

Purification was performed by using PhyTip 200 µL volume columns, containing 20 µL of ProPlus (MabSelect SuRe™) affinity resin (Biotage GB Limited, Hengoed, United Kingdom.) operated on a Tecan Freedom EVO® 200 robotic liquid handling platform (TECAN Group Ltd.) operated on Freedom EVOware®, Version 2.7 (TECAN Group Ltd.). Consumables included 25, 100, and 300 mL troughs (TECAN Group Ltd.), 96-well DWP, 2.2 mL, deep-well plates (Thermo Fisher Scientific), Greiner 96-well plates with u-bottom (Greiner Bio-One Ltd.), and V-squared bottom 96-well plates (Agilent Technologies Inc.). Gibco™ 1x DPBS (Thermo Fisher Scientific) was used as column equilibration and first wash step (wash 1) buffer whilst the second wash step (wash 2) buffer was composed of 25 mM sodium acetate, 120 mM sodium chloride, pH 5.5. Elution was carried out by using 100 mM glycine buffer pH 2.6 or 25 mM sodium acetate buffer, pH 3.6. Equipment and software packages for UHPLC analysis were all purchased from Agilent Technologies Inc. and involved Agilent 1260 Infinity II UHPLC system including a degasser, quaternary pump, thermostatted multi-sampler, and diode array detector (DAD) in conjunction with a multi-column compartment with a column selection valve. All UHPLC parts were joined by 1.6 mm OD, 0.12 µm ID stainless steel capillary tubing with stainless steel fittings. System control and data analysis were accomplished with Agilent OpenLAB CDS ChemStation Edition, version C.01.07. The Acquity UPLC Protein BEH SEC 200 Å, 1.7 µm (Waters) and Unix™-C SEC-300 300 Å, 1.9 µm (Sepax Technologies, Inc.) columns, both with 2.1 × 150 mm (ID x length) dimensions were tested. Mobile phase recipes and separation gradients for method development were created using the Agilent Buffer Advisor Software with ZedGraph Library, version 5.0.8.20000, and DotNetZip Library, version 1.9.1.5. All the buffers were prepared by using deionized water dispensed by Barnstead Nanopure Diamond Lab Water System and filtered before use with Nalgene™ Rapid-Flow™ Sterile Disposable Filter Units with a 0.2 µm pore size PES Membrane (Thermo Scientific). The pH of the buffers was confirmed by Benchtop pH meter (Mettler Toledo, Greifensee, Switzerland). The JMP® Pro 15.0 statistical software (SAS Institute Inc.) was used for assisting method development by design of experiment (DoE) models and data analysis together with Python 3.7 scripting language (Python Software Foundation).

2.4. Cell culture

96-DWPs (Greiner #780,271 with Duetz #SMCR1296a lids) screen was carried out in a Khuner shaking incubator at 350 rpm, 5% CO2. Liquid handling was carried out using a Hamilton Star Plus. Titre was performed on an Octet with Protein A biosensors.

3. Results and discussion

3.1. Development of an automated microscale purification method for 96-DWP cell cultures

A Protein-A affinity chromatography method using PhyTip technology was designed to be suitable for the purification of mAbs and BsAbs expressed in 350 μL cell cultures volume 96-DWPs. Two model molecules, mAb-1 and BsAb-1 were selected as representative of two classes of biopharmaceuticals and used throughout method development. During the early stages of the CLD process, expression titres are often low, and this is particularly accentuated when both transient expression and microscale bioreactors systems such as shaken 24- and 96-DWPs are used. Maximizing the sample recovery during purification whilst maintaining process throughput is key for enabling successive analytical testing. A design-of-experiment (DoE) strategy was adopted to identify and subsequently optimize the purification parameters that enhanced protein recovery. Protein purification by PhyTips is governed by the fundamental principles of Dual Flow Chromatography (DFC) characterized by a controlled bidirectional flow of sample and mobile phase travelling in and out/back and forth the resin-filled pipette tip column in all the steps of the process including sample loading (capture), washing, and elution [56]. This is fundamentally different from traditional flow-through chromatography where samples and mobile phases are pumped through the column unidirectionally. In DFC, the time of protein-ligand interaction can be controlled by adjusting the volume of the fluids processed during each aspirate/dispense cycle (processing volume), the number of cycles, and the flow rate in each purification step. In this context, the use of a programmable robotic system was key for controlling the flow rate of liquid aspiration and dispensing to modulate the time of interaction of the target protein with the resin bed to maximize sample recovery as well as enable high-throughput applications [55].

Based on the fundamental understanding of the DFC principles, a Main-Effects Screening Design (MESD) was used to identify the process parameters that most importantly affect sample recovery. The process parameters studied for each purification step were the number of aspirating/dispensing cycles and the flow rate [56]. Additionally, the volume of elution buffer was included as a factor in the MESD to balance the efficiency of the disassociation with an appropriate final concentration of eluted product to perform further analytical testing. To generate a consistent starting material for method development, representative of the host cell culture fluid (HCCF), the mAb-1 and BsAb-1 samples were diluted in cell culture media in bulk at a concentration of 2 mg/mL. This allowed for a column loading equal to ~75% of the resin capacity for a total sample volume of 300 µL. Post purification sample concentration was measured by UV absorbence at 280 nm and used for calculating the per cent protein recovery (%R). Linear regression analysis was used to build an empirical model to identify the factors that most significantly improved%R (Fig. S1). Increasing the number of capture cycles and the final elution volume as well as decreasing flow rate, significantly improved the%R of both molecules (Fig. 1A). Subsequently, a Box-Wilson Central Composite Design (CCD) was adopted to optimize these three process parameters. Linear regression analysis was used to generate an empirical model for identifying optimal process conditions necessary to enhance%R without compromising the method throughput (Fig. 1B). Desirability profiling [57] was carried out to identify optimal process conditions. Although increasing the number of capture cycles improved%R, the method throughput was considerably reduced due to the impact on processing time. An acceptable compromise between%R and purification time was found with 20 capture cycles at 3 µL/s and a final elution volume of 200 µL (Fig. S2). The maximum%R

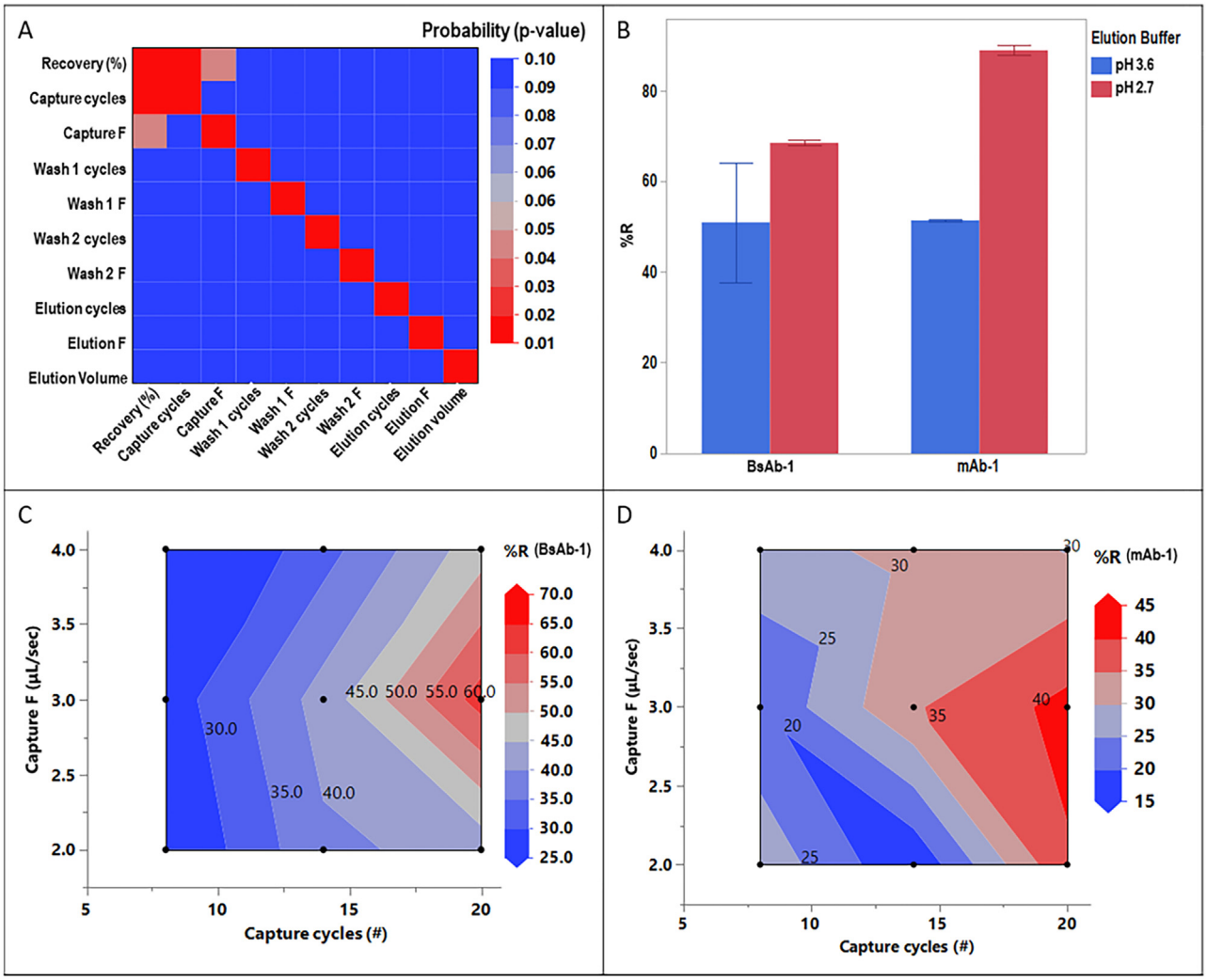

Fig. 1. DoE-assisted PhyTip purification method development. Color map displaying the correlation probability between the purification process parameters studied in the main effect screening design (MESD) and the final protein per cent recovery. The probability is reported in terms of *p*-value indicating how significant each factor affects the protein recovery. (A) Comparison of BsAb-1 and mAb-1 percent recovery obtained when using elution buffer with pH 3.6 (blue) and pH 2.7 (red). The bar chart reports the mean percent recovery and standard deviation (error bar) of the BsAb-1 and mAb-1 samples purified in triplicates. (B) and (C) Contour plot showing the variation of the final protein recovery in relation to changes in capture flow rate (D) and capture cycles optimized during the central composite design (CCD) for both BsAb-1 and mAb-1, respectively. The CCD data points, and the mean replicate values of the protein percent recovery estimated by the model were represented together with the contour areas colored with blue and red shades based on low and high percent recovery, respectively.

estimated by the CCD model was 64 and 47% for BsAb-1 and mAb-1, respectively, demonstrating some improvement from 60% and 36% achieved in the initial MESD. Sample recovery was further improved by using glycine hydrochloride, pH 2.7 as elution buffer, achieving consistent 70 and 90% for BsAb-1 and mAb-1 within 2 h without compromising PQ (Figs. 1C and S3). When compared to previously published work, this microscale PhyTip purification method exhibited a sufficient sample recovery for its final application, desirable throughput and enhanced capability for purifying proteins expressed in low-volume cell cultures [54,57].

One of the key aspects of platform methods is their fitness for routine use therefore, thorough method validation was carried out. A repeatability study including 6 replicates for both BsAb-1 and mAb-1 samples demonstrated a%R relative standard deviation (RSD) of 2.1% and 0.5%, respectively, indicating consistent recovery. A 5-levels linearity study was performed over a column loading range of 70–900 µg, corresponding to ~10–110% of the resin capacity. Linear regression analysis of the column loading (μg) *vs.* the protein recovery (μg) demonstrated a coefficient of variation $R^2 \geq 0.99$ for both molecules analyzed in duplicates (Fig. S4). The%R obtained across the column loading range investigated from the minimum loading point up to resin capacity was 80% and 85% for BsAb-1 and mAb-1, respectively with RSD < 20%. This confirmed consistent recovery over a wide sample concentration range. The data demonstrated that the microscale purification method was fit for purpose, in processing as little as 300 µL of cell culture supernatants in 96-DWP achieving 80 ± 5%R within 70–900 µg column loading range without compromising the aggregates profile and providing 200 µL of purified product for subsequent analytical testing (Fig. S3).

3.2. Development of a sensitive high-throughput method for aggregate analysis by narrow-bore sec

The development of a platform HTSEC method for monitoring aggregation in mAbs and BsAbs was achieved by DoE. The molecules chosen for method development as representative models of each biopharmaceutical class were two mAbs (mAb-1 and mAb-2) and one bispecific antibody (BsAb-1). The DoE served to

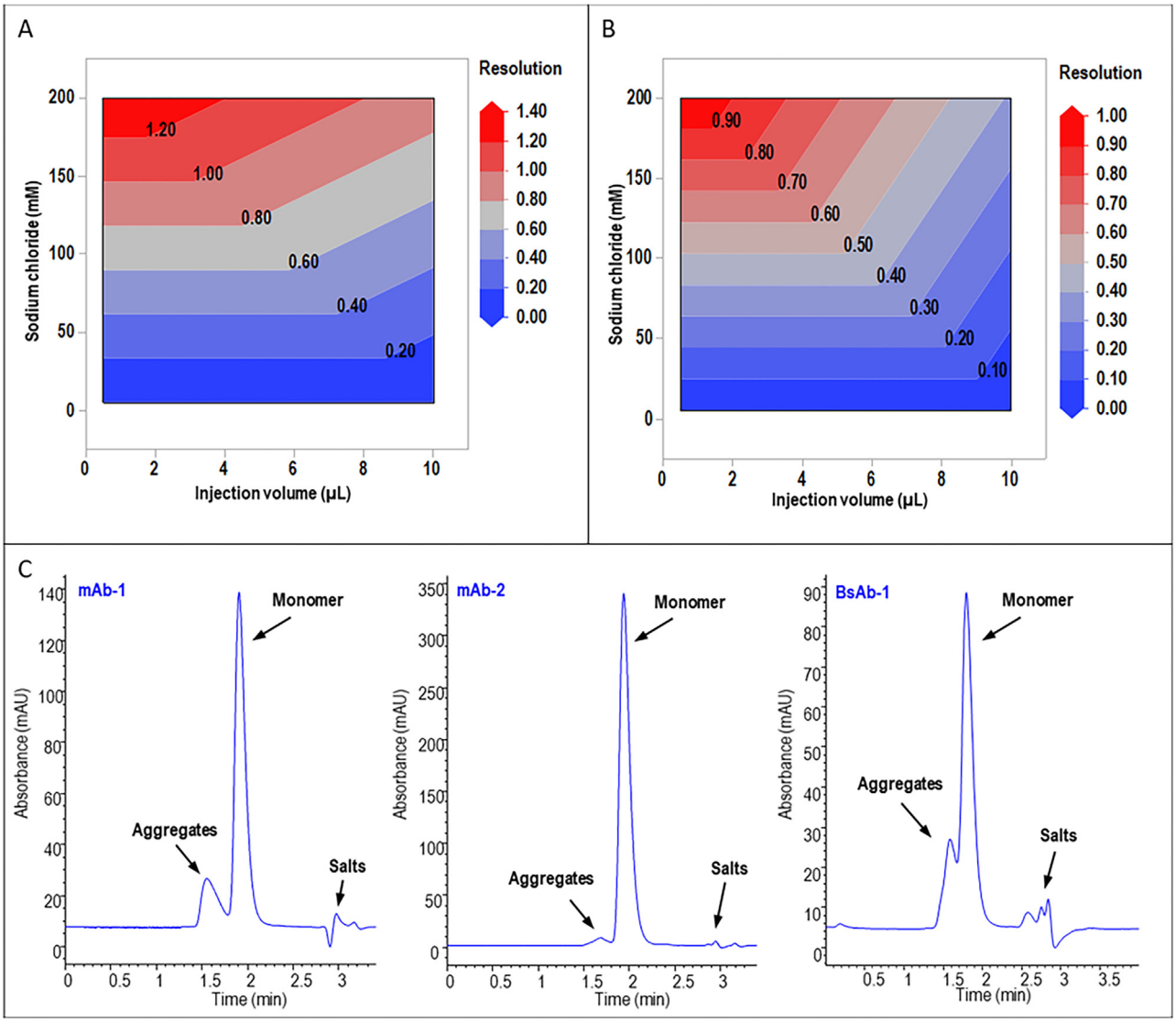

Fig. 2. DoE-assisted HTSEC method development. At the top, a contour plot displaying the variation of the resolution between the monomer and aggregates peaks in relation to changes in injection volume and sodium chloride concentration obtained with the Waters (A) and Sepax (B) columns. The contour plot areas representing low and high-resolution values were presented in blue and red shades, respectively. C) Chromatograms of mAb-1, mAb-2 and BsAb-1 samples were obtained with the optimized HTSEC method conditions.

efficiently identify the optimal column chemistry, flow rate, injection volume and mobile phase composition. Column temperature and mobile phase pH were kept constant at 25 °C and 6.8 respectively to maintain analysis under native conditions. Two types of narrow bore SEC columns were assessed, a Waters (ethylene-bridged hybrid (BEH) with diol-coated spherical particles presenting 1.7 μm diameter and 200 Å pore size) and a Sepax (a lay-down monolayer hydrophilic film bonded to spherical silica particles of 1.8 μm diameter and 300 Å pore size) both of equal dimensions, 2.1 ID x 150 mm length [50]. The quality of the separation was assessed through the calculation of the resolution between the monomer and aggregates peaks. Amongst the factors under investigation, the concentration of sodium chloride, the injection volume and column chemistry had a statistically significant effect on the separation efficiency (p-value ≤ 0.05) (Fig. S5). The main contributor was the content of sodium chloride in the mobile phase. As shown in Fig. 2, the resolution was improved by decreasing the injection volume and increasing the concentration of sodium chloride in the mobile phase. A minimum of 100 mM sodium chloride was needed to resolve the aggregates from the monomer peak with consistent%aggregation obtained using 200 mM sodium chloride (Fig. S6). This trend was consistent across the two columns tested; however, the aggregate and monomer peaks were better resolved with the Waters column which was therefore selected as the optimal stationary phase. Increasing the method flow rate was fundamental for improving the analysis speed and this study showed that higher flow rates did not affect peak resolution. Furthermore, these experiments demonstrated that higher flow rates improved peak shape by reducing peak width and distortion. The conditions estimated by the DoE model were sufficient to obtain a desirable separation between aggregates and monomer peaks in both model mAbs, therefore, no further optimization was undertaken. The chromatographic profiles of mAb-1, mAb-2 and BsAb-1 samples obtained with the optimized HTSEC method are shown in Fig. 2C.

To ensure that the HTSEC method was suitable to support sample testing for process development, further optimization was performed. During early-stage CLD, the expression titres are often low,

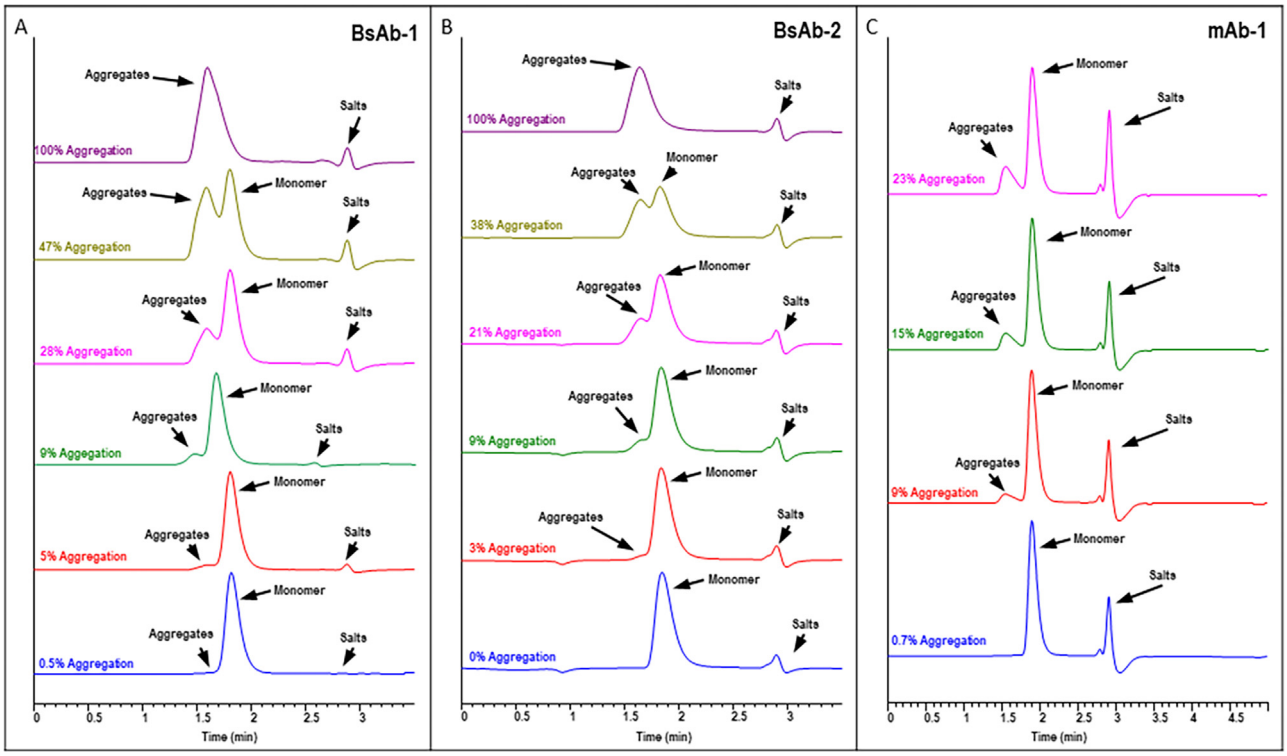

Fig. 3. HTSEC analysis of samples containing various levels of aggregates. HTSEC stacked chromatograms of (A) BsAb-1, (B) BsAb-2, and (C) mAb-1 samples show the presence of different levels of aggregation. The%aggregation of BsAb-1, BsAb-2, and mAb-1 was analyzed by a benchmark SEC method, reporting 35, 22, and 17% aggregates, respectively. Subsequently, the aggregates and monomer peak fractions were collected and blended at different ratios to generate samples with various levels of aggregates. Namely, the BsAb-1 and BsAb-2 samples, the aggregate fraction (100% aggregates) was spiked into the monomer peak fraction to form samples containing 0% (blue), 10% (red), 20% (green), 40% (pink), 60% (gold), and 100% aggregates. The mAb-1 stressed sample (20% aggregate) was spiked into the reference sample (99% monomer) to create samples with aggregate levels of 1, 7, 13 and 20%. The samples were diluted to 1 mg/mL prior to analysis by HTSEC with an injection volume of 5 µL.

and the samples are at low concentrations even after purification. Large injection volumes contribute to the increase in extra-column dead volume (V_{ec}) which ultimately affects the SEC separation. This phenomenon is more pronounced when small ID columns such as 2.1 mm are adopted. Therefore, subsequent experiments were carried out to identify the injection volume limit and optimal column loading range for this method (Fig. S7). The total run time of the HTSEC method from injection to data acquisition was 3.5 min per sample, enabling complete analysis of 96 samples in 5.6 h. Method assessment was performed to validate the HTSEC method for its intended use. Specificity was assessed by comparing the UV profile of mAb-1 against those obtained by injecting process buffers and no interfering peaks were observed (Fig. S8, A left). Repeatability was evaluated over 6 replicates and reported no more than 0.2% RSD for both monomer retention time and aggregates relative peak area, indicating excellent method precision (Fig. S8, A right). The sample concentrations for BsAb-1 were 0.2, 0.4, 0.8, 1.7, and 2.5 mg/mL with an injection volume of 10 μL, whilst sample concentrations for mAb-1 and mAb-2 were 1, 2, 4, 8, and 10 mg/mL with an injection volume of 0.1 μL. This evaluated the linearity of the method at the extremes of the injection volume range, over a range of concentrations, and different molecules. Linear regression analysis confirmed the linear response of the variation of the aggregates and monomer peak areas against the sample concentration, with $R^2 > 0.99$ (Fig. S8, B). The method limit of detection (LOD) and limit of quantification (LOQ) inferred from the linearity study for the aggregates peak were 0.77 µg and 2.3 µg, respectively (Fig. S8, C). To demonstrate the capability of the method to discriminate amongst samples with different aggregation levels, samples containing various degrees of aggregates were generated with thermal stress. As shown in Fig. 3, the HTSEC was able to discriminate between samples containing different levels of aggregates confirming its suitability for use as a ranking tool in clone selection. The optimized HTSEC method parameters are summarised in Table 1.

Table 1
Optimized HTSEC method.

HTSEC Method	
Column	Waters Acquity UPLC Protein BEH SEC column, 200 Å, 1.7 µm, 2.1 mm x 150 mm
Mobile phase	50 mM sodium phosphate, 200 mM sodium chlorife, pH 6.8
Flow rate	0.15 mL/min
Temperature	25 °C
Injection Volume	0.5–10 µL
Column loading range	2–15 µg
UV	210 nm, 218 nm

It is worth noting that further improvements in the separation of size variants were limited by the low resolving power of the SEC separation which is accentuated by the use of a narrow bore column. Further attempts at increasing peak resolution may be focused on reducing the instrument V_{ec} by optimizing the fluidics path. This would entail reducing the length and diameter of the capillary tubing, optimizing the fittings, and replacing instrument parts to lower the internal dispersion volume. Despite the instrument limitation, the resolution between aggregates and monomer peaks was considered sufficient for the final method application.

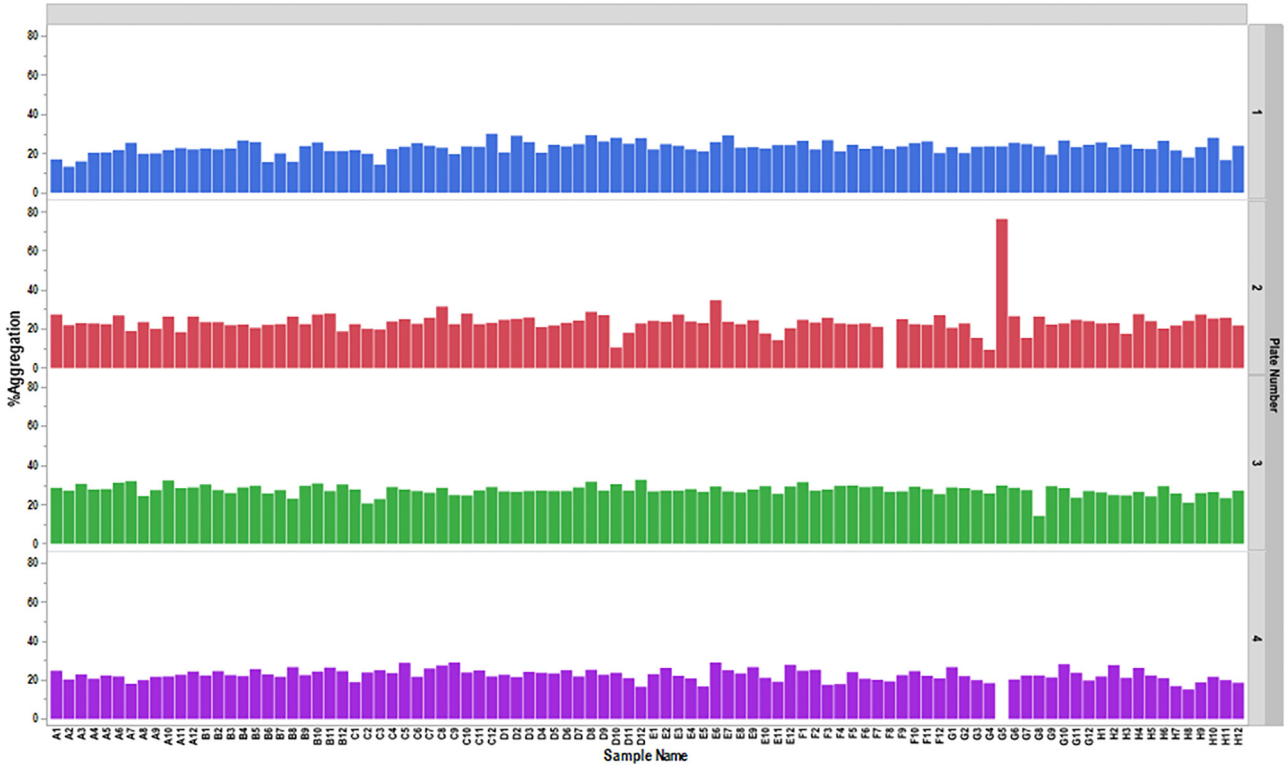

Fig. 4. High throughput PhyTip purification/HTSEC analysis of% aggregation in each 96-DWP analysed during the SPOG screen. The bar chart displays the%aggregation impurities present in 96-DWP analysed in the SPOG screen. The%aggregation range of plate 1 (blue) varied from 13.3 to 30%, with both mean and median values of 23%; The%aggregation range of plate 2 (red) varied from 9.3 to 76%, with both mean and median values of 23.4 and 22.9, respectively. The concentration of sample F8 was below the method LOD, thus no data were acquired. The%aggregation range of plate 3 (green) varied from 14.2 to 32.7%, with both mean and median values of 27.4 and 27.3, respectively. The%aggregation range of plate 4 (purple) varied from 15.2% to 29.1%, with both mean and median values of 22.6 and 22.4, respectively. The concentration of sample G5 was below the method LOD, thus no data were acquired.

3.3. PhyTip purification/HTSEC application to high-throughput shake-plate overgrow (SPOG) screen

A research study comparing conventional fed-batch CLD screens to semi-continuous perfusion and adaptive feeding to mimic the NGM platform was used as a test model for the aggregation screen. A CHO host cell line and a hydrogen peroxide adapted CHO-K1 derived cell line (H_2O_2 host) (Mistry et al. 2020) were transfected with a vector to produce BsAb-1, a difficult-to-express (DTE) bispecific antibody. A SPOG screen was set up to compare the performance of the CHO CAT-S and H_2O_2 host clones when cultured in both standard fed-batch and semi-continuous perfusion systems. In this study, 192 clones were cultured in duplicate within shaken 96-DWPs with 350 μL total cell culture volume per well. Cell culture operations for inoculum in 96-DWPs, feed supplementation, media exchange, and sampling were automated on a robotic liquid handler. On day 11, the expression titre range of the cell cultures was 7.64–1372.3 mg/L with a mean titre value of 390.46 mg/L, corresponding to ~78 μg average of product in a volume of 200 μL HCCF available for analysis. Relevant clones were selected for aggregation analysis. After harvesting, the clarified supernatants were analysed by PhyTip-purification/HTSEC in series. Firstly, PhyTip purification was performed to purify the BsAb-1 from the HCCF and at a sufficient concentration for aggregation analysis. The PhyTip purification method was integrated with a Python script (py1) that served to monitor the purification performance throughout the process by calculating each sample%R, adjusting the required injection volume to obtain a consistent column loading of 5 μg, and flagging the samples with concentration below the method LOD and LOQ. The py1 script introduced a process control element for monitoring and feeding back PQ data analysis in an automatic fashion. Following PhyTip purification, the 96-DWP was loaded into the UHPLC system autosampler for HTSEC analysis. A blank, system suitability and control sample were added at the start and end of each 96-DWP analysis sequence to monitor the system performance during the analysis. Therefore, each 96-DWP HTSEC run counted a total of 102 injections, requiring < 6 h of analysis time per plate. Processing one 96-DWP by PhyTip purification took ~8 h in total, thus, the 384 samples could be processed within 32 h. The sensitivity of the HTSEC method enabled quantification of aggregates using < 5 μg of product. Product quality information was obtained for samples with expression titres < 200 mg/L and culture volume of ~200 μL. Data analysis was automated by a Python-JMP combined script (py2) for the final data analysis, and the data were finally visualized in a bar chart displaying the%aggregation present in each plate well. Automation of the data analysis workflow allowed the processing of 384 samples of data in a single click. Fig. 4 shows the results of the PhyTip purification/HTSEC workflow applied for the processing of the SPOG screen.

The SPOG screen was used to select the top clonal cell lines by titre to proceed to a more controlled and scaled-up screen in the ambr micro-bioreactors (Sartorius). By including the aggregate analysis for BsAb-1, a number of clones with high aggregate (27–31.4%) were removed and the selection of the top 24 was extended marginally to capture a few additional mid-ranking clones between 60 and 70% of the titre of the top clone (Fig. 5).

This experiment compared replicate clones in two different process conditions, a fed-batch and a semi-continuous perfusion screen. While the titre showed a high correlation between the two

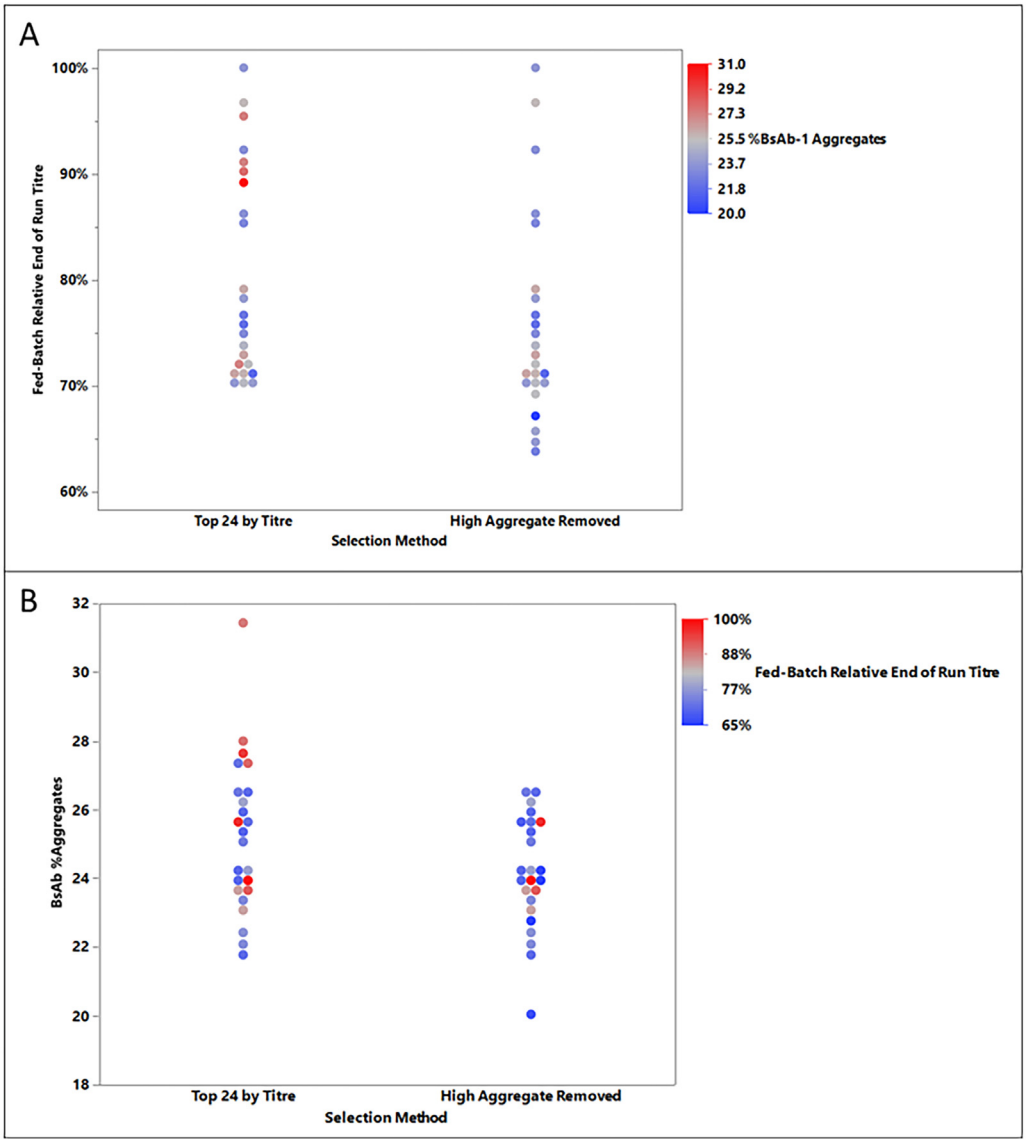

Fig. 5. Effect of removing samples with BsAb aggregate within the fed-batch process greater than 27% on the cell lines selected for micro bioreactor evaluation. By including the aggregation data in the selection of cell lines to proceed to the microbioreactors, a small compromise on titre (A) shows a large result in the level of aggregation of the clones (B).

processes, the aggregate data did not (Fig. 6). There were clones which showed high aggregate in both processes but also others which showed a lower aggregate in the semi-continuous perfusion, suggesting a difference in response to process conditions and advocating for a scale-down process which is more closely matched to the proposed manufacturing strategy for that molecule. The cross hairs in Fig. 6B, indicate quadrants where clones were high or low in both processes, extending the low category to 25% and below. In this case, those low in the semi-continuous perfusion and low to moderate aggregation in the fed batch are still of interest. The perfusion process demonstrated a reduction in BsAbs aggregation [59]. Choosing clones which show low aggregation in a semi-continuous scale-down model may have an improved chance to respond to a manufacturing perfusion situation.

4. Conclusion

This study demonstrated the development and application of a PhyTip purification/HTSEC workflow as an automated high throughput ranking tool for clone selection, providing quantitative aggregation analysis of proteins expressed in 96-DWP cell cultures. The purification method employed Protein-A filled PhyTip columns and was successfully automated on a robotic liquid handler for the parallel processing of 96 samples at a time. Method development aimed to maximize the protein%R to provide sufficient material for analytical testing within a suitable time for high throughput operations. DoE-assisted method optimization helped the identification of both optimal purification and aggregation analysis conditions. The PhyTip purification enabled the processing of as little as 300 µL of HCCF providing high protein recovery (> 80%) despite low titre levels (< 200 mg/L). Fast and sensitive aggregation analysis was achieved with a 2.1 mm ID SEC column in only 3.5 min with a sub-2 µg sample requirement. Although a decrease in resolution was observed in comparison to conventional SEC separations run on larger column dimensions, the HTSEC method could discriminate between samples containing various levels of aggregation, providing a valuable high-throughput tool for supporting clone selection during large screening experiments. Automation of the experimental procedure for both PhyTip purifications and HTSEC enabled the processing of 384 cultures within 32 h with

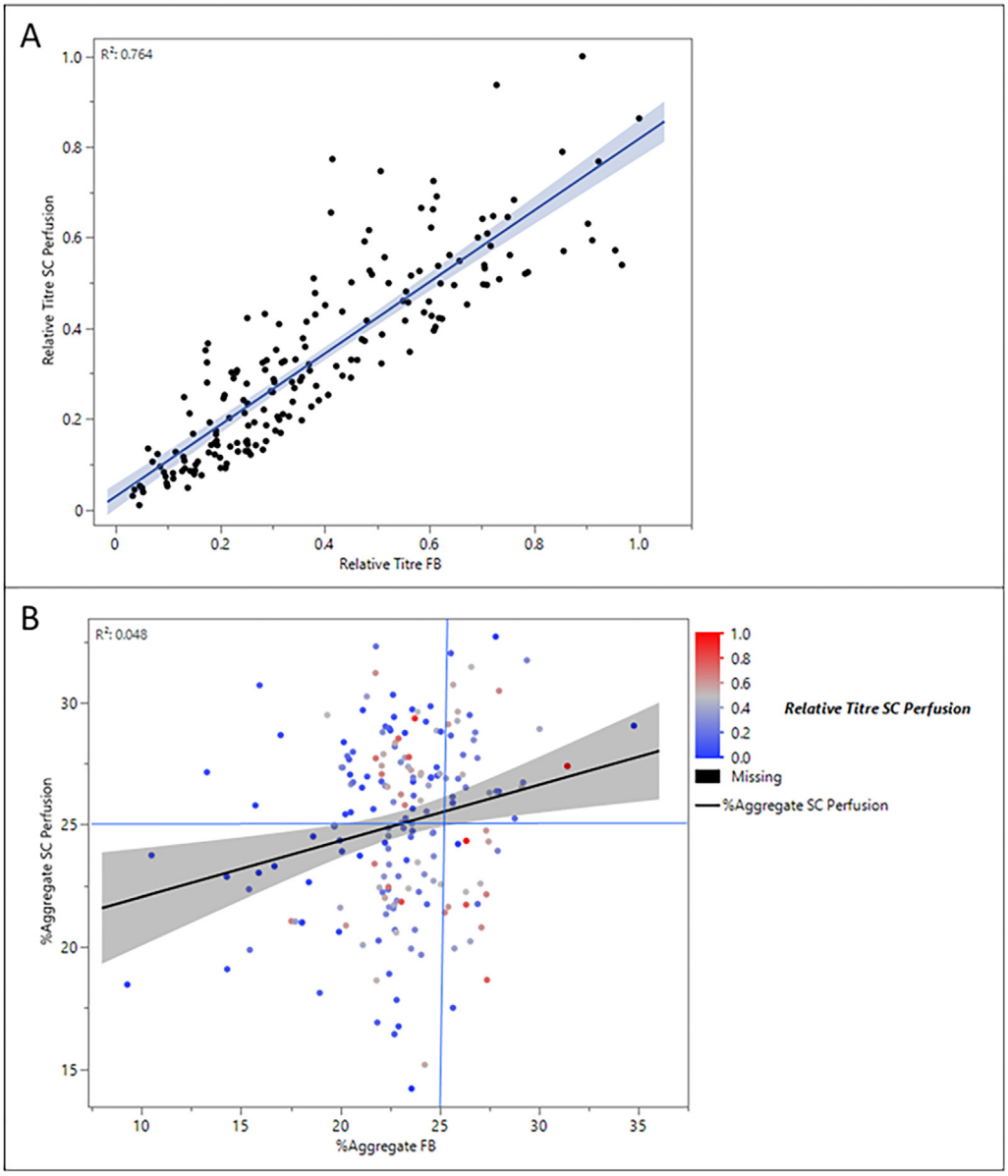

Fig. 6. Relative titre and percentage aggregate expressed by clones cultured in the fed-batch and semi-continuous perfusion processes. (A) Correlation of BsAb 1 titre at the end of the fed-batch (FB) and semi-continuous (SC) perfusion (R^2 0.764). (B) Correlation of BsAb1% aggregation observed during both processes (R^2 0.048). Correlation analysis was performed using linear least squares regression. The blue lines indicate 25% aggregation as a threshold for clones with lower aggregation, highlighting those with a low aggregation in both processes. The relative final titre values from the semi-continuous perfusion are coloured on a scale of blue to red shades to highlight those with low BsAb aggregation in perfusion or perfusion and fed-batch conditions.

minimal sample consumption (2 μg of protein per sample). Automated data analysis provided the generation of quantitative protein aggregation reports compatible with the CLD timelines. The PhyTip purification/HTSEC workflow met the throughput demands of SPOG screens analytical testing of protein aggregates. The present analytical strategy demonstrated a higher throughput in comparison to previously published mD-LC methods [25,44,45] due to the ability to perform purification for 96 samples at a time in parallel and reduce the scale of aggregation analysis by SEC. This enabled faster analysis time and lower sample requirements with adequate automation capability [60]. High throughput aggregation analysis of SPOG screens provided an extra dimension alongside the titre data for clone selection and led to a difference in the choice of clones to investigate in the microbioreactors at a stage where there is a high cut-off for clones for further evaluation. The PhyTip purification/HTSEC workflow offers a rapid small-scale platform solution for aggregates analysis of next generation mAbs and can be easily integrated into HTPD platforms accelerating process optimization and clone selection.

Declaration of Competing Interest

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

CRediT authorship contribution statement

Giulia Lambiase: Conceptualization, Methodology, Validation, Investigation, Formal analysis, Data curation, Visualization, Writing – original draft. **Kerensa Klottrup-Rees:** Conceptualization,

Methodology, Investigation, Formal analysis, Data curation, Visualization, Writing – original draft. **Clare Lovelady:** Conceptualization, Methodology, Writing – review & editing. **Salma Ali:** Investigation, Formal analysis, Data curation. **Samuel Shepherd:** Methodology, Validation, Investigation, Writing – review & editing. **Maurizio Muroni:** Conceptualization, Methodology. **Vivian Lindo:** Supervision, Writing – review & editing, Project administration, Funding acquisition. **David C. James:** Conceptualization, Supervision, Writing – review & editing, Project administration, Funding acquisition. **Mark J. Dickman:** Conceptualization, Supervision, Writing – review & editing, Project administration, Funding acquisition.

Data Availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2023.463809.

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