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Fed-batch production assessment of a tetravalent bispecific antibody: A case study on piggyBac stably transfected HEK293 cells

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ABSTRACT

The transition from preclinical biological drug development into clinical trials requires an efficient upscaling process. In this context, bispecific antibody drugs are particularly challenging due to their propensity to form aggregates and generally produce low titers. Here, the upscaling process for a tetravalent bispecific antibody expressed by a piggyBac transposon-mediated stable HEK293 cell pool has been evaluated. The project was performed as a case study at Testa Center, a non-GMP facility for scale-up testing of biologics in Sweden, and encompassed media adaptation strategies, fed-batch optimization and a novel antibody purification technology. The cell pool was adapted to different culture media for evaluation in terms of cell viability and titers compared to its original Expi293 Expression Medium. These parameters were assessed in both sequential stepwise adaption and direct media exchanges. By this, a more affordable medium was identified that did not require stepwise adaptation and with similar titers and viability as in the Expi293 Expression Medium. Fed-batch optimizations resulted in culture densities reaching up to 20 × 10^6 viable cells/mL with over 90 % viability 12 days post-inoculum, and antibody titers three times higher than corresponding batch cultures. By implementing a novel high-speed protein A fiber technology (Fibro PrismA) with a capture residence time of only 7.5 s, 8 L of supernatant could be purified in 4.5 h without compromising the purity, structural integrity and function of the bispecific antibody. Results from this study related to medium adaptation and design of fed-batch protocols will be highly beneficial during the forthcoming scale-up of this therapeutic antibody.

Introduction

Antibody therapeutics constitute the largest segment of biological drugs. To date, up to 570 different monoclonal antibodies (mAbs) have been evaluated in clinical trials, of which 79 have been approved by the U.S. Food and Drug Administration (FDA) [1]. To ensure ample quantities of high-quality protein for use in clinical trials, preclinical biological drug development requires an efficient upscaling process. In this context, bispecific antibody drugs are particularly challenging due to their inherent structural complexity, which often results in lower titers and a higher proportion of aggregates compared to the corresponding parental antibody format [2]. Consequently, upscaling process

Abbreviations: mAb, monoclonal antibody; CMO, contract manufacturing organization; CHO cells, Chinese hamster ovary cells; PTMs, post-translational modifications; HEK cells, human embryonic kidney cells; ITR, inverted terminal repeat elements; scFv, single-chain variable fragment; GMP, good manufacturing practice; MCS, multiple cloning site; CMV, cytomegalovirus; GFP, green fluorescent protein; ELISA, enzyme-linked immunosorbent assay; HRP, horseradish peroxidase; TMB, 3,3',5,5'-Tetramethylbenzidine); SPR, surface plasmon resonance; LDH, lactate dehydrogenase; CIP, cleaning-in-place; CV, column volume; SEC, size exclusion chromatography; DBC, dynamic binding capacity.

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development has become increasingly important, and often involves dedicated contract manufacturing organizations (CMOs).

Mammalian cell lines are the principal expression system for biotherapeutic proteins because these cells will provide human-like post-translational modifications (PTMs). At present, Chinese hamster ovary (CHO) cells are the most used host platform for the manufacture of recombinant therapeutic proteins, mainly due to their capacity to maintain good growth kinetics in high-density serum-free suspension cultures, high yields of protein with human-like glycosylation moieties, and the low the risk of infection by human pathogenic viruses [3]. Nevertheless, glycoproteins produced in CHO cells lack certain human glycans, as well as producing non-human glycan structures that may provoke an immune reaction when used as a therapeutic drug [3,4]. The growing number of intrinsically complex proteins, such as bispecific antibodies, entering preclinical and clinical development, has made the human embryonic kidney (HEK) 293 cells an attractive alternative for biologicals requiring a human glycosylation profile [4]. HEK293 derived cell lines are generally used for transient protein production, but they have also lately been reported as an efficient platform for the stable expression of therapeutic proteins [5,6].

Consistent large-scale protein production requires stably expressing cell lines, which has led to the development and implementation of an increasing number of various genetic integration systems for stable expression, *e.g.* transposon-mediated DNA integration. The piggyBac system has proved to have the highest transposition activity and general flexibility among the most frequently used transposon-based systems, surpassing Sleeping Beauty, Tol2, and Mos1 [7,8]. The concept involves a helper plasmid encoded by the piggyBac transposase, which cuts the host DNA and transposon flanked by inverted terminal repeat elements (ITR) at the 5′ and 3′ends containing the gene of interest. When expressed in the cell, the transposase then recognizes the ITRs on the transposons where it subsequently initiates excision to mediate integration into the host genome [7]. The piggyBac system has been used for stable gene integration in both CHO and HEK293 cells [8–10] as a reliable and efficient method for expression of monoclonal and multivalent bispecific antibodies [11,12]. Although single cell clone screening and selection post transfection generates higher yields and control, heterogeneous piggyBac cell pools are reported to reduce

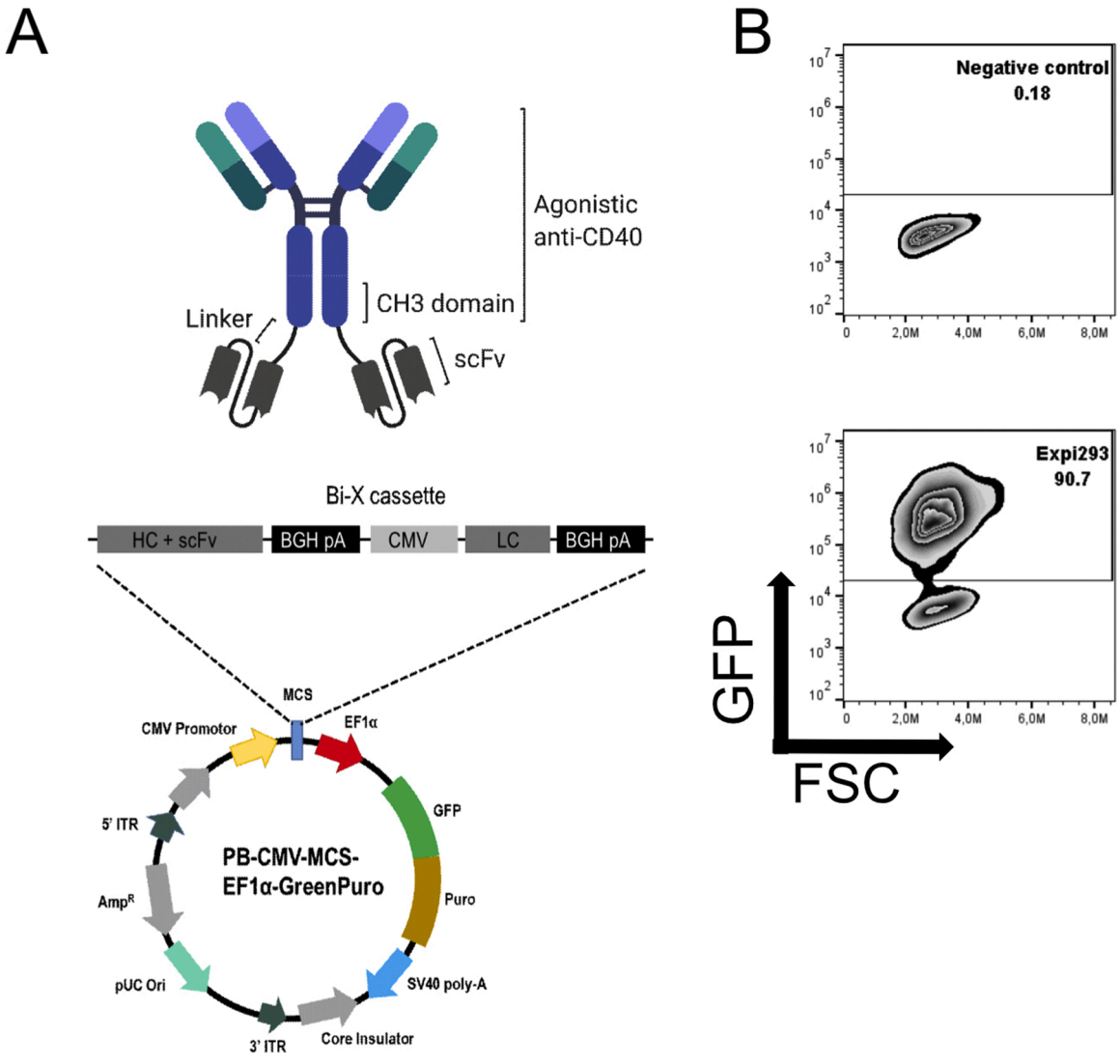

Fig. 1. PiggyBac HEK293 cell pool establishment. A) The piggyBac pB513B-1 vector with the Bi-X cassette coding for the heavy chain (HC) + single-chain variable fragment (scFv) and the light chain (LC) of the tetravalent bispecific antibody used in the study. B) GFP expression of cell pool (>90 % GFP positive) measured by flow cytometry in Expi293 Expression medium at the final stages prior to media adaptation.

production start from 9 months down to 6–8 weeks while retaining a high and stable expression [13,14].

Maintaining high cell viability, proliferation and a stable metabolic cell profile is essential to improve antibody titers. One of the most popular strategies is semi-continuous nutrient supply during the culture, known as a fed-batch process. Advances in fed-batch protocols through changes in feed composition and feed rates can be considered among the most significant contributing factors for the increased protein yields observed over the last 20 years [15].

Here, the ambition was to explore potential upscaling challenges of a candidate immunotherapy drug based on a tetravalent bispecific antibody design that is able to bind the CD40 protein through its Fab-region, and a specific linear peptide epitope *via* its scFv-domains (Fig. 1A). The project was performed as a case study at Testa Center, a non-GMP facility for biologics production in Sweden, covering cell pool generation, media adaptation strategies, fed-batch optimization and the implementation of a novel antibody purification technology. So far, protein expression and purification of this molecule have been limited to transient lab-scale production, which is not representative of the future workflow and expression systems with this drug. Knowledge around challenges in the scale-up production phase and functional properties when stably expressed in human derived cells, even if minor, prior to setting the final candidate drug design is very valuable. Consequently, results from this test case will be taken into consideration for further drug design and cell line development, as well as upstream and downstream protocols.

Material and methods

Vector construction

The piggyBac pB513B-1 (Cat. PB513B-1) donor vector and the pB200A (Cat. PB220PA-1) helper vector (coding for PB transposase) were purchased from System Biosciences (Palo Alto, CA, US). The bispecific antibody expression cassette, denoted Bi-X, was cloned in the multiple cloning site (MCS) of the piggyBac PB513B-1 vector by In-Fusion cloning (Clontech, Shiga, Japan, Cat. 638909) with heavy and light chains controlled by separate Cytomegalovirus (CMV) promoters. Concomitant GFP and puromycin expression is regulated by the EF1α promotor (Fig. 1A).

Cell culture

Suspension-adapted Expi293 F cells (Thermo Fisher Scientific, Waltham, MA, USA, Cat. A14635) were cultured in chemically defined, serum and protein-free Expi293 Expression Medium (Thermo Fisher Scientific, Cat. A1435102) at 37 °C in shaking incubators (80 % humidified, 125 rpm and 8% CO_2).

Cell transfection and stable cell pool generation

Transfection (FectoPRO DNA transfection kit, Polyplus Transfections, Illkirch-Graffenstaden, France, Cat. 116-001) was carried out with a DNA donor to helper vector ratio of 1:3. The cell pool was established after four weeks of puromycin (Thermo Fisher Scientific, Cat. A11138-03) selection (10 μg/mL) pressure until cells showed viability of >96 %. The transfection efficiency and the establishment of the stable cell pool at that point were evaluated by their GFP expression by flow cytometry (Beckman Coulter CytoFLEX Flow Cytometer, Brea, CA, USA).

ELISA assay

Supernatant titers and functionality of the produced bispecific antibody were determined by sandwich ELISA. Briefly, 96-well high binding ELISA plates were coated at 1 μg/mL with a mouse anti-human-Fc IgG antibody (MabTech, Nacka Strand, Sweden, Cat. MT145), or respective cognate targets of the bispecific antibody (0.1 μg/m biotinylated CD40-Fc and 1 nM biotinylated peptide epitope sequence, respectively) and incubated overnight at 4 °C in PBS. Biotinylated CD40-Fc and biotinylated peptide were incubated on streptavidin pre-coated plates (Thermo Fisher Scientific, Cat. 15500). Following 1 h blocking (PBS with 3% BSA, 0.05 % Tween-20) at room temperature (RT), samples were added and incubated 1 h at RT. An anti-human kappa HRP (1:10000) (Southern Biotech, Birmingham, AL, USA, Cat. 9230-05) was used for detection (incubated for 1 h at RT) before adding 3,3',5,5'-Tetramethylbenzidine (TMB) (Thermo Fisher Scientific, Cat. 37574). The reaction was stopped after 4–6 min by 1 M of H_2SO_4 and the optical density (OD) was measured at 450 nm and 650 nm. Plates were washed three times in PBS 0.05 % Tween-20 between each incubation step in the assay. As a reference for the qualitative binding sandwich ELISAs, a transiently produced Bi-X in Expi293 F cells in Expi293 Expression Medium was used. In short, transient Bi-X was purified by a HiTrap Protein A HP column (Cytiva, Cat. 17-0402-03) followed by a desalting step (HiTrap Desalting column, Cytiva, Cat. 17-1408-01) before a final size exclusion chromatography step (HiLoad 16/600 Superdex 200 pg SEC column, Cytiva, Cat. 28-9893-35) equilibrated with PBS (Sigma-Aldrich, D8537-500ML). 6 M Guanidine HCl was used for column cleaning-in-place and 0.1 M Glycine-HCl for elution (1 M pH 2.7 ± 0.1, 10X concentrate, Polysciences Inc. Warrington, PA, USA, Cat. 24074-500).

Surface plasmon resonance (SPR)

Antibody titers were measured by surface plasmon resonance (SPR) using a Biacore T200 instrument (Cytiva, Marlborough, MA, USA). Briefly, culture supernatants were filtered (0.45 μm) and analyzed on a Series S Sensor Chip Protein A (Cytiva, Lot. 10260138) with HBS-EP+ (10 mM HEPES, pH 7.4; 150 mM NaCl; 3 mM EDTA; 0.05% Tween 20) buffer used as running buffer and 10 mM Glycine (pH 1.5) used as regeneration buffer. The standard curve was prepared from 1 mg/mL Herceptin (Trastuzumab) and diluted in the same cell culture medium as the samples in a dilution series starting from 50 μg/mL to 0.5 μg/mL. All measurements were performed in duplicate. A detailed protocol of the Biacore setup is outlined in Supplementary Information 1.

Adaptation to a low-cost culture medium

The cell pool was evaluated in different HEK293 dedicated cell culture media (adaptation media) against the reference Expi293 Expression Medium before the upscaling process. The media selected were CD293 (Thermo Fisher Scientific, Cat. 11913019), 293SFM (Thermo Fisher Scientific, Cat. 11686029), ExCell 293 (Sigma Aldrich, Saint Louis, MO, USA, Cat. 14571C) and BalanCD HEK293 Medium (Irvine Scientific, Santa Ana, CA, USA, Cat. 91165). The cell pool was cultured in increasingly higher ratios of adaptation media to Expi293 Expression Medium. For comparison, the cell pool was also directly re-suspended in 100 % adaptation media. During the adaptation, the cultures were passaged at a higher seeding density during adaptation (0.8–1.2 × 10^6 cells/mL). Cells were considered fully adapted once growing in 100 % new medium and meeting the growth rate and maximum cell density expected values.

Feeding solutions

The feeding strategy adopted for the fed-batch cultures involved the use of BalanCD HEK293 Feed (Irvine Scientific, Cat. 91166), and the peptones Difco TC Yeastolate, UF (BD Biosciences, San Jose, CA, USA, Cat. 292804), BBL Phytone (BD Biosciences, Cat. 15ABP195). Each peptone was prepared using a 100 g/l stock solution in ultrapure water to a final concentration of 6 g/l. A fourth feed solution referred to as "Peptone Mix" was prepared by equally blending the two previous

peptone stock solutions with a third one made up of Bacto Malt Extract (BD Biosciences, Cat. 15ABP201) to a final concentration of 6 g/l.

Batch and fed-batch process in shake flasks

Batch and fed-batch experiments were performed in duplicates in 250 mL shake flasks (Corning, Corning, NY, USA, Cat. 431144) with 50 mL initial working volume. Cultures were inoculated at a cell density of 0.5×10^6 cells/mL in BalanCD HEK293 medium and incubated at 37 °C (80 % humidified, rotation at 125 rpm and 5% CO_2). Starting from day 3, cultures were supplemented with different feed solutions on a daily basis in equal volume until day 9 before harvesting on day 11 – 12. The optimal feed volume was determined by pilot experiments. A total of 28 % (4% per event) and 21 % (3% per event) were optimal for the BalanCD HEK293 Feed and peptones, respectively. Glucose was added to the cultures in order to maintain a culture concentration of 1−3 g/l. Media supernatants were collected, and after centrifugation (2000 x g for 10 min), stored at −20 °C for subsequent analyses. Daily measurements were taken to evaluate cell density, viability, offline pH, pCO2, pO2, osmolality, nutrient and metabolite (glucose, lactate, glutamine, glutamate, NH_3/NH_4 and lactate dehydrogenase) concentrations prior to adding feed.

Fed-batch cultures in bioreactors

Bioreactor cultures were performed in 50 L Cellbag WAVE Bioreactor (Cytiva, Marlborough, MA, USA, Cat. CB0050L11-31) with cells inoculated at a density of 0.5×10^6 cells/mL in 15 L BalanCD HEK293 medium. An agitation speed of 18 rpm–22 rpm was used to improve Dissolved Oxygen (DO) control and reduce cell aggregation. The cultures were fed from day 3 for 8 consecutive events to day 10 with 4% of initial inoculation volume. CO_2 into the surface aeration was used for maintaining pH 7.0. DO was set to 40 % of air saturation by surface aeration at 0.30 lpm. Supernatant collection and daily culture measurements were taken as previously described for shake flask fed-batch cultures. Osmolality, glucose, lactate, and ammonia were evaluated twice a day from bioreactor samples taken at 6 h intervals, as this sampling frequency ensured a good control of the feeding regime and to calculate the feed requirements to maintain the desired concentration of 280–380 mOsm/kg, 1−3 g/l, < 4 g/l and < 9 mM respectively. Cultures were supplemented with 4 mM L-Glutamine (Thermo Fisher Scientific, Cat. 25030081) only at day 0.

Osmolality, gas, nutrient and metabolite analysis

Glucose, lactate, glutamine, glutamate, NH_3/NH_{4+}, and lactate dehydrogenase (LDH) concentrations were evaluated using the Cedex Bio metabolite analyzer (Roche, Basel, Switzerland). The osmolality was measured by OsmoPRO (Advanced Instruments, Norwood, MA, USA) and the offline pH, pCO_2, pO_2 was measured using the ABL9 gas analyzer (Radiometer, Copenhagen, Denmark).

Antibody purification by high-speed fiber-based protein A affinity chromatography

Affinity purification was carried out using a 3.75 mL HiScreen Fibro PrismA prototype unit (Cytiva) on the ÄKTA pure 150 chromatography system (Cytiva) equipped with the Unicorn 7.3.0 software (Cytiva). The system and the tubing were washed with 1 M NaOH (Sigma Aldrich, Cat. S8045-500 G) to limit endotoxins.

Cells were removed from supernatant by sequential depth filtration, a 1.6 m^2 capsule with SP series media (3 M, Saint Paul, MN, USA, Cat. E16E07A10SP02A) followed by a 0.23 m^2 capsule with ZB series media (3 M, Cat. E16E071A90ZB05A). After depth filtration, supernatant was filtered into 20 L bags (Cytiva, Cat. 12410224) through a 10" 0.6/0.2 µm filter capsule (Cytiva, Cat. 12410097). Approximately 40 L of cell culture was processed using this filter set-up with a Watson-Marlow 630 peristaltic pump (Watson-Marlow Fluid Technology Group, Cornwall, UK) and SciLog pressure sensors and monitor (Parker Hannifin, Cleveland, OH, USA)

The supernatant was filtered through 0.22 µm filters (Cytiva, Cat. 12410096) and equilibrated to RT before purification. The removal of particles and contaminants was necessary to achieve the optimal performance of the Fibro PrismA. The Fibro PrismA prototype was equilibrated with 6 column volumes (CV) of 20 mM Na_2HPO_4 (Sigma Aldrich, Cat. S9390-500 G, S9638-500 G), 150 mM NaCl (Merck, Kenilworth, NJ, USA, Cat. 1.06404.1000), pH 7.4 buffer. After loading, two consecutive post-load wash steps were performed with 10 CV of 20 mM Na_2HPO_4, 500 mM NaCl buffer (pH 7.4) and 10 CV of 50 mM acetate buffer (Sigma Aldrich, Cat. W302406-1KG-K, 33209-1L-M) (pH 6), respectively. Samples were eluted with 10 CV of 50 mM acetate buffer (pH 3.5) followed by a cleaning-in-place (CIP) with 1.0 M NaOH. All buffers were pre-filtered with 0.22 µm bottle-top vacuum filters (Corning, Cat. 430513). A flow rate of 30 ml/min (residence time of 7.5 sec) was used for equilibration, sample application, washing and elution. CIP and re-equilibration were run at 15 mL/min (residence time of 15 s).

Analytical size exclusion chromatography

Analytical size exclusion chromatography was performed on the ÄKTA pure 25 chromatography system using a Superdex 200 Increase 10/300 G L (Cytiva) column run at 0.750 mL/min in 1X, pH 7.4 PBS buffer (Medicago, Quebec, Canada and Thermo Fisher Scientific).

Large scale preparative size exclusion chromatography (SEC) and buffer exchange

Samples were filtered through 0.22 µm Sterivex filters (Merck) prior to large-scale preparative SEC (BPG 100/950 column packed with Superdex 200 prep grade resin (Cytiva) with a column volume of 5220 mL). PBS (Thermo Fisher Scientific) was used as buffer and flow rates varied between 15 and 20 mL/min depending on sample. Eluted samples were concentrated in PBS by Amicon Centrifugal Filter Units (Merck, Kenilworth, NJ, USA) and filtered through 0.22 µm Sterivex filters (Merck) prior to storage at -80 °C. Purified sample concentration was measured with Optizen NanoQ Lite's (K LAB, Daejeon, Republic of Korea) IgG measurement program.

Endotoxin test

Samples were diluted 5 times (= 1 + 4) with endotoxin-free mQ-H2O and analyzed with a Charles River Endosafe PTS (portable test system) unit (Charles River, Wilmington, MA, USA) using Limulus Amebocyte Lysate (LAL) cartridges (Charles River), standardized with U.S. Std. Endotoxin, sensitivity 5−0.05 EU/mL (Charles River, Cat. PTS20F).

Antibody stability studies

Antibody stability was evaluated at 40 °C, 4 °C and several freeze-thaw cycles by NanoDSF using a Tycho NT.6 instrument (Nanotemper München, Germany) and analytical SEC. In short, 1 mL of purified antibody at 1 mg/l was incubated at 40 °C (sampling at 5 h, 24 h, 4 d and at weeks 1, 2, 3 and 4) or at 4 °C (sampling at weeks 1, 2, 3 and 4). Freeze-thaw cycles when stored at -20 °C were repeated 7 times. Baseline measurements prior to the start of the experiment served as reference. Samples analyzed by NanoDSF were diluted to 0.1 mg/mL in PBS prior to analyses. For analytical SEC, 67 µg of sample diluted with PBS was loaded.

Statistical software

Statistics were performed using GraphPad Prism v7.04.

Results

Generation of stable cell pool for bispecific antibody expression

The Bi-X bispecific antibody was expressed in Expi293 cells following transfection with the piggyBac PB513B-1 vector at different donor to helper pDNA ratios (Fig. 1A). A ratio of 1:3 achieved the highest titers, which subsequently was used for further cell transfections, and following four weeks of 10 μg/mL puromycin selection pressure in Expi293 Expression medium, resulting in an established Expi293 stable cell pool (>90 % GFP positive cells) that was used for the subsequent cell medium adaptation and upscaling process application (Fig. 1B).

Cell medium adaptation process

Different commercially available HEK293 culture media were compared to the dedicated Expi293 Expression medium, either sequentially adapted to the new media as an iterative process or directly into 100 % new adaption medium. BalanCD and ExCell 293 produced the highest titers and cell number during sequential adaptation (data not shown). However, at direct media replacement, only cells cultured in BalanCD performed similarly as in the original Expi293 Expression medium in terms of viability and proliferation (Fig. 2A and Supplementary Fig. 1). The sequential adaptation approach exposed several disadvantages, the foremost being the high number of passages required during the long media substitution. The high passage number also resulted in a statistically significant decrease in antibody titers (Fig. 2B). As a result of this difference, the subsequent upscaling process involved exclusively the cell pool directly adapted to the BalanCD medium.

Batch and fed-batch experiments in shake flasks

Here, a set of different fed batch strategies was explored. In addition to a dedicated BalanCD Feed matching the identified medium, used as per the manufacturer's suggestion, and the study of [16], three peptone solutions (autolyzed yeast, an enzymatic digest of soy, and combination of the previous two together with malted barley) were also assessed. The feeding strategy is outlined in Fig. 2C.

Batch cultures reached a maximum cell density of 7.9×10^6 cells/mL at day 6 before culture viability and proliferation started to decrease (Fig. 3A). With the exception of Yeastolate (9×10^6 cells/mL) and BalanCD feed (18×10^6 cells/mL), the remaining fed-batch cultures reached a similar cell density to the reference batch cultures (Fig. 3A). However, compared to the batch cultures, all feeds resulted in lower lactate dehydrogenase (LDH) levels and higher viability during the culture (Fig. 3B,C). Based on the evaluated parameters, the BalanCD HEK293 Feed surpassed the others in terms of maximum viable cell density, stable osmolality level below 300 mOsm/Kg, reduced accumulation of metabolic by-products and higher antibody titers (Fig. 3C–G).

As a result of the finite glucose source, and subsequent shift to lactate

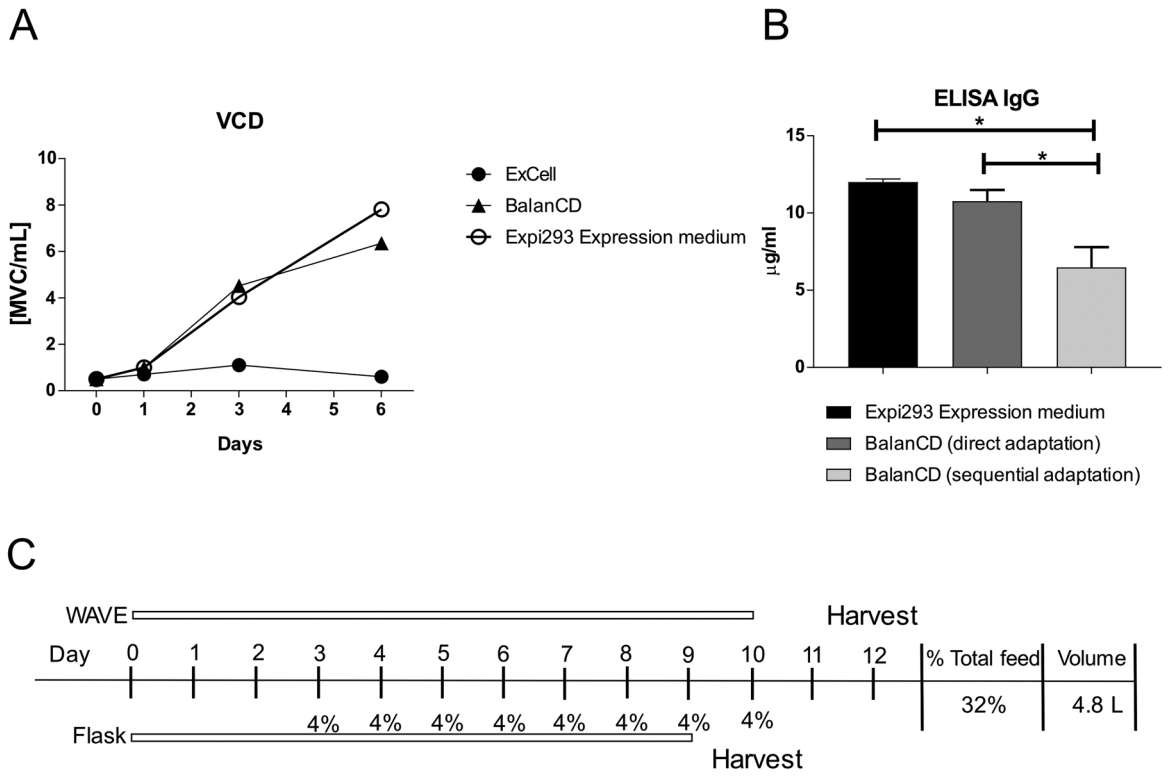

Fig. 2. Cell media adaptation performance assessment. A) Viable cell density during direct adaptation experiment showing BalanCD medium performing similarly to the Expi293 Expression medium in contrast to ExCell 293 medium. Cells were resuspended in 100 % new medium and passaged 4 times before the batch experiment was performed. B) IgG titers from the cell pool at different passage numbers illustrating the decline in titers upon prolonged adaptation. The cell pool at fewer passages, in either Expi293 original and BalanCD media (direct adaptation), produced similar titers compared to the sequentially adapted cell pool with higher passage numbers ($p < 0.05$, ANOVA, Tukey's multiple comparison test). Subsequent fed-batch studies were conducted with the early passaging of the cell pool. C) Feeding strategy for the WAVE fed-batch cultures. Cells were fed with 4% of initial starting volume from day 3 up until day 9 (flask) or day 10 (WAVE). The total volume of feed for one WAVE bioreactor for a total feed volume of 4.8 L (32 % of initial inoculation volume). Flask cultures were harvested at day 10 and WAVE at day 12.

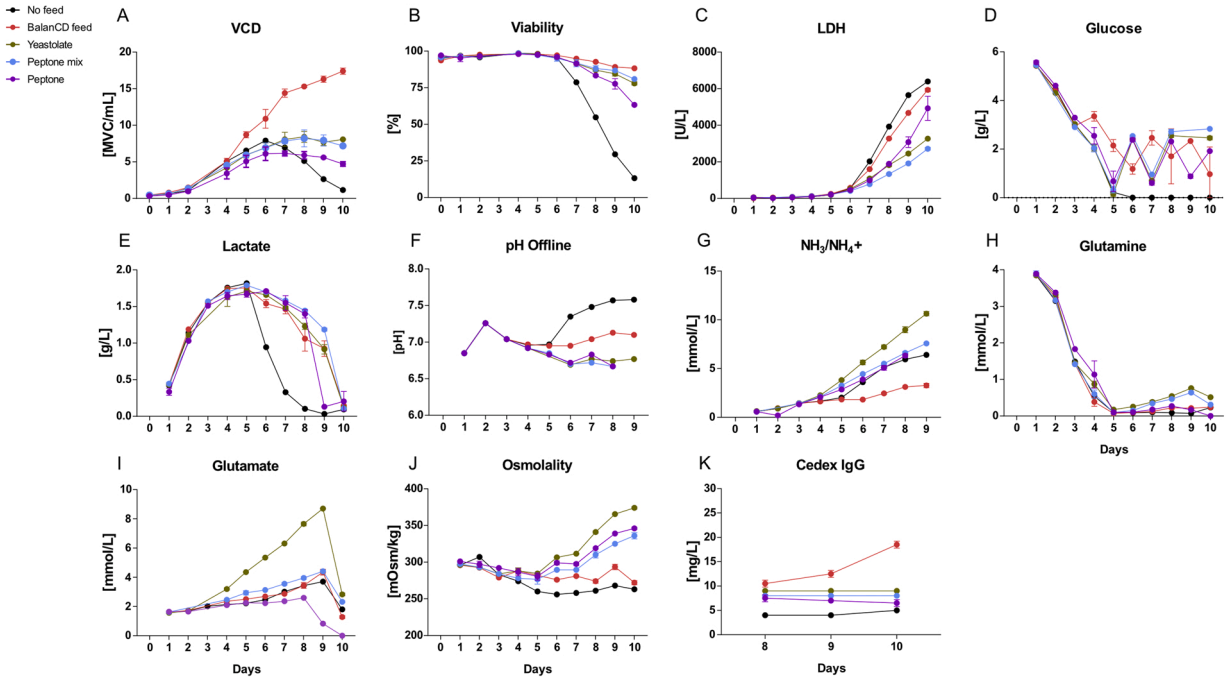

Fig. 3. Fed-batch pilot study with selected feeds in shake flasks using BalanCD Feed, Yeatolate, Peptone mix and Peptone feeds. The cell pool was cultured in fed-batch over 10 days testing in duplicate for different feed solutions with a normal batch culture as negative control. The cultures were characterized and metabolic profiled on daily bases for: A) vable cell density (MVC/mL), B) cell viability (%), C) lactate dehydrogenase (U/L), D) glucose (g/L), E) lactate (g/L), F) offline pH, G) ammonium (mmol/L), H) glutamine (mmol/L), I) glutamate (mmol/L), J) osmolality (mOsm/Kg), K) IgG titers by Cedex over the duration of the fed-batch cultures (mg/L).

as primary energy source, the fed-batch cultures experienced a lower final culture pH of 7.1 compared to pH 7.5 in the batch cultures (Fig. 3F). To minimize ammonia levels in the cultures, additional glutamine was not supplemented, a strategy previously shown to reduce glutaminolysis [17]. The cultures supplemented with BalanCD Feed showed the lowest levels of ammonia (<4.00 mmol/L). Interestingly, during the mid-exponential phase of the cultures, the glutamine level increased independently of feed solution (Fig. 3I). A control fed-batch experiment with constant glutamine supplementation was carried out in parallel with no observed effect on titers, although a significant increase in ammonia production was observed (data not shown).

Compared to the different feeds used and the regular batch culture, the BalanCD Feed maintained stable osmolality levels in the optimal range of 275–290 mOsm/kg for Expi293 cells (Fig. 3J). Moreover, the BalanCD Feed also resulted in the highest antibody titers at the end of the cultures (Fig. 3K).

Fed-batch experiments in bioreactors

Based on its high viable cell density, optimal osmolality range, low metabolic by-products and highest antibody titers, the BalanCD Feed was selected for the large-scale bioreactor experiments. A feeding regime based on offline measurement by the Cedex metabolite analyzer was developed in two WAVE bioreactor experiments, an initial pilot experiment BRX1, followed by a process-optimized BRX2 (Fig. 4).

The pilot bioreactor (BRX1) that was fed reached 28 % of the initial culture volume starting from day 3. Interestingly, compared to the shake flask fed-batch cultures with glucose concentration kept at 2 g/L, the pilot BRX1 lactate accumulation was twice as high, resulting in a decrease of pH to below pH 6.8 (Fig. 5A–C). The acidification of the culture medium led to an automatic compensation by the upward pH-control through increased $NaHCO_3$. As a result, osmolality increased to over 350 mOsm/Kg and pH to above pH 7.1 at day 8 post-inoculum (Fig. 5C, D). However, after adjustment, the culture recovered to values similar to BRX2 (Fig. 5E–H). To prevent excessive increase in pH, glucose feed was reduced to 1−2 g/L and pH adjustment limited to CO_2 modulation in BRX2, which also reduced lactate concentration by almost 50 % (1.7 g/L compared to 3.2 g/L) and retained osmolality levels below 300 mOsm/Kg (Fig. 5B, D). Bioreactor fed-batch cultures were not supplemented with additional glutamine, which also reduced ammonia (Fig. 5I, J). Compared with BRX1, the small adjustments in BRX2, although the differences for some parameters, did not significantly improve the antibody titers when assessed by ELISA at day 12 of harvest (Fig. 5K, L).

Affinity chromatography and preparative size exclusion chromatography

Supernatant from the respective bioreactor was processed in two purification runs using a HiScreen Fibro PrismA 3.75 mL prototype unit with a sample application flow-rate of 30 mL/min (residence time of 7.5 s). To adhere to the dynamic binding capacity (DBC) for Fibro PrismA of 30 mg/CV, each bioreactor was processed in two rounds of 8 L. A representative affinity chromatogram of a Fibro run and its following analytical SEC showing 4.2 % aggregates is depicted in Fig. 6A, B, with bispecific antibody eluted as a single peak. This shows that the high flow-rate used with the unit can be applied to aggregate-sensitive bispecific antibodies. A preparative SEC step was used as a final polishing for the combined bioreactors, which resulted in an estimated recovery of 170 mg antibody with purity of >99 % (Fig. 6C). The exact yield was difficult to assess accurately due to use of different analytical methods at different steps. Endotoxin test determined the purified product to be safe to use in *in vivo* experiments (data not shown).

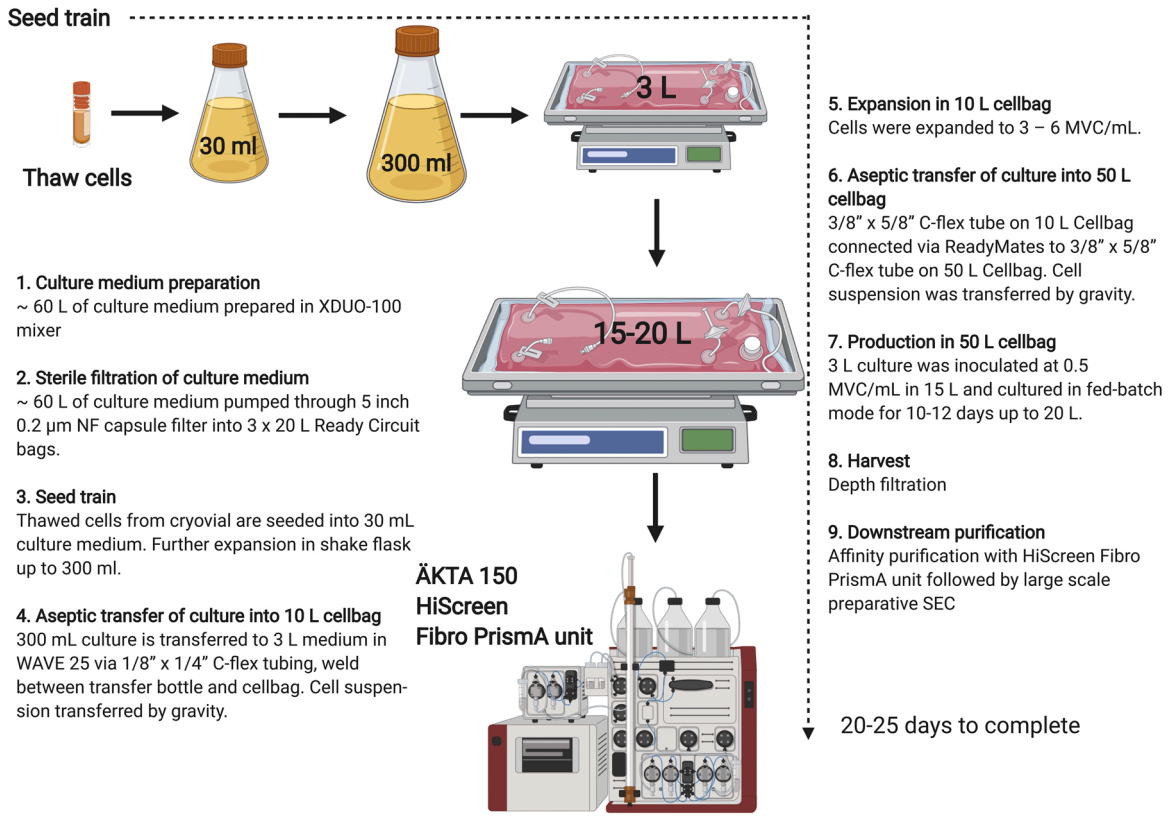

Fig. 4. Workflow of the upscaling process development. Illustration generated in Biorender.

Antibody characterization and stability evaluation post production

Qualitative ELISA confirmed functional properties of the purified Bi-X with similar target affinity to that of a transiently expressed protein with the identical protein design (Fig. 6D). Thus, structural and functional binding integrity was not impaired despite the high flow rates used at the Fibro affinity purification step. The structural integrity of the bispecific antibody was also monitored over 4 weeks by analytical SEC and NanoDSF under the following conditions: 40 °C, 4 °C and through 7 freeze-thaw cycles. Within the first few hours at 40 °C, the antibody experienced up to 40 % increase in aggregation, which stabilized after the first day (Fig. 7A). It was, however, apparent that the sample concentration was reduced over time as the analyte and aggregate peak area decreased proportionally to each other. Compared to SEC post 5 h incubation at 40 °C, NanoDSF did not show any initial significant unfolding or change in protein structure as indicated by a shift in the inflection temperature. Instead, the inflection temperature, when incubated at 40 °C, remained constant at 81 °C for all 7 time point measurements (Fig. 7B). Notably, despite the complex nature and propensity to aggregate, the antibody also remained stable during long time storage at 2% aggregation at 4 °C (Figs. 7C,D) and 4% following the 7 freeze-thaw cycles (Fig. 7E,F).

Discussion

Assessing manufacturing challenges in early drug development projects is an opportunity rarely considered, especially when it comes to complex protein-based drugs, as human resources, economy, and time generally are limited. The benefit of combining two drugs in one has made bispecific antibodies an attractive option with a large potential as biopharmaceutics. However, as of now, only two bispecific antibodies (Blinatumomab and Emicizumab) are approved for clinical use [18]. One contributing factor, among others, is the difficulty in producing bispecific antibodies in sufficient amount and purity. There are even reports of instances where clinical trials have run out of material due to production problems [19]. Thus, for future clinical applications of the drug, knowledge on how to successfully transition from lab-scale to large-scale production early on in the project is vital. Therapeutic antibodies also require correct post-translational modification when produced, both for their safety profile and biological function [20,21]. The bispecific molecule here had previously exclusively been transiently expressed in the Expi293 cell line (manuscript in preparation), hence, the use of the same expression cell line enabled benchmarking the results from this initial proof-of-concept upscaling project to previously produced material antibody. However, it is acknowledged that in a GMP setting, a GMP classified CHO cell line would be used.

In Uppsala, Sweden, a novel bioprocess pilot-scale testbed for non-GMP production of biologics was recently established to provide and accommodate academic institutions and companies that are conducting early upscaling processes development. Here, the facility was used in a proof-of-concept study for the production processes of our tetravalent bispecific antibody drug. Due to limited funds and time, several decisions were made to enable the project. As upscaling was the focus of the project, transient production was not considered. Given the restricted timeline, a stable cell pool was proceeded with in order to invest more time in learning the upscaling process and challenges for this specific protein design. Compared to establishing a cell line, a cell pool significantly reduces the time and cost from the cloning step to a purified and available product, which is important when conducting large-scale pilot production [22]. However, the trade-off is generally lower titers [23], as was apparent here. So far, piggyBac cell pools have shown high stability during prolonged culture, but also a viable

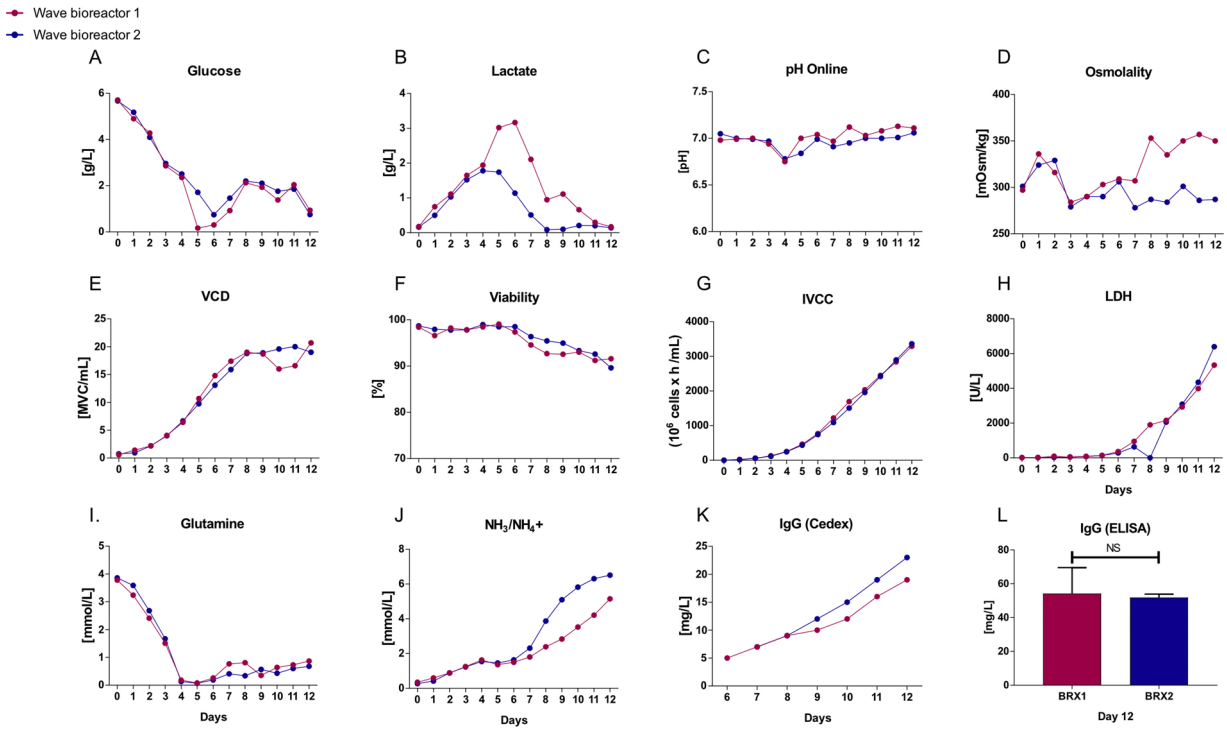

Fig. 5. Productivity and cell performance assessment in fed-batch experiments in WAVE bioreactors. BalanCD medium and BalanCD Feed were used for the upscaling process in respective WAVE bioreactor. The cell pool was cultured in fed-batch over 12 days and characterized daily for A) glucose levels (continuous supply), B) lactate, C) pH in the cell culture medium, D) osmolality, E) viable cell density (MVC/mL), F) cell viability, G) integral viable cell concentration, H) lactate dehydrogenase (cell death), I) glutamine, J) ammonium, K) IgG titers measured by Cedex over the duration of the culture, L) IgG titers measure by ELISA at harvest. There was no significant difference in antibody titers (2-tailed unpaired *t*-test based on 3 separate measurements of the BRX supernatants).

intermediate step for clonal cell line selection [13]. In this project, the fluorescent marker present in the piggyBac vector served as a selection marker for integrated vector, which can aid future cell line generation by single-cell sorting of highly fluorescent cells from the cell pool [24].

To investigate differences in culture performances, the project proceeded with the screening of different standard HEK293 cell media to complement the original transfection medium. A negative impact on viability, cell growth rate and protein production is often the risk during media adaption [25], which is why a sequential replacement was performed in parallel with direct medium replacement. In terms of antibody titer, the fully adapted cell pool was largely unaffected compared to the dedicated expression medium at the same passage number. However, a notable decline in antibody titer upon increased passage numbers was detected for any media used, and thus the decision to abandon the sequential adaption step. Instead, the cell pool was cultured directly into the candidate medium from the same passage number as when the sequential adaptations were initiated. Surprisingly, it did not negatively impact cell growth kinetics, antibody titers, or biological function in the BalanCD Medium when referenced to the original Expi293 Expression Medium. Thus, evaluating direct adaptation may be an option for other projects involving media optimization.

Although high density cultures burden the glucose consumption rates, it was found that maintaining glucose at the lower end of the spectra at 1−2 g/L resulted in a glucose to lactate metabolism shift during the exponential growth phase. The metabolic profile obtained coincides with the results shown in [26], where it was hypothesized that the acidification of the culture medium in the mid-exponential phase due to lactate secretion might trigger HEK293 cells to shift from glucose to lactate metabolism, and thus better culture performances [27].

The fed-batch strategy was developed by testing the BalanCD HEK293 Medium in combination with BalanCD Feed or peptone feed solutions. The BalanCD system has previously been used to improve cell growth and volumetric productivity for HEK293 high-density cultures in a perfusion bioreactor system [16]. Compared to other media and peptone feeds assessed here, the system performed well in this study as well. The peptones used were selected from commercially available peptones of yeast, soy, and malt origin. Peptones are widely used as a supplement for serum-free culture media, individually or by blending strategies, with positive effects on volumetric protein productivity in both transient and stably transfected CHO and HEK293 cell cultures [28–31]. However, the dedicated BalanCD Feed outperformed autolyzed yeast (Difco TC Yeastolate), the enzymatic digest of soy (BBL Phytone) and the combination of the two blended with malted barley (Bacto Malt Extract) in terms of accumulated metabolic byproducts, desired osmolality level, maximum viable cell density, and most importantly, enhanced antibody expression. The BalanCD Feed was subsequently selected for the large-scale bioreactor experiments, where the protocol optimization led to an improved fed-batch process by reaching a higher cell density of up to 20×10^6 cells/mL and increased culture longevity. Although cultures were not continuously supplemented with glutamine, levels were increased independently of batch or any feed solution, an effect potentially related to the increase in cell density and the resulting rise in glutamine synthetase concentrations over time [5].

In this testbed case, we also assessed a well-engineered high-speed Protein A fiber technology (Fibro PrismA) that is designed to compensate for high volume and low-titer antibody levels, as in the case of this project. This was the first time that the unit was tested for low titer supernatant and a bispecific antibody. Despite the high flow-rate used in the capture step, the tetravalent bispecific antibody showed high purity and stability, as well as retaining antigen binding. Loading of 8 L cell

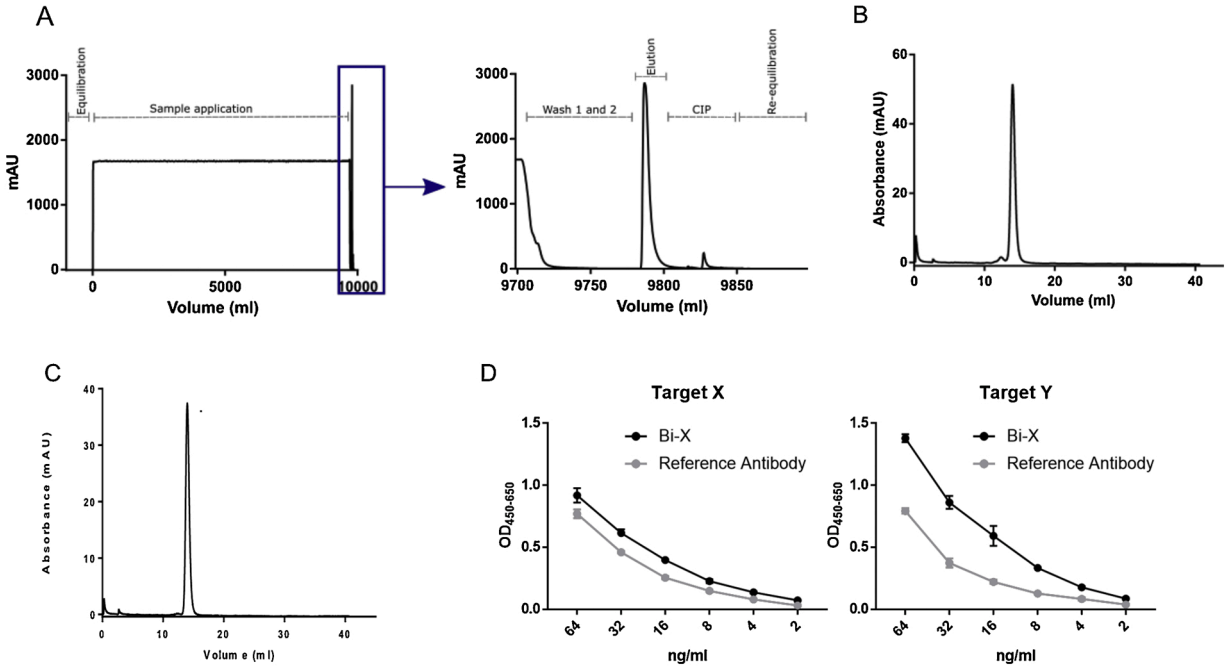

Fig. 6. Downstream affinity purification and functional evaluation of purified sample. A) Representative chromatogram for one Fibro affinity run with 8 L of harvested and filtered supernatant. The different stages of the procedure are indicated in the chromatogram with equilibration, sample application, washes, elution, cleaning-in-place (CIP) and re-equilibration. The end of the chromatography is shown to the right. B) Affinity purified sample from (A) analyzed by analytical size exclusion chromatography (SEC) with a Superdex 200 Increase 10/300 G L column. C) ELISA assessing antigen target binding post Fibro purification of Bi-X in a two-fold dilution series ranging from 2 to 64 ng/mL (x-axis). The results from optical density (OD) measurements at 450 nm with correction for background at 650 nm show retained binding to both targets (X and Y). The results are comparable with that of a transiently produced version of Bi-X here used as the reference antibody (n = 3).

culture supernatant to the Fibro PrismA, 3.75 mL unit at a flow rate of 30 mL/min resulted in a cycle time of approximately 4.5 h with the given protocol. Compared to a conventional resin-based Protein A column, the majority of the cycle time was spent on sample loading with washes, elution and CIP only accounting for a few minutes. This allowed sample purification to be reduced to 1−2 days for the combined volume of BRX1 and BRX2. A resin-based purification alternative with a relevant load and residence time (load of 30 mg/mL at a residence time of 4 min) would have required almost a week. However, the Protein A column could be increased in size for faster purification, but at a higher consumable cost. Optionally, a separate concentration step prior to the capture step could be performed to reduce the volume of culture supernatant. In contrast, the very short residence time of the Fibro PrismA unit essentially allowed sample concentration and analyte capture to be performed as a single step, illustrating a novel way to combat low titer cultures without sacrificing the stability, quality, and yield of the expressed protein. Further, it is also a fast and cost-efficient alternative to current affinity purification strategies.

The pre-packed Fibro PrismA format also eliminates the time spent on column packing, which after CIP and strip can be reused up to 300 cycles based on current Cytiva data [32]. Despite its complexity, the purified sample following preparatory SEC and sample concentration contained less than 1% aggregates. To be noted, although suitable within the realm of the current project, the downstream workflow will have to be adjusted for industrial high-titer cultures. The Fibro PrismA affinity capture-step is robust for titers in the range of 1−10 g/L. However, the constraints of the purification process relate to the preparative SEC, which is not feasible for large-scale manufacturing. High volumes and titers require very large columns, which are very expensive to pack, and only permit low flow-rates. Consequently, industrial scale preparative SEC will result in long and expensive purification cycles. To accommodate future post-capture polishing steps more tailored to fit industrial applications, an ion exchange or mixed-mode chromatography resin would be the next step in optimizing the downstream process.

Functional and structural studies were subsequently performed under different conditions. Following confirmation of a retained dual binding capacity, the structural integrity was investigated at different temperatures and throughout several freeze-thaw cycles. At 40 °C, a fast initial degradation was observed after which the remaining fraction maintained a more uniform structural integrity. Interestingly, this degradation was only apparent in the analytical SEC measurements, but not in the corresponding NanoDSF measurements. In the prospect of future *in vivo* experiments, this increased basic structural information under different conditions can be indicative for how stable the drug might be upon injection, or its shelf-life and storage stability.

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Author contributions

LD, OA, AN, IL, TL and ML carried out the experiments with support from HP, AM and AA. OA, LD, AN, IL, TL and ML processed the experimental data and performed the analysis. MH aided TL and ML in protocols and data interpretation. SM, LD, HP, OA, AO, GH, PF provided material and advise on antibody upscaling and purification work. AN, ML, SM, IL, TL wrote the initial manuscript draft. All authors provided

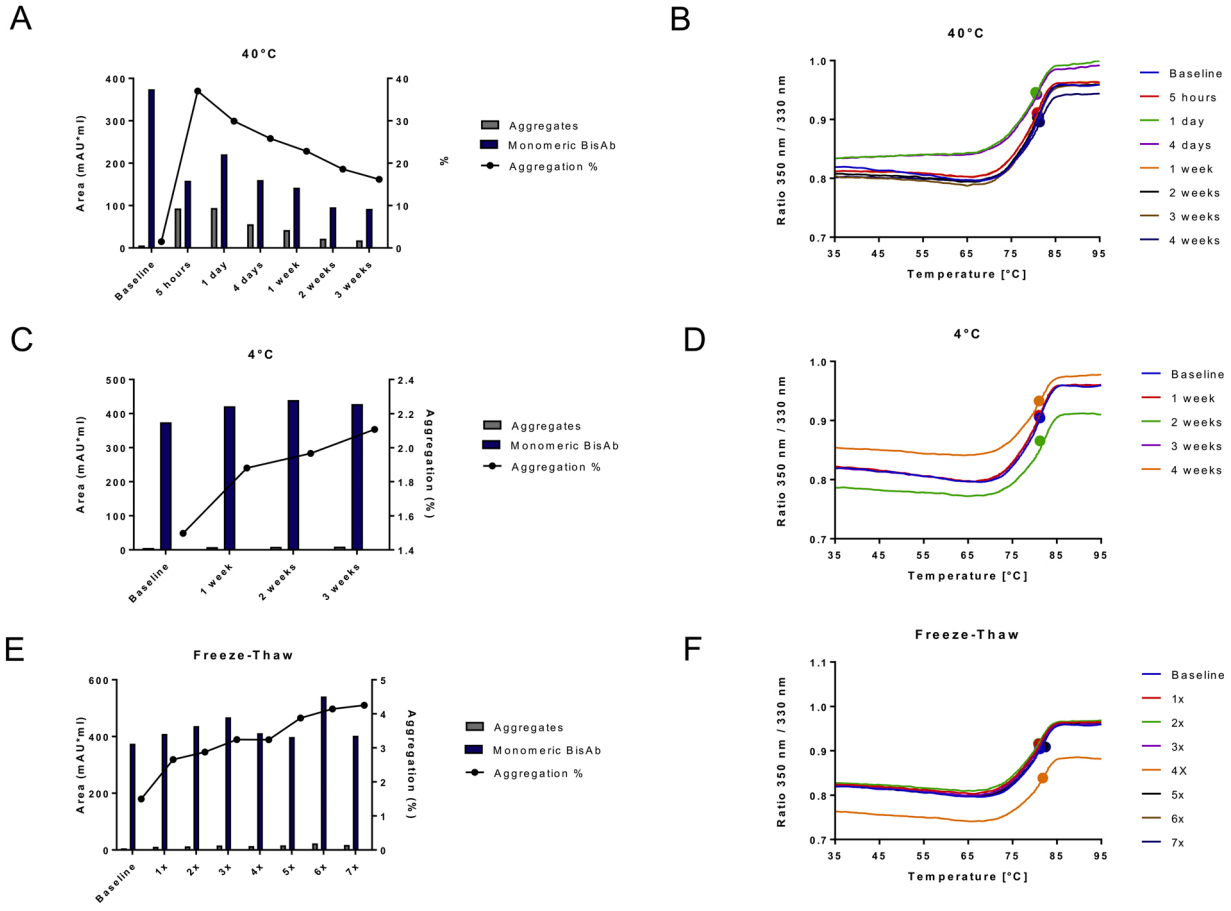

Fig. 7. Post production protein stability assays. Aggregation levels were analyzed by analytical SEC and presented as areas (ml*mAU) of aggregate (grey) and analyte (blue) peaks as bars (right Y-axis) with percentage of aggregation as a line graph (right y-axis). Aggregation at A) 40 °C, C) 4 °C and E) 7 freeze-thaw cycles. Structural integrity was analyzed by NanoDSF at B) 40 °C, D) 4 °C and F) 6 freeze-thaw cycle. The lines depict protein folding state while the dots show inflection temperature for each measurement. There is no shift to left or right on the x-axis and the inflection temperature is about 81 °C for all samples.

critical feedback and helped shape the research, analysis and manuscript. SM and HP conceived the original idea. ML, SM, HP, GH, PF, AA, MH, AM, AA, AO supervised the project.

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Declaration of Competing Interest

SM is the founder and shareholder of Immuneed AB and Vivologica AB and is the Chief Development Officer and shareholder of Ultimovacs ASA/AB. None of the mentioned companies have taken part in the study nor do they have an interest in the specific subject matter described herein. SM, ML and IL are founders and shareholder of Strike Pharma AB. SM and HP are inventors of a patent application related to the antibody design and function, but not related to the process development described herein. None of the other authors declare any competing interests.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.nbt.2021.07.002.

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