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Development of a platform-based approach for the clinical production of HIV gp120 envelope glycoprotein vaccine candidates

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ABSTRACT

Preclinical development of vaccine candidates is an important link between the discovery and manufacture of vaccines for use in human clinical trials. Here, an exploratory clinical study utilizing multiple gp120 envelope proteins as vaccine antigens was pursued, which required a harmonized platform development approach for timely and efficient manufacture of the combined HIV vaccine product.

Development of cell lines, processes, and analytical methods was initiated with a transmitted founder envelope protein (CH505TF), then applied to produce three subsequent gp120 Env (envelope) variants. Cell lines were developed using the commercially available Freedom CHO DG44 kit (Life Technologies). The fed-batch cell culture production process was based on a commercially-available medium with harmonized process parameters across the variants. A platform purification process was developed utilizing a mixed mode chromatography capture step, with ceramic hydroxyapatite and ion exchange polishing steps. A suite of analytical methods was developed to establish and monitor the Quality Target Profile (QTP), release and long-term stability testing of the vaccine products.

The platform development strategy was successfully implemented to produce four gp120 envelope protein variants. In some cases, minor changes to the platform were required to optimize for a particular variant; however, baseline conditions for the processes (cell line type, media & feed system, chromatography resins, and analytical approaches) remained constant, leading to successful transfer and manufacture of all four proteins in a cGMP facility.

This body of work demonstrates successful pursuit of a platform development approach to manufacture important vaccine candidates and can be used as a model for other vaccine glycoproteins, such as HIV gp140 trimers or other viral glycoproteins with global health implications.

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1. Introduction

HIV-1 infection continues to be a global health threat [1,2]. Development of combination anti-retroviral drugs has saved millions infected with HIV-1 from progressing to acquired immunodeficiency syndrome (AIDS) [1,2]. However, control of the epidemic depends on the development of an effective vaccine [1–3]. Unlike most viruses that cause disease in humans, HIV-1 poses unique challenges to vaccine development [4]. The primary difficulties encountered with the virus are the speed at which it establishes a persistent lifelong infection, the extent of genetic variability, the lack of protective immune correlates and the lack of animal models that predict vaccine efficacy in humans. The expectation of an effective HIV-1 vaccine is to raise broadly neutralizing, protective antibodies against the circulating virus and block further infection. The development of a vaccine candidate to elicit such antibodies remains a challenge [4] as traditional approaches to vaccine development (like that of the many licensed viral vaccines) have failed to protect against Human Immunodeficiency Virus (HIV) infection [1]. Hence, strategies to design and produce novel immunogens capable of both activating antibodies and inducing a lasting immunity are an area of active research.

Discovery of broadly neutralizing antibodies (bnAbs) and protective non-neutralizing antibodies (pnnAbs) and the identification of interactions of these antibodies with the virus have paved the way for a new field of structure-based vaccine design [5,6].

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Challenges have been addressed with the use of primary virus isolates with limited passaging to retain the neutralization phenotype and the design of novel soluble stable trimer immunogens as more suitable candidates for an HIV-1 vaccine [3]. The primary target of the bnAbs is the outermost component of the HIV virus, the envelope protein [5].

The design of stable immunogens to induce bnAbs was achieved with a thorough knowledge of the structure of the HIV-1 envelope (Env). Determination of the precise structure of the envelope protein was hampered by the unstable trimeric protein. Recent advances elucidating the structure of the envelope protein along with the use of mutagenesis and chemical crosslinking helped stabilize Env. These advances have allowed for development and evaluation of engineered vaccine immunogens that retain the structure of Env [5].

Many forms of Env are being developed and tested for the ability to elicit both bnAbs and pnnAbs [1] with an emphasis on those that bind CD4 [7]. The different forms of envelope glycoproteins that are currently being evaluated include gp160, gp150, SOSIPs, gp120, and a few other forms of engineered envelope proteins; whereas, only a few of the vaccine candidates have been tested in clinical studies [8].

An important hurdle in candidate evaluation is the transition of a putative molecule from research laboratories to clinical manufacture. That transition, earmarked as 'development', represents a milestone activity in realizing the potential of life-saving therapeutics and vaccines. Manufacture of multiple, related candidates in series poses several challenges – low expression levels of envelope glycoproteins; inherent biochemical and structural complexity and instability of the target contributing to expression and purification challenges; and high cost and time investments. In this study, we describe approaches to overcome such challenges while developing a platform and carefully implementing analytical tools for novel antigens in a contract development and manufacturing (CDMO) setting. The result of this work demonstrates the ability to manufacture a series of complex vaccine antigens of suitable quality and with this efficiency, to expedite clinical trials.

2. Materials and methods

2.1. Stable pool generation and single cell cloning

The primary sequence of all four gp120 envelope proteins CH505TF, CH505w53, CH505w78, and CH505w100 were codon optimized for expression in Chinese Hamster Ovary (CHO) cells [9]. Two independent transfections of CHO-DG44 cells for each vector were performed in parallel [10]. A single round of amplification with methotrexate (MTX) concentrations of 0.5, 1 and 1.5 μM was performed for all stable pools. Protein expression of amplified pools was assessed from cell banks in a batch shake flask study. Shake flasks seeded at 3×10^5 viable cells/mL in 60 mL CD OptiCHO selection medium without MTX were harvested. Amplified pools based on ELISA titers were advanced to single cell cloning by limiting dilution cloning (LDC). LDC was performed to generate the top clonal cell lines. For the first round of cloning, cells from two producing pools were cultured in exponential growth phase and seeded at 0.5 cells/well into a total of 40 × 96 well plates. All wells were imaged by Clone Select Imager (CSI) on days 0, 1, 2, 3, 7 and 14 for confirmation of monoclonality. Twenty-five clones with the highest titer at the 6 well plate stage were expanded to 125 mL shake flasks. Overall, following LDC, 161, 144, 105 and 114 clones were determined to be of monoclonal origin for CH505TF, CH505w53, CH505w78 and CH505w100, respectively. The top 8 clones were selected based on titer assessment.

2.2. Expression stability

An expression stability study was performed for the top eight clones for each Env. At generations 20 and 40, interim RCBs were generated for each cell line. Significant changes to population doubling time (PDT) and expression titers were monitored to assess the stability of the top clones.

2.3. Fed-batch cell culture production

Vial thaw was performed in non-baffled shake flasks (Corning Life Sciences, NY) containing CD OptiCHO medium supplemented with L-glutamine and cultivated in an incubator shaker (Multitron, Infors HT) set at 37 °C, 8% CO_2, and 80% relative humidity. Cells were passaged every 3–4 days prior to fed-batch production. Fed-batch production was performed in non-baffled shake flasks and Ez-Control 3 L bioreactors systems (Applikon Biotechnology, The Netherlands). CD EfficientFeed A and CD EfficientFeed B (Thermo Fisher Scientific, MA) were added in 1:1 ratio during fed-batch production. Production bioreactors were also supplemented with HT supplement (Thermo Fisher Scientific, MA), soy hydrolysate (MilliporeSigma, MA) and yeastolate (Thermo Fisher Scientific, MA). CHO Bioreactor feed supplement (MilliporeSigma, MA), cystine and tyrosine were added, but not in all four processes (Table 1). Temperature was set at 37 °C and downshifted to 33 °C during exponential growth phase, typically on day 6. Dissolved oxygen was maintained at 30% with pure oxygen and pH was controlled at a setpoint of 6.8 – 7.0 using 1 M Na_2CO_3 and CO_2 gases. Offline pH and gases were measured using ABL80 Flex blood gas analyzer (Radiometer, CA); cell count and cell viability using Vi-CELL XR automated cell counter (Beckman Coulter, CA); metabolites including glucose, lactate, ammonia, glutamine and glutamate were measured using Nova Bioprofile 400 (Nova Biomedical, MA) and CEDEX Bio HT analyzer (Roche, Switzerland). Production cultures were harvested upon cultivation for 12–14 days using POD depth filters (MilliporeSigma, MA) and 0.22-μm PES filters (MilliporeSigma, MA) and supplemented with additional soy and yeast hydrolysate (2:3 ratio) to prevent proteolytic cleavage. Cell culture supernatant and clarified harvest samples were analyzed for titer using a RP-UPLC method. In-process quality attributes were assessed only if clarified harvest samples were purified by capture chromatography.

2.4. Purification process

The purification process was developed using AKTA Avant chromatography systems (Cytiva, Sweden). Resins incorporated into the final platform purification process include Capto MMC (Cytiva, Sweden), Ceramic Hydroxyapatite, Type I, 40 μm (Bio-Rad, CA), CM Sepharose HP (Cytiva, Sweden) and SP Sepharose HP (Cytiva, Sweden). All resins were packed into Vantage columns (Millipore Sigma, MA) to a bed height of 10–22 cm and operated at a linear velocity of 150–300 cm/hr during development. Column loading for all chromatography steps was 1.0–2.0 g product per L resin. Concentration adjustment and buffer exchange were achieved within the platform using Pellicon ultracel, 30 kDa molecular weight cutoff membranes (Millipore Sigma, MA) operated at a transmembrane pressure of 15 psig across > 6 diavolumes for ultrafiltration/diafiltration (UF/DF) #1 and across 10 diavolumes for the final UF/DF. All Envs were formulated as drug substance at 1.2 g/L in 20 mM phosphate, 150 mM NaCl, 0.02% polysorbate 80, pH 6.5.

Dedicated viral clearance steps are included in the platform. Viral inactivation for CH505TF was achieved by acidifying the product to pH 3.7 with 2 M acetic acid and holding for 30 min prior to neutralizing to pH 7.2 with 2 M Tris base. During development of

Table 1
Differences in cell culture production processes.

Parameter	CH505TF	CH505w53	CH505w78	CH505w100
Cystine supplement	0.35% each on Day 4 and 8	None	None	None
Tyrosine supplement	0.10% each on Day 4 and 8	None	None	None
CHO Bioreactor Feed (C1615)	None	0.5% w/w each on Day 0, 3, 6 and 9		
Harvest criteria	Day 12–18	Day 12	Day 14	Day 14

Table 2
Quality attributes, assays, and product target/specification for CH505 Envs.

Attribute	Assay	Purpose	Target/Specification
Product Titer	ELISA	Clone Selection	N/A
Product Titer	RP-HPLC	In Process Control	N/A
Identity	SDS-PAGE	Release	Comparable to Reference
Identity	Peptide Mapping	Release	Comparable to Reference
Residual CHO Protein	ELISA	Release	$\leq$ 100 ppm
Residual CHO DNA	Extraction & qPCR	Release	$\leq$ 1 ng/mg
N-Glycosylation Profile	HILIC-HPLC-FLD	Release & Stability	Comparable to Reference
Aggregate Content	SEC-HPLC	Release & Stability	$\geq$ 95% Main Peak
Hydrophobic Character	RP-UPLC	Release & Stability	> 90% Main Peak
Clipping	SDS-PAGE	Release & Stability	Comparable to Reference
Clipping	RP-UPLC	Release & Stability	> 90% Main Peak
Clipping	SEC-HPLC	Release & Stability	$\geq$ 95% Main Peak
Clipping	Western Blot	Development support only	No observable clipping
Charge Heterogeneity	IEF	Development support only	Comparable to Reference
Antigenicity	SPR	Development support only	Comparable to Reference
Antigenicity	Octet	Development support only	Comparable to Reference

follow-on Envs, the platform was updated to include detergent inactivation whereby 10% Triton X-100 was added to the Capto MMC eluate to a final concentration of 1% and held for 30–90 min. For viral filtration, a Planova 15 N filter (Asahi Kasei, Japan) was loaded to $\leq$ 50 L/m^2 at 12 psig. Supplementary Table 1 outlines process details for each of the four Envs.

2.5. Analytical method development

Detailed descriptions of analytical methods can be found in Supplementary Material, Analytical test methods. Briefly, reversed-phase ultra-high performance liquid chromatography (RP-UPLC) was utilized to analyze hydrophobic variants using a Waters Acquity UPLC BEH300 column with mobile phase A & B consisting of 0.1% v/v trifluoroacetic acid (TFA) in water or acetonitrile, respectively. Charge heterogeneity evaluation was performed using Novex pH 3–10 IEF (isoelectric focusing) Protein Gels and Serva IEF pI Markers. Focused gels were fixed using 12% TCA, 3.5% sulfasalicylic acid, stained with Invitrogen Colloidal Blue stain for 30 min, destained, then imaged with Quantity One software (BioRad, Hercules, CA). The Cygnus Generation 1 CHO HCP ELISA kit (Cat. No. #F015, Cygnus Technologies, Southport, NC) was used to monitor residual host cell protein, according to the manufacturer's instructions. Antigenicity of the vaccine product was measured by evaluating binding affinities of the Envs for HIV-neutralizing antibodies using biolayer interferometry (BLI) with the Octet® platform (Sartorius ForteBio, Fremont, CA). This made use of anti-human IgG (Fc capture) biosensors, which were dipped into samples containing Env, where both association and disassociation were measured. The binding response (in nanometers) was plotted vs the concentration of product. To measure Env from cell culture supernatants, an indirect Enzyme-Linked Immunosorbant Assay (ELISA) was developed. The ELISA used two anti-Env antibodies, one to capture and one to detect, with a final secondary HRP conjugate for amplification and quantitation. A standard curve using known quantities of Env was used to measure product titer. RP-UPLC was also utilized to analyze the concentration of Env as a second titer method. For this assessment, a Phenomenex Jupiter C4 column was used with mobile phase A & B consisting of 0.1% v/v TFA in water or acetonitrile, respectively. Purity of the envelope samples was assessed using size exclusion high performance liquid chromatography (SEC-HPLC) with a TSKgel G3000SWxl column (Tosoh Bioscience, PA) and a mobile phase system of 20 mM sodium phosphate, 300 mM sodium chloride, pH 7.4.

3. Results

3.1. Quality target profile

A QTP was generated for the glycoproteins in order to collect and compare product quality attributes for each individual Env drug substance. The quality attributes evaluated throughout cell line, upstream and downstream development are outlined in Table 2. These attributes were assessed using available methods throughout the development and manufacturing programs to inform quality of the Envs produced. Some attributes were critical to the final profile outcome or the performance of the processes (i.e. RP-UPLC, residual HCP, binding potency), whereas others were less informative to product quality (i.e. SDS-PAGE, IEF; Fig. 1).

3.2. Analytical method development

Three methods were critical for driving decisions around the purification process development. When samples of product were separated on analytical RP-UPLC, a hydrophobic variant pre-peak could be observed resolving from the main peak in some samples (Fig. 2). In some cases, this variant was fractionated, collected and demonstrated to exhibit lower potency by SPR binding than the RP-UPLC main peak. The variant was thought to have an altered glycosylation profile and/or conformational structure that reduces its ability to bind the broadly neutralizing antibodies used in the potency assay. As a result, RP-UPLC was used throughout process development as a critical method to inform on product quality. The target for purity varied by product, but generally $\geq$ 90% main peak purity was targeted through purification. The RP-UPLC

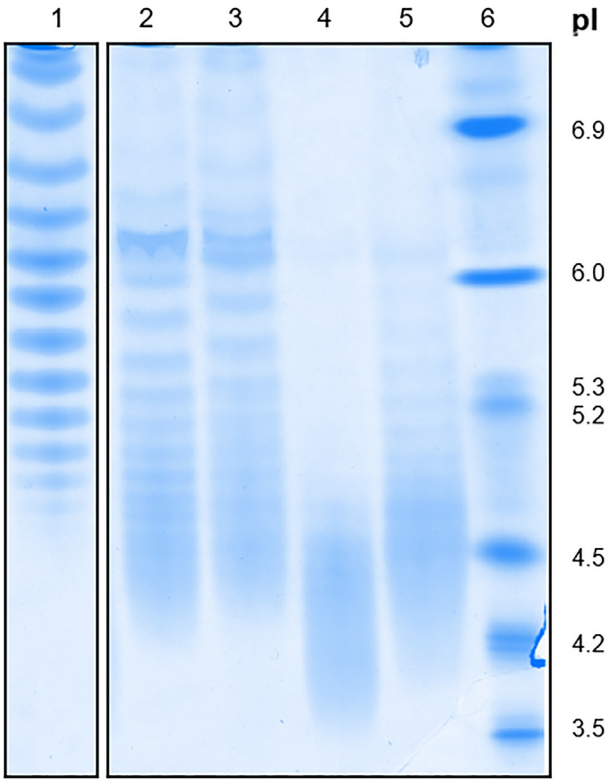

Fig. 1. Isoelectric Focusing of purified gp120 variants demonstrating the heterogeneity of charge profiles. Depicted are CH505TF BDS (Lane 1), CH505w53 interim reference (Lane 2), CH505w78 interim reference (Lane 3), CH505w100 development sample (Lane 4), CH505w100 interim reference (Lane 5).

method was also demonstrated to be stability-indicating, so it was used for all envelopes as part of their bulk drug substance stability programs.

Residual CHO protein ELISA was another important method that was employed routinely to monitor the clearance of contaminating proteins from the CHO cell host. The lack of a protein affinity capture step in the downstream process made the host cell protein (HCP) content of the feed stream for subsequent polishing steps a more significant challenge than more standard bioprocesses. Additionally, the broad charge heterogeneity of the target added to the complexity of separating host cell proteins limiting more classic ion exchange approaches. Extensive resin screening, on-column wash strategies, and fractionation steps were explored to reduce HCP levels to industry acceptable levels for a protein vaccine component. In the end the purification process was able to achieve 3–4 logs clearance of HCPs (Fig. 3) and resulted in the detection of less than 100 ppm of CHO HCP to Env.

Because antibody-binding based neutralization of these Envs is fundamental to their ultimate use as a vaccine candidate, binding affinity of the Env to various antibodies was also carefully monitored as the process was developed. Surface plasmon resonance (SPR) provided early assessments of binding affinity to well-characterized CD4 binding site lineage mAbs; however, BLI was explored as a faster and higher throughput means to attain comparable binding potency assessments. A binding method was successfully developed by immobilizing the neutralizing antibody on the Octet® biosensors, then dipping into increasing concentrations of product-containing samples. Binding responses as a function of product concentration were measured, and this analysis enabled both fast and high throughput testing, with the evaluation of > 50 samples in a single day. For CH505w53, BLI was used to balance product purity and target potency for the capture step. This was achieved by excluding fractions that exhibited poorer binding potency via Octet® BLI (Fig. 4).

3.3. Cell line development and single cell cloning

Cell line development utilized the Freedom CHO DG44 platform to generate top clones expressing the prototype HIV envelope glycoprotein variants CH505TF, CH505w53, CH505w78 and CH505w100. Following pool generation under MTX selection, limiting dilution based single cell cloning identified eight top clones per envelope (Fig. 5). Overall, the productivity of the cell lines expressing each Env in small scale shake flask fed-batch cultures was determined to be between 0 and 120 mg/L (Fig. 6). The productivity was sustained over cell aging and the top 50 percent cell lines determined to be suitable for GMP manufacturing. The lead cell line for each of the prototype Envs was identified based on a combination of expression stability, small-scale manufacturability assessment and product quality attributes including glycan analysis, SEC-HPLC, SDS-PAGE, and potency.

3.4. Cell culture production process development

Differences in growth rate were observed among stable clones expressing the four envelope proteins, but no major changes were made to the process of generating inoculum in shake flasks for all four Envs. Shake flask cultures were passaged every 3–4 days, cell viability was targeted to remain > 90%, ensuring highly viable cells

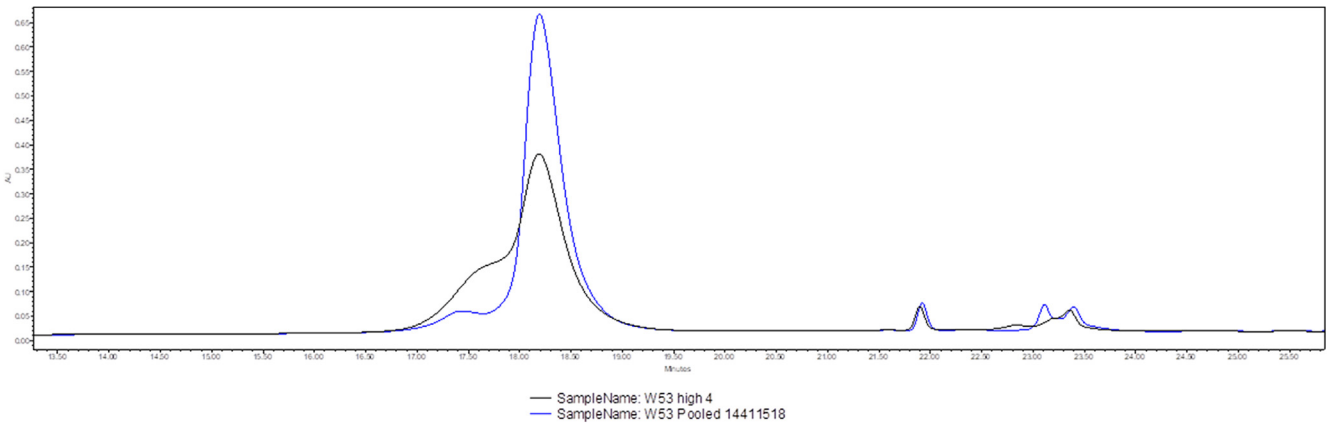

Fig. 2. RP-UPLC analysis of CH505w53 samples. Black trace represents a sample of CH505w53 held at a pH of 10.0 for 2 h, buffer exchanged, then run via RP-UPLC to evaluatewhether RP-UPLC is a stability indicating method. Blue trace represents the control. The pre-peak observed was a key quality attribute monitored during development of the purification process.

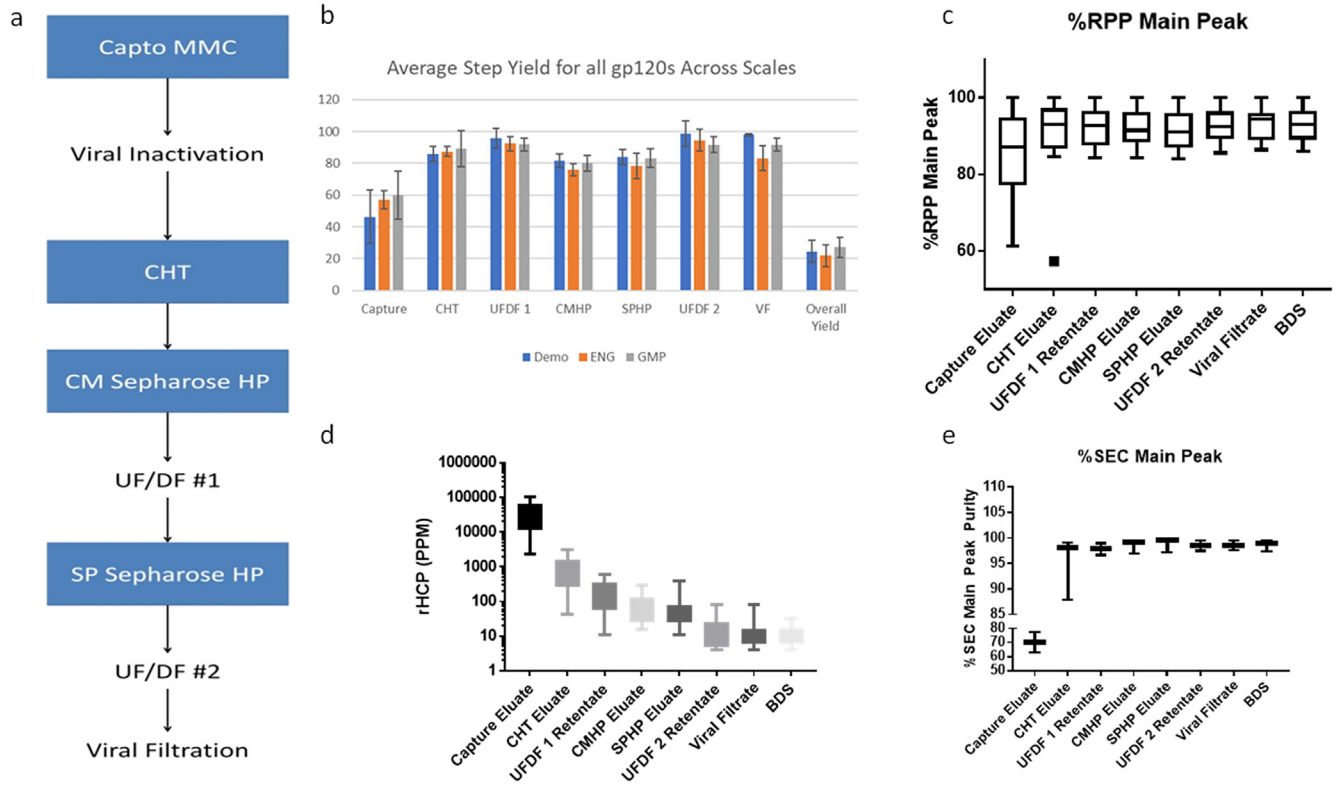

Fig. 3. Downstream platform process outline and performance. Demonstration run scale utilized 10 – 20 cm ID columns. ENG and cGMP scales utilized 14 – 45 cm ID columns. Depicted are (a) process overview, (b) average step yield, including standard deviation, for all four Envs across the downstream process unit operations, (c) purity of process intermediates as measured by RP-UPLC, (d) resHCP levels of each process intermediate and (e) main peak purity as measured by SEC-HPLC.

were used for production. The process of generating inoculum initially used for CH505TF is consistent for the other three Envs.

In the upstream platform, inoculum from shake flasks was transferred to fresh CD OptiCHO medium supplemented with L-glutamine at a fixed target viable cell density to initiate production. Cells were cultured in a bioreactor with temperature, pH and dissolved oxygen control. Chemically defined feed media (CD EfficientFeed A and CD EfficientFeed B) were added to the bioreactor culture on specified days and culture was sampled daily to test for cell viability and glucose concentration. Bioreactor temperature waschanged to 33 °C during exponential growth phase to reduce growth rate, to promote product formation and to ensure high cell viability. This process was developed for CH505TF and consistently used for the other three Envs. Glucose and antifoam were supplemented as necessary. No other feed supplement was added to the CH505TF bioreactor culture, but supplement additives were included for the other three Envs. Three feed supplements (HT supplement, hydrolysates and CHO bioreactor feed) were evaluated for modulating product concentration and antigenicity. After 14–18 days in the production bioreactor (>80% cell viability criteria), bioreactor culture was harvested.

3.5. Product capture and purification process development

The downstream platform was established using CH505TF as the model envelope. With the other Envs some platform evolution and process specific optimization was required. Overall, the platform consists of four chromatography unit operations, viral inactivation, viral filtration and two buffer exchange steps by ultrafiltration/diafiltration (Fig. 3a). For the ion exchange-based chromatography steps Env specific binding and elution conditions had to be defined per Env given the differences in charge heterogeneity (Fig. 1); however, the overall approach was maintained resulting in faster process definition.

With the goal of avoiding lectin affinity for the capture step, a series of ion exchange and mixed mode resins were screened. From initial resin screening, Capto MMC was selected for additional development. In the platform process, the product was captured from clarified cell culture harvest on Capto MMC. Following product binding, an intermediate wash was performed prior to product elution. For CH505TF, the product underwent low pH viral inactivation; however, subsequent Envs exhibited low pH instability as measured by the observation of prepeak in RP-UPLC. Therefore, the platform viral inactivation strategy was altered to utilize detergent inactivation. Viral inactivation was completed using 1% Triton X-100 upon loading of the product on ceramic hydroxyapatite in the absence of phosphate. Elution from CHT was achieved by 5 mM phosphate and 200 mM sodium chloride. Across all Envs evaluated, the CHT step required the fewest molecule specific changes. The third chromatography step utilizes CM Sepharose HP, a weak cation exchange resin and was used to primarily separate Env from rHCPs. For this unit operation, product was bound at approximately pH 5.0 and low conductivity and eluted using sodium chloride and, in some cases, a lower pH. Despite the heterogeneity of charge across the Envs, residual HCP clearance across this step was achieved by modulation of a pH transient that occurs during elution. Each Env required optimization of pH, buffer concentration and sodium chloride amount for elution from CM Sepharose HP. The final polishing step is SP Sepharose HP, a strong cation exchange resin and was used for additional and/or redundant rHCP clearance. Each Env was bound at approximately pH 5.0 and low conductivity and eluted from the column using sodium chloride. Following the SP Sepharose HP step, the Env was buffer exchanged into the defined formulation buffer and concentrated to 1.5–2.0 g/L prior to viral filtration using a Planova 15 N viral filter. After viral filtration, the product

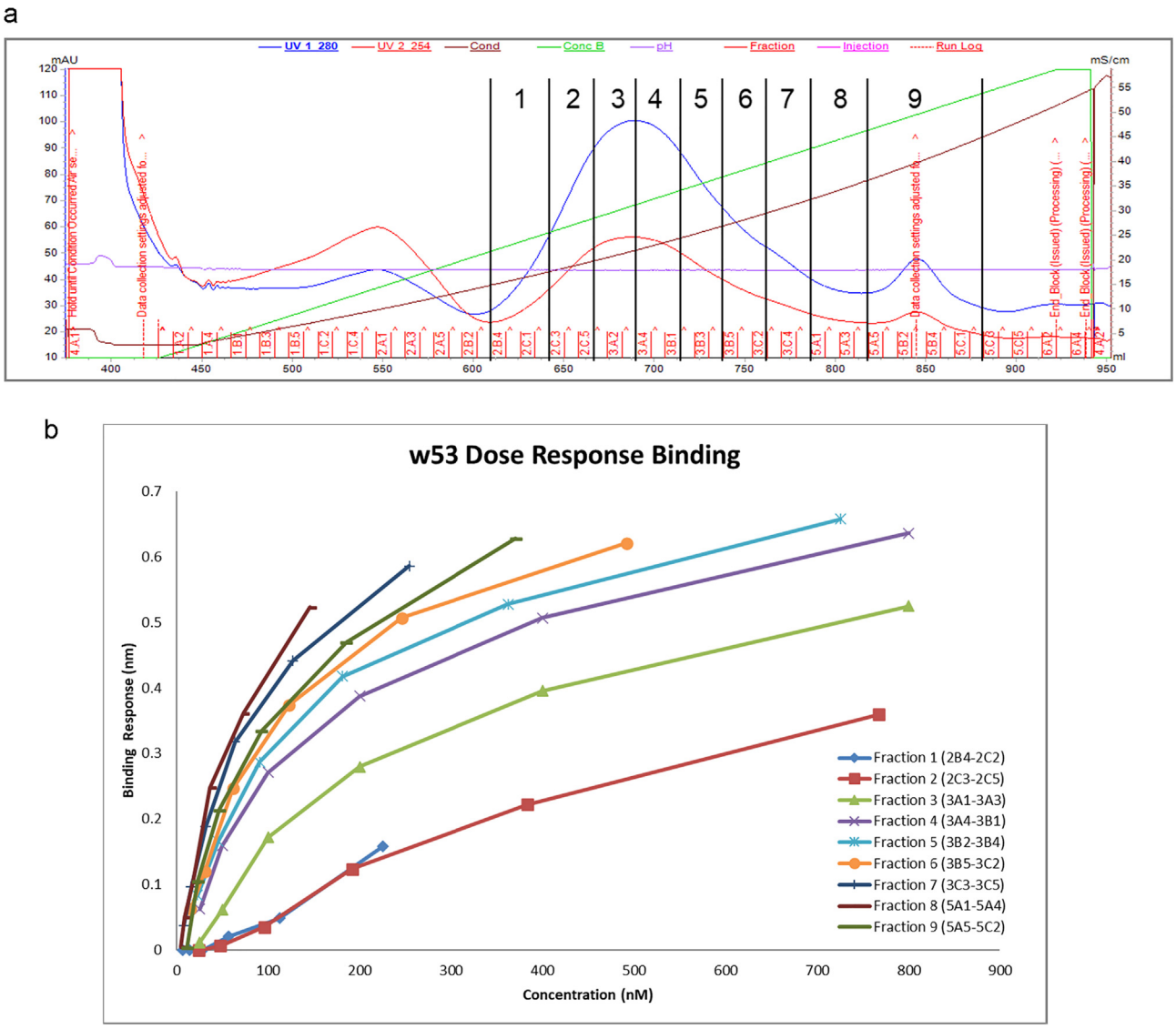

Fig. 4. Use of a high throughput biolayer interferometry method to inform purification process decisions for Env development. Fractions from a Fractogel SO3- purification step (a) were tested for their binding potency using an Octet method (b) where end point binding response were plotted versus Env concentration. Based on these analyses, a fractionation strategy was designed as part of the purification process for this step for CH505w53 variant.

concentration was adjusted to 1.2 ± 0.2 g/L and polysorbate 80 was added to a final concentration of 0.02%. Bulk drug substance was filled in polycarbonate bottles and stored at ≤ -65 °C.

3.6. Process outputs

The Freedom CHO DG44 platform used by cell line development generated pools and clones that exhibited similar growth profiles with peak viable cell density of 6–10 × 10^6 cells/mL. Multiple concentrations of MTX were evaluated for each Env allowing for the selection of the highest titer pools to be selected for single cell cloning (Fig. 5). The platform process offered repeatable timelines for each of the four Envs.

The cell line development protocols used to generate the top clones expressing each Env were assessed to be consistent in performance. This included transfection efficiency, stable pool recovery and generation and stability of expression of the top clones over milestone generations.

The upstream platform achieved consistent cell growth in the production bioreactor with a peak viable cell density of 8–12 × 10^6 cells/mL and a cell viability of approximately 85% at harvest. Bioreactor titers for the four Env variants ranged from 100 to 210 mg/L with 80–90% product recovery. Process performance across scales was consistent for all the four Envs (Fig. 7). Cell growth and viability in seed and production bioreactor were the main challenges in development of a robust, scalable process. Expected cell growth and viability in seed bioreactor were achieved by avoiding use of WAVE Cellbag and same was achieved in production-stage by using liquid feed media directly procured from the manufacturer. Additionally, a specified quantity of feed media was added prior to inoculation of the production bioreactor to increase nutrient levels. These measures in seed and production bioreactor were applied to all four Envs.

The downstream gp120 Env platform achieved an overall process yield of 17–35% for the Envs evaluated. For all Envs the capture step had the lowest yield – in the range of 40–60%. These low capture step yields were attributed to the large degree of charge and/or hydrophobic heterogeneity of the product and in some cases, product with reduced binding potencyless potent forms of the target. The remaining unit operations deliver a step yield > 70%.

Comparable performance of the platform downstream process was achieved from lab scale to GMP manufacturing scale. Consis-

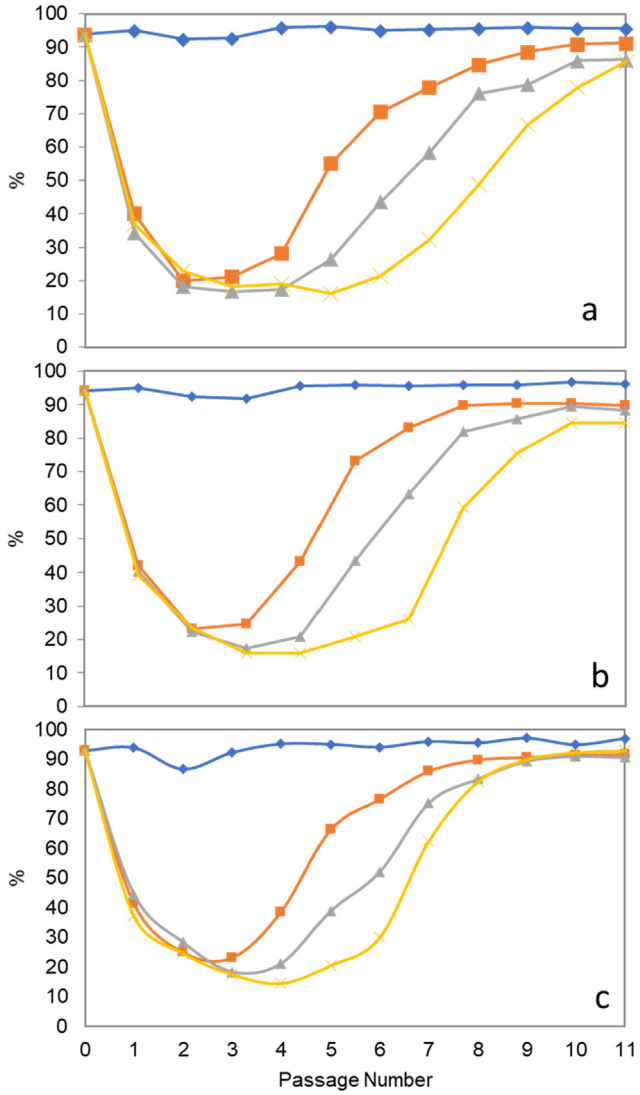

Fig. 5. Recovery and generation of stable pools expressing the HIV-1 envelope variants (a) CH505w53, (b) CH505w78 and (c) CH505w100.

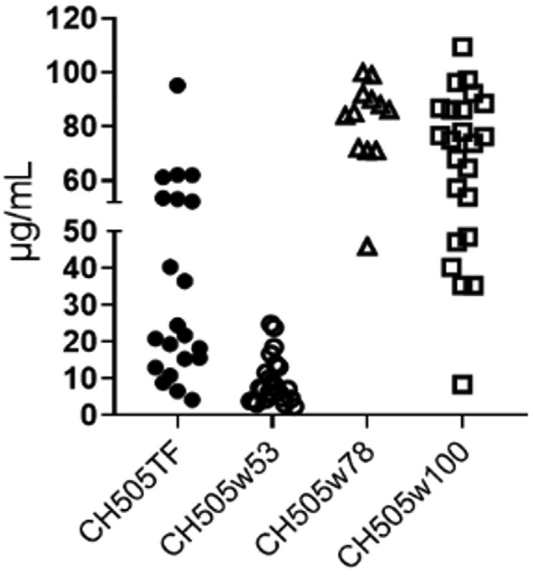

Fig. 6. Productivity of top clones in shake flask fed-batch study for each of the Env variant.

tent unit operation and overall process yields were observed when directly comparing demonstration run and manufacturing scales (Fig. 3). The most yield variability was observed for the capture step and viral filtration across scales; however, this variability did not impact overall process yield. Similarly, product quality was maintained with scale up. The main peak purity by SEC-HPLC and RP-UPLC were similar across scales and among envelope proteins. Process resHCP clearance was also consistent with scale up – in all cases the target specification of <100 ppm was achieved.

4. Discussion

Throughout establishment and assessment of the platform process, various challenges were encountered that required resolution prior to the scale up and cGMP manufacture of each Env. This is one of the key functions in the development of a product and in the refinement of production and characterization techniques to ensure suitability for manufacture. Several of these obstacles were a function of the complexity of the molecules, including biocompatibility issues, low productivity, biochemical heterogeneity and product quality challenges as highlighted below.

Standard methods and analytical strategies for monoclonal antibodies were insufficient to address the needs of these complex proteins. The cell lines exhibited low productivity and the Envs were not amenable to affinity capture, had a heterogeneous N-glycosylation profile, and were sensitive to proteolytic cleavage. Multiple product titer methods were attempted and needed throughout the development of these programs, include multiple ELISAs, Octet, SDS-PAGE, SEC-HPLC, and RP-HPLC methods. Additionally, the complex and heavy glycosylation of the protein made application of any methods using fused-silica capillaries (CE-SDS, icIEF) challenging, leaving only lower resolution and less quantitative methods available for purity and charge variant analysis, i.e. SDS-PAGE and IEF. Despite the setbacks, a panel of analytical methods was developed that provided the needed assessment and monitoring of product quality. A list of the methods used to monitor product quality and process performance can be found in Table 2.

The use of disposable technologies for cell culture has occasionally shown to cause biocompatibility issues due to leachable/extractables from the single-use film or due to absorption of nutrients from the basal medium. During process development for CH505TF, poor cell growth and viability were initially observed upon scaling the production stage in single-use bioreactor bags, but several measures were implemented to prevent impact. Inoculum for the production bioreactor stage is typically generated in seed bioreactors (WAVE Bioreactor™, stirred tank bioreactor, etc.), but this strategy was not used for the Envs due to highly variable cell growth in the WAVE Cellbag. Instead, culture from multiple shake flasks was combined to inoculate the production bioreactor. As a precaution, inoculum from shake flasks was gravity drained into the bioreactor bags to prevent shear damage. The production bioreactor bag was filled with water for injection (WFI) and held at process temperature and agitation for a day prior to replacing with basal medium. Fixed amounts of feed media (CD EfficientFeed A and B) were added to the production bioreactor prior to inoculation to replenish any adsorbed nutrients. Two other upstream process modifications were required involving media formulation and media storage. Basal medium and feed media were directly procured in liquid form from the manufacturer to prevent incomplete dissolution and improper filtration during rehydration of powders (Fig. 8). Lastly, use of single-use storage containers for feed media

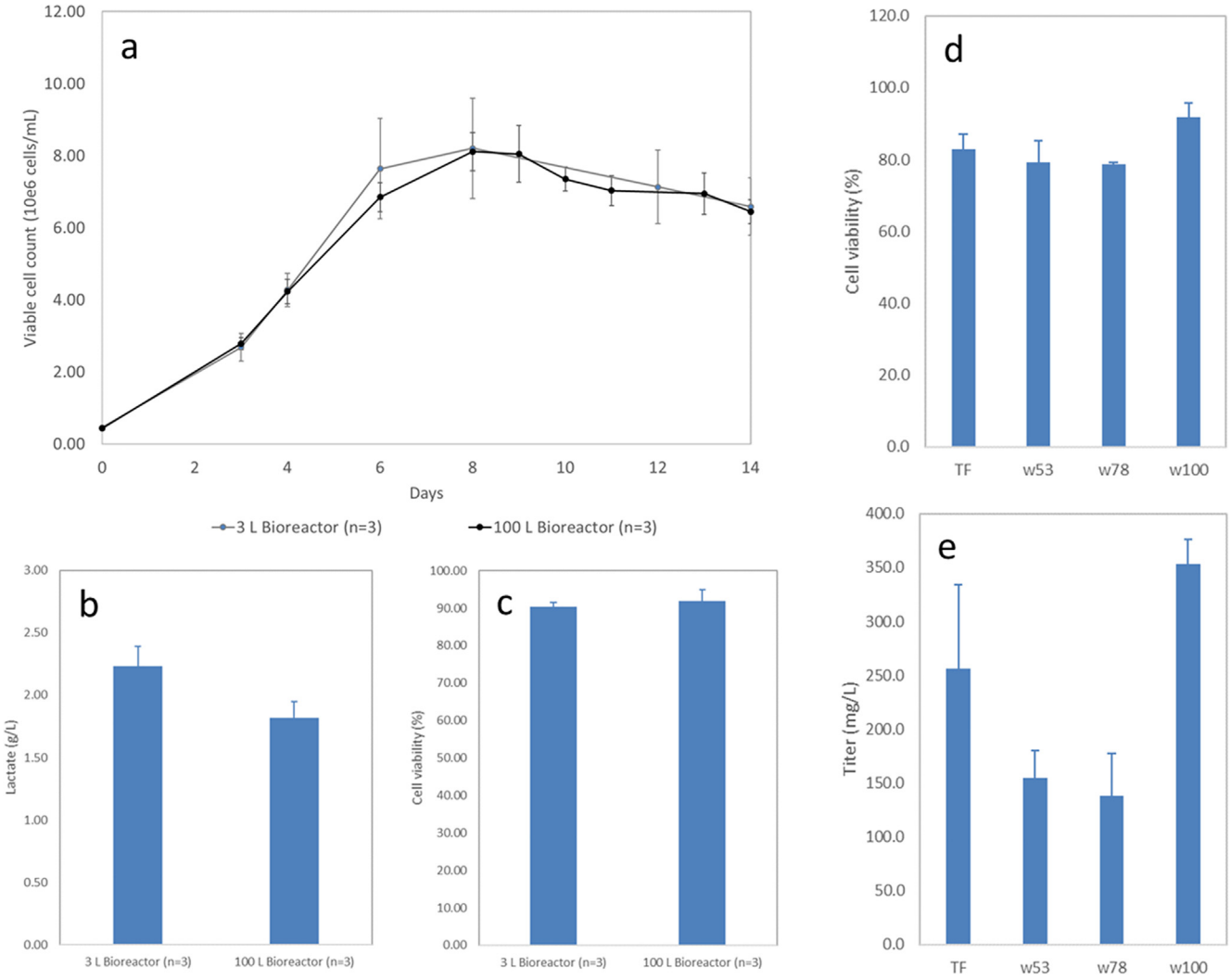

Fig. 7. Fed-batch production process performance in lab-scale to manufacturing-scale bioreactors. Viable cell count (a), peak lactate (b) and cell viability at harvest (c) for CH505w100 Env variant. Cell viability at harvest (d) and production bioreactor titer (e) comparison for all four CH505 Env variants. Error bars represent 1 standard deviation.

was avoided. Autoclaved glass bottles (10 L and 20 L) were used to re-filter the liquid feed media prior to addition in the production bioreactor. All these precautions were first tested for CH505TF, and subsequently implemented for all remaining Envs. These preventive measures avoided the need to use traditional non-disposable bioreactor technologies without impacting process performance (Fig. 8).

Due to the low titer in the stable pools and clones for CH505TF, several feed supplements were screened in shake flask experiments. A combination of CD EfficientFeed A and B along with addition of protein hydrolysates (10 g/L soy and yeast mixture) were identified as the top choice. Even though titer was increased, upon further testing of the production process in bench-scale bioreactors, product cleavage was observed in clarified harvest. Given that protein hydrolysate was already being added to the production process, 10 g/L soy and yeast hydrolysate was supplemented in clarified harvest also. This second hydrolysate addition was considered to provide an alternate protein source (substrate) to the CH505TF Env protein. This hydrolysate addition strategy was successful in eliminating proteolytic cleavage of product and was implemented for all other Envs.

Even though the important production process parameters like temperature, pH, dissolved oxygen, medium and feed media type and hydrolysate supplement were platform based on CH505TF, other supplements were tested for the remaining Envs for further increase in titer. Supplements like cystine, tyrosine, and CHO bioreactor feed supplement were assessed with the CH505w78 and CH505w100 Env cell lines. Addition of cystine and tyrosine with CH505w78 and CH505w100 Envs showed improvement in titer but led to lower antigenicity (Table 3). Some evidence of increased product aggregation was also seen for CH505w100 with the cystine and tyrosine supplementation cultures.

As introduced above, during establishment of the gp120 Env downstream purification platform, resHCP reduction was a challenge. For the development of CH505TF, resins from all modes of chromatography were screened and an initial process was defined. Loading on all chromatography steps was kept low at 1.0–2.0 g/L resin given the low productivity, BDS material needs to support clinical Phase I studies (target 1 g) and need for HCP clearance. During this effort, initial conditions for Capto MMC and CHT were defined. The initial process then incorporated a weak anion exchanger, DEAE, in flow-through mode in lieu of CM Sepharose HP. The DEAE step was followed by SP Sepharose HP. Interestingly, other strong anion exchangers were not as effective in reducing HCP, likely a function of particle size and resulting resolution. In addition to establishing four chromatography steps in the process,

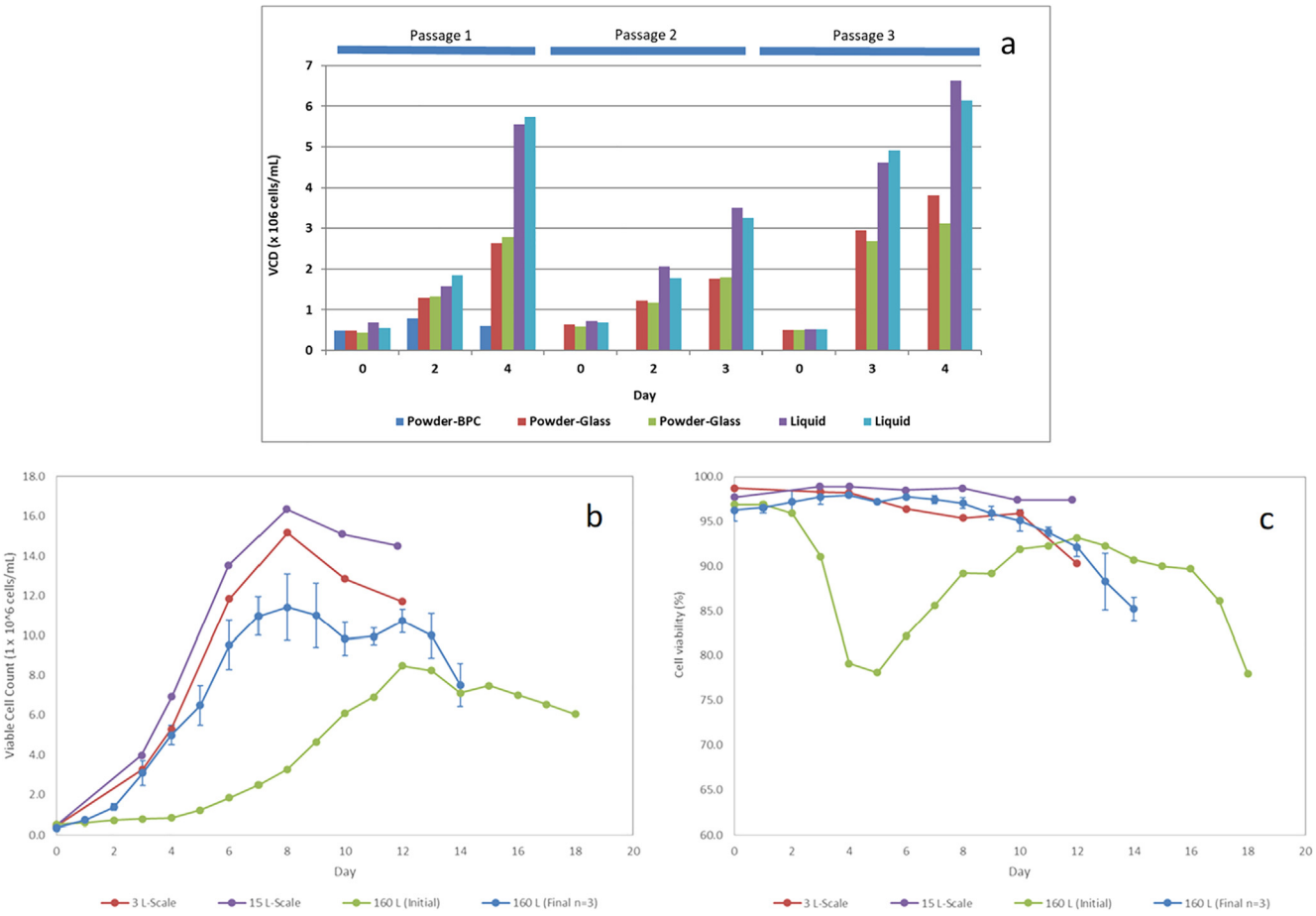

Fig. 8. Effect of raw materials and single-use bags on cell culture process performance. Differences in viable cell count (a) in three shake flask passages using powder-rehydrated basal medium stored in single-use bag, glass bottle, and liquid medium directly procured from the manufacturer. Powder-BPC cultures did not advance into passage 2 due to low cell growth. Viable cell count (b) and cell viability (c) comparison for 3 L, 15 L and 160 L production-scales. Production in 160 L (initial) did not include biocompatibility risk mitigation whereas production in 160 L (Final n = 3) incorporated biocompatibility risk mitigation strategies.

Table 3
Impact of cell culture production process and productivity on aggregate and antigenicity for CH505w100. Antigenicity (data not shown) ranking derived from CD4 binding, CD4 binding relative to ConS gp120 and CD4i upregulation. +++ is highest and + is lowest antigenicity.

Bioreactor duration (days)	Feed Type	C1615 Feed	Cystine/Tyrosine supplement	Titer (g/L)	HMW (%)	Antigenicity
14	ActiCHO	6.5%	Yes	0.68	4.1%	+
12	EfficientFeed	No	No	0.29	0.1%	++
14	EfficientFeed	No	No	0.36	0.0%	+++
14	EfficientFeed	2%	No	0.37	0.2%	++

a series of intermediate, modulator washes were also evaluated across Capto MMC. An intermediate wash containing 0.5 M urea and pH 8.8 was included in the process [11]. This initial baseline process, containing DEAE, achieved a resHCP level of 200–300 ppm.

Further reduction in HCP was evaluated by 1) investigating the impact of production bioreactor duration on resHCP levels in bulk drug substance, 2) implementing modulator washes on CHT and SP Sepharose HP and 3) assessing alternatives for the DEAE flow through step. Based on silver stained SDS-PAGE gels, production bioreactor duration did not dramatically impact the contaminants in the product following four chromatography steps and the day of harvest was not altered. Modulator washes were selected for incorporation on CHT and SP Sepharose HP, although neither had a dramatic effect on HCP reduction. The largest process improvement resulted from replacing the DEAE step with CM Sepharose HP. A head to head comparison between DEAE and either IMAC or CM Sepharose HP showed that CM Sepharose HP provided more resHCP clearance, thus lowering the burden on SP Sepharose HP. With these added optimization efforts, resHCP levels were consistently below 50 ppm.

Product stability was another challenge that was encountered during platform implementation for the follow on Envs. During the platform assessment for CH505w100 low pH viral inactivation was identified as an issue based on the generation of a pre-peak by RP-UPLC and reduced antigenicity by SPR. As a result, low pH viral inactivation was replaced with detergent inactivation using Triton X-100. This platform change was also applied for CH505w78 and CH505w53.

In addition to resHCP levels and Env purity, the antigenicity of each Env was monitored during development. While some platform optimization and process specific conditions were defined based on SPR results for CH505TF, CH5050w78 and CH505w100, antigenicity was essential during the definition of the CH5050w53 process. Following platform implementation and optimization where required, the binding affinity for several important broadly neutralizing antibodies was markedly lower than the

reference material produced transiently in 293 T cells and purified by Lectin affinity chromatography. Through various troubleshooting studies, it was hypothesized that only a subpopulation of the CH505w53 in the BDS sample had the intended binding affinity to CD4 and the intermediate mAbs and that the act of binding and eluting from Capto MMC altered CH505w53 product quality. As such, an alternative capture step, Fractogel SO3-, was developed and implemented. During the implementation of Fractogel SO3-, a BLI based method was developed and implemented (Fig. 4) to support high throughput antigenicity evaluation. Process conditions were defined such that active species were enriched in the capture eluate. The remainder of the downstream process followed the Env platform and the resulting product quality of the BDS was aligned with expectations.

5. Conclusions

In summary, we have demonstrated that it was possible to develop a suite of analytical test methods, a quality product profile and a platform for stable expression and purification for four gp120 Envs. This approach represents a proof of concept for cGMP production of HIV vaccine proteins using a platform strategy, and that expression of gp120 vaccine proteins is feasible in stable CHO cell lines while targeting impurity levels like that of monoclonal antibody products. Finding conditions for growth and expression and impurity clearance along with appropriate analytical methods was critical in process establishment. Once the platform was established for CH505TF, faster and more efficient process development and decisions were enabled. Similar approaches for other candidate molecules should help shorten development timelines preceding clinical trials and is well suited for HIV vaccine development.

Declaration of Competing Interest

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

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2021.05.073.

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