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Impact of next-generation high productivity perfusion cell culture process on host cell protein profile and a comparison with fed-batch cultures.

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Abstract

Fed-batch culture currently represents the typical choice for the production of monoclonal antibodies (mAbs) in the biopharmaceutical industry. However, the implementation of perfusion culture process combined with continuous manufacturing has gained attention due to increased productivity and resource savings. In this paper, we compared the host cell protein (HCP) production and profile of mAb1 between fed-batch and perfusion culture processes. Our work demonstrated differences in HCP production based on the type of cell culture process for the first time. We focused on HCPs that get carried through the purification process and are present in the final drug substance at levels impacting antibody quality and stability. Perfusion process had lower HCP levels and enabled higher clearance of problematic HCPs compared to fed-batch suggesting a viable alternative process. Furthermore, our work demonstrates proof of concept of the impact of cell culture process on specific product quality and help to navigate the process design when we move from traditional fed-batch to next-generation perfusion cell culture.

Impact of Next-generation High Productivity Perfusion Cell Culture Process on Host Cell Protein Profile and a Comparison with Fed-batch Cultures

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Abstract

Fed-batch cell culture processes is widely used in biopharmaceutical industry for monoclonal antibodies (mAbs) manufacturing. As cell culture platforms continue to evolve, perfusion process stand out an attractive alternative to fed-batch process due to the high productivity, manufacturing flexibility and low cost. In this study, the host cell proteins (HCPs) that accumulated extracellularly in fed-batch and perfusion process of the antibody (mAb1) were identified and quantified using liquid chromatography-tandem mass spectrometry (LC-MS/MS), followed by functional analysis. Due to the high viability and low cell concentration, less HCPs were detected in steady state (SS) perfusion process than fed-batch culture and non-steady state (NSS) perfusion. In perfusion process of mAb1, altered distribution of charged variant species may provide

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an opportunity to remove problematic HCPs, including lipoprotein lipase (LPL). The HCP profile studies could provide guidance on selecting the appropriate process to maintain good mAb quality for a therapeutic antibody.

Keyword: perfusion, host cell protein, profiling, downstream process development, polysorbate degradation

Introduction

Chinese hamster ovary (CHO) cells are the most common host organisms for production of recombinant therapeutic proteins, including monoclonal antibodies (mAbs). Fed-batch (FB) cell culture processes using CHO cells have been established as a platform for most mAbs in the biopharmaceutical industry (Zhu, 2012). As cell culture platforms continue to evolve, perfusion process stands out as an attractive next generation alternative to fed-batch process. High demand, competitive landscape and fast-paced biopharmaceutical markets require higher productivity, manufacturing flexibility and reduction in development time (Rodrigues et al., 2010). In order to accept the current market need, continuous downstream manufacturing combined with perfusion upstream process offers great advantage on the intensification of the volumetric productivity with limited investment. In the perfusion process, media is fed on a regular basis and the product containing media is harvested continuously. The continuous product removal and short residence time reduce the variability in product modification and lead higher product heterogeneity. Short residence time adds another advantage for unstable molecules such as enzymes or bispecific antibodies (Karst et al., 2018).

Perfusion cell culture have diverged to two mode of operations, steady state (SS) and non-steady state (NSS) (Bielser et al., 2018). SS perfusion mode maintains constant cell density over a lengthier culture duration. The harvest stream is used to remove excess cells to keep a steady concentration. NSS perfusion mode focuses on a higher intensified cell density, which varies during the culture duration. Therefore, perfusion rate must be adjusted dynamically to meet the cellular demand and allow cells to grow at the maximum rate (Karst et al., 2017). Changing the upstream processes can impact the host cell protein (HCP) profile and the relative abundance of particular proteins in harvest cell culture fluid (HCCF), as well throughout the downstream purification (Hogwood et al., 2013; Park et al., 2017). Despite great research interest in HCPs, there are no published investigations to compare perfusion and fed-batch processes for differences in HCP production and profiles, as well as the two modes of perfusion cell culture process.

In this study, we collected HCCF from various bioreactor cultures (FB, SS perfusion, NSS perfusion) at different growth phases of a mAb1-producing CHO DG44 cell line. A complete analysis of HCPs will establish the early proof of concept to understand the differences in HCP production based on differences in cell culture processes. We utilized liquid chromatography-mass spectrometry (LC-MS) based proteomics throughout the study, which have recently shown great promise for HCP characterization (Bracewell et al., 2015; Walker et al., 2017). Discovery-based proteomics by data-dependent acquisition (DDA) is a powerful technique for unbiased identification of proteins in a sample. However, this method is not ideal for the consistent detection of low abundance proteins. HCPs co-exist with highly concentrated mAb product (dynamic range of concentrations [?] 5 orders) in the HCCF and purification intermediates, which puts a great challenge for detection. Here, to meet our needs, we developed a parallel reaction monitoring (PRM) approach for the target proteins of interest. PRM uses targeted tandem MS to simultaneously monitor product ions of a targeted peptide with high mass resolution and accuracy (Kreimer et al., 2017).

In our HCP profiling studies, we focused on the comparison of one particular group of HCPs that are known to affect product stability. Particle formation in the polysorbate (PS)-formulated biotherapeutics that has become a major quality concern and potential risk factor in the industry. Recent published data and our own investigation have provided clues for the leading cause of PS degradation by HCPs remaining in drug product. Low levels of residual HCPs that have lipase activity, such as lipoprotein lipase (LPL) (Chiu et al., 2017), hydrolyze polysorbates cleaving them into fatty acids. The accumulation of free fatty acids ultimately precipitates to form particles upon long or in some cases short-term storage.

Particle formation in drug substances was observed in mAb1 and this molecule was used in this study. Source materials were generated by three different modes of cell culture processes, subsequently the enzymes that

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potentially can hydrolyze polysorbates were identified, quantified and compared. The aim of this body of work was to establish proof of concept and methodology to compare FB and perfusion cell culture processes. Perfusion process is shown to be a viable alternative to utilize on the problematic molecules, such as mAb1 that have product quality issues when cultured in traditional FB mode.

Materials and Method

Cell line and cell culture

A suspension-adapted recombinant CHO-DG44 cell line expressing mAb1 in the dihydrofolate (dhfr) selection system was cultivated in FB, SS perfusion and NSS perfusion in Boehringer Ingelheim's proprietary in-house media. Fed-batch material was derived from a 12-kL stainless steel bioreactor inoculated at a seeding density of 3.0 x 10^5 cells/ml. Dissolved oxygen (DO) was maintained at 60% and the temperature and agitation were 36.8°C and 29 rpm, respectively. Nutrient feed media was continuously fed at 10% cell culture volume starting from day 3 and repeated every 48 hours with a targeted final glucose concentration at 4 g/L. Culture supernatant collected from the bioreactor was centrifuged at 5000 rpm for 55 L/min and stored at -80°C pending further analysis.

Perfusion cultures (SS, NSS) were established with a 2 L working volume and subsequently seeded at 12.4 x 10^6 cells/ml and 12.1 x 10^6 cells/ml, respectively. In-house perfusion growth medium (1x concentration) started perfusing immediately following inoculation on day 0 at the rate of 1 vessel volume per day (1 vvd) and increased gradually to 2 vvd on day 2. The SS reactor maintained a constant 2 vvd perfusion rate from day 3-14 using a combination of three concentrated feeds and diluent, the respective rates thereof adjusted accordingly to maintain a residual culture osmolality of 310 ± 15 mOsm. A culture bleed controlled by an Incyte permittivity probe (Hamilton, Reno, NV) was used to maintain the viable cell density (VCD) target of 40 x 10^6 cells/ml. The NSS reactor did not utilize a cell bleed; cell culture proliferated to the maximum VCD attainable by the system (feed, DO control, etc.). The addition rate of the three concentrated feeds remained constant at a total of 0.5 vvd; the addition rate of the diluent varied accordingly to maintain a residual culture osmolality target of 330 ± 50 mOsm. Permeate was harvest by 0.2 μM hollow fiber filtration throughout culture.

Viable cell concentration and mAb assay

Total cell counts and cell viability were measured daily by Trypan Blue exclusion using a Vi-CELL cell counter (Beckman Coulter Life Sciences, Indianapolis, IN). Daily permeate samples were collected for titer and metabolite analysis on the Cedex BioHT (Roche Diagnostics GmbH, Mannheim, Germany).

Purification of mAb1

Laboratory scale chromatographic separations were performed using an ÄKTA explorer chromatographic system (GE Healthcare, Uppsala, Sweden). mAb1 is an IgG1 antibody with *isoelectric point (* pI) value of 8.5. The antibody was captured from the HCCF by recombinant ProA chromatography using MabSelect Sure (GE Healthcare, Uppsala, Sweden).

Host cell protein quantification by ELISA

HCPs were quantitatively determined by Gyrolab xP Workstation using Gyrolab CHO-HCP E3G kit (Gyros Protein Technologies, Uppsala, Sweden). The Gyrolab xP workstation is an automated analytical platform based on microfluidic technology (Heo et al., 2014; Mora et al., 2010).

HCP profile analysis using mass spectrometry

Protein (1.5 mg) from HCCF and ProA pool was diluted in 50 mM ammonium bicarbonate (pH 8.0) to 1 mg/ml final concentration. Trypsin was added into the solution at the ratio by weight of 50:1 (substrate to trypsin), and incubated overnight at 37°C. After adding 7.5 mM dithiothreitol, the digested samples were boiled at 90°C for 10 min. They were then cooled down rapidly and precipitates were removed by

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centrifugation at 16000 x g for 5 min. The remaining supernatants were applied to Pierce Peptide desalting spin columns (Thermo Scientific, Rockford, USA).

Digested peptides were separated using a ACQUITY UPLC CSH C18 column (130 A 1.7 μm, 2.1 x 150 mm) on an ACQUITY Arc high-pressure liquid chromatography (HPLC) (Waters, Milford, USA). The column was held at 60°C. A 200-min gradient from 98/2 to 55/45 water/acetonitrile (0.1% formic acid added) was used with a flow rate of 0.3 mL/min. The column eluate was analyzed on a Thermo Q Exactive Plus mass spectrometer (Thermo Scientific, Sunnyvale, CA) using DDA mode where the top 20 most abundant peptide ions were subjected to MS/MS analysis (dynamic exclusion 60 second, resolution 17500) after a survey scan (m/z range: 300-2000, resolution: 70000). Data analysis was performed by Proteome Discoverer 2.2 (Thermo Scientific, Sunnyvale, CA) using SEQUEST algorithm against CHO K1 proteome database (GCF_000223135.1 CriGri_1.0 from www.ncbi.nlm.nih.gov) by adding decoys and common contaminants. The XCorr confidence thresholds were set as 1.2, 1.9, 2.3, and 2.6 for charge state z = 1, 2, 3 and z [?] 4 individually. The delta C_n was set as 0.05, and the target false discovery rate (FDR) was set as 0.01. The searched results were further filtered by criteria such as unique pep # [?] 2 and FDR Confidence=High. The semi quantitative label-free quantification was performed using MS peptide signals where the Minora algorithm performed untargeted feature detection.

Three lipases targeted using MS

Three lipases were selected as HCP targets for PRM analysis. They are lipoprotein lipase (LPL), putative phospholipase B-like 2 (PLBL-2), and lysosomal phospholipase A2 isomer X1 (LPLA2). For each lipase, at least three unique tryptic peptide sequences were selected for analysis. The PRM LC-MS instrument setting was similar to the DDA analysis, but the LC gradient was shortened to 80 min. The MS2 signal responses were analyzed by Skyline 4.1 software (MacCoss Lab, University of Washington) where four fragment ions were extracted for each peptide. The PRM quantification was based on the signal intensities comparison to the standard lipase proteins.

GO and pathway analysis

Gene enrichment and functional annotation analyses were performed using the DAVID gene functional classification tool (http://david.abcc.ncifcrf.gov.home.jsp) to elucidate the biological functions of identified proteins (Huang da et al., 2009a, 2009b).

Fluorescence micelle assay (FMA)

FMA was used to determine the concentration of PS in the samples. The FMA reagent contains 0.15 M sodium chloride, 0.05 M Tris, 5% ACN, 5 μM *N* -phenyl-1-naphtylamine and 15 ppm (parts per million) Brij35 with a pH 8.0. 10 μl of sample was mixed with 240 μl of FMA reagent in a 96 well plate (Greiner Bio-One, Monroe, USA) and shaken for 60 sec at 35°C. The fluorescence detector, Tecan Spark (Tecan, Mannedorf, Switzerland) was set to an excitation wavelength of 350 nm and an emission wavelength of 420 nm. For quantitation, a calibration curve ranging from 0.1mg/ml to 0.6 mg/ml PS was used as standard.

Lipase activity assay

Lipase activity assay buffer was prepared with sodium phosphate, sodium chloride, 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate hydrate (CHAPS) and 4-methylumbelliferyl decanoate (MUD4) at pH 7.4. After the mixing, the plate was analyzed in the fluorescence detector, Tecan Spark. The maximum excitation/emission wavelengths were determined to be 340 nm and 450 nm. The kinetic measurement was reported over 1.5 hours with 45 seconds interval. The slope of fluorescence over time is directly proportional to the hydrolytic activity of the sample used within the linear measuring range. The hydrolytic activity allows determination of lipase content in the sample.

Results and Discussion

Cell culture performance

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The current industry standards for the production of stable mAbs is a fed-batch process in stirred tank bioreactors. Perfusion methods offer not only process advantages but also possibly low HCPs released into the condition media due to high viability and less apoptosis in the culture. Three culture processes, FB, SS perfusion and NSS perfusion, have been compared from same host cell line, producing the same recombinant product, mAb1. FB culture was harvested 11 days after the production bioreactor was inoculated and perfusion supernatants were sampled daily.

Figure 1 shows the profiles of cell growth, viability and mAb1 production in culture supernatants during FB, SS and NSS perfusion cultures. In FB cultures, a maximum viable cell concentration (MVCC) of 15.6 X 10^6 cells/ml was achieved on day 7 (Fig1a). In NSS perfusion, the trend for MVCC was very similar to fed-batch but reached around 221.7 X 10^6 cells/ml on day 7, which was 14 times more cell density than FB culture. In SS perfusion, the viable cell density was maintained at 40 X 10^6cells/ml throughout the culture days with ˜95% viability (Fig1b). Fed-batch viability was reduced to ˜25% and NSS perfusion viability was also reduced to ˜37% on day 11. Perfusion cultures were intended to have high productivity therefore average productivity is 5.7 times greater in SS and 18.5 times greater in NSS compared from FB (Fig1c).

Identification and quantification of HCPs by LC-MS

Total HCPs measurements of HCCF from different modes of cell culture processes, FB, SS and NSS are listed in Table 1. Samples from both unpurified (i.e. HCCF) and Protein A purified materials were analyzed. For the perfusion systems, HCP counts across the harvest duration provides insights to the HCP profile changes from early to late growth stage.

During the early stages of cell culture growth, there is a significant lag between central metabolism and post-translational modification. A strategy was developed to exclude harvest product from the early days (1-5 days) of production to ensure stable product quality. Hence, the mAb1 quality and composition of HCPs were less varied depending on culture days but maintained significant changes with the different culture processes. The level of HCPs in HCCF were greater than ˜10^5 ppm for FB and NSS perfusion but approximately 10^4 ppm for SS perfusion. Low cell viability for both FB and NSS perfusion culture processes can mean high levels of HCPs released in the feed stream due to cell lysis.

General patterns of HCP distribution was visualized in 2D-PAGE shown Figure 2. The loading of the gel is normalized by the amount of product. Different culture processes show a comparable spot pattern and intensity with mAb1 spots. For HCP comparison, SS shows less overall HCP population. Day 5 perfusion product also shows less HCPs detected compared to perfusion day 14. Interestingly, NSS perfusion demonstrates additional HCPs in the HMW area (across pI range) while the FB shows additional HCPs across the MW and PI ranges. There are several HCP species (spots) and abundance (spot volume) varied among the culture processes. These minor variations in HCP composition and abundance between different processes suggest influence of cultivation process on HCP in cell culture supernatants.

ELISA and 2D-PAGE have limited capacity to detect HCPs that are in low abundance or poorly immunogenic. Orthogonal detection and monitoring technique using MS offers the opportunity not only to arrive at an accurate quantification of total HCPs but also provides the ability to identify the individual protein species with high sensitivity (Levy et al., 2014). Samples were analyzed by high-resolution LC-MS and HCPs were identified by SEQUEST search against the CHO K1 proteome database (GCF 000223135.1 CriGri 1.0 from www.ncbi.nlm.nih.gov) through Thermo Scientific Proteome Discoverer v2.2. Table 1 summarizes the group number of HCPs in HCCF in the cultures with at least 2 identified unique peptides. A total of 1310 HCPs were identified in FB culture supernatants. Perfusion SS contained fewer detected HCPs (˜300) and NSS perfusion shows comparable numbers of HCPs (˜1700) as FB that were likely due to the high cell concentration. Approximately 83% of fed -batch, 70% NSS perfusion, 92% SS perfusion were commonly identified with other culture processes using. HCP composition was significantly more diverse in the FB and NSS perfusion culture than in the SS perfusion culture. These data confirm similar trends in HCP population using ELISA.

The HCCF from the three types of cultures were later purified by ProA chromatography and analyzed

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for HCP population. As indicated in Table 1, results from both MS and ELISA method show drastic decrease on the HCP level for both perfusions, but HCP levels remain relatively high for FB. This result indicates that it might be easy to remove host cell protein from perfusion process by ProA column. To investigate any significant differences in product quality from FB, SS and NSS and their potential impacts on methodologies used in the study, product quality indicators such as aggregation, fragmentation, N-linked glycosylation patterns and charge variants were evaluated after ProA purification (Supplementary Table 1). Both aggregation and fragmentation were slightly reduced in perfusion cultures compared to fed-batch. N-glycosylation showed similar pattern for both types of culture. Perfusion NSS has been observed a simpler glycosylation (G0F) increased as the runs progress and cell densities go up due to the decrease in nutrient availability in all culture processes. At the same time, high mannose (Man5) of NSS perfusion increased and a similar correlation has also been reported in other publications, which indicate that this could be a general phenomenon (Pacis et al., 2011).

The charged variants analysis of mAb1 shows that FB has 24.4% acidic peaks, and 8.4% basic peaks (Figure 3). With perfusion processes, the abundance acidic species is significantly reduced to 13.6% for SS and 15.8% for NSS while more basic forms are produced to 14.6% for SS and 13.4% for NSS compared to FB. Other mAbs also exhibited similar trends in charged variants with perfusion processes due to the increase C-terminal lysine and/or less light chain N-terminal glutamine cyclization (Gomez et al., 2020; Walther et al., 2019). The general consensus according to a number of scientific publications and various conventions is that C-terminal lysine and N-terminal glutamine cyclization, are likely non-CQAs (critical quality attributes), which may not have substantial effects on antigen binding affinity, efficacy and safety of the antibody product (Du et al., 2012; Sing h et al., 2016). However, charged variants can alter the composition of HCP impurity profile if HCPs are binding to mAbs.

The relatively minor charge differences have been shown to have a large impact on the total HCP association due to the single abundant charge species that bind substantially more strongly to HCPs (Levy et al., 2014). For ProA chromatography, post load wash is a critical step to reduce HCPs. In the mAb1 purification process, neutral pH (pH 7.4) was employed for the capture and wash step. Since HCPs tend to have lower isoelectric points (pI) (Lavoie et al., 2019), the majority of HCPs are anionic whereas mAb1 (pI 8.5) is cationic at the neutral pH. Thus, strong hydrophobic and electrostatic interactions can form between mAb1 and HCPs. Based on our proteomic analysis after ProA purification, FB culture contains HCPs that have theoretical pI below neutral pH, pI $<$ 6 [?] 50%, pI $<$ 7 [?] 74%, and pI $<$ 8 [?] 82%. This result showed that these low isoelectric points tend to bind with acidic species of mAb1 in FB culture. Our work brings up the awareness of potential differences of charged variants between fed-batch and perfusion and the needs for further characterization of the product, as well as HCPs. In general, the differences we have observed in all product qualities tested, *i.e.* aggregates, N-glycan, charged variants, are not expected to have a noticeable impact on the methodologies used in this work.

Comparison of HCP distribution and Cluster analysis of quantified HCP

The relative fold changes of quantified HCPs were calculated by comparing the protein abundance between the perfusion processes to fed-batch and visualized through a heat map shown in Figure 4. Each band represents one HCP species as identified by mass-spec based proteomics. Blue corresponds to fold decrease and red to fold increase in abundance comparing FB to perfusion processes. HCPs were further classified in three clusters according to their concentration profiles during the cultures. Cluster 1 and cluster 3 represents HCPs that have a large fold increase and decrease (>2.5 fold) respectively from the fed-batch cultures. Cluster 2 contains HCPs that show inconsistent fold changes throughout the culture days. Although there were many HCPs that did not show large fold changes from FB to perfusion processes, distinct red and blue represent the HCPs that show a significant increase and decrease in protein abundance regarding the specific culture processes. SS perfusion culture shows large portions of HCPs with reduced abundances. Depending on the manufacturing processes, these prevalent differences in HCP population suggest to develop "process-specific" HCP assays. Commercially available ELISA kits, typically used the reagents derived from FB, can be limited in their utility by poor sensitivity and coverage.

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A cutoff of 2.5 fold was used for the absolute fold changes based on the %CV of the dataset. HCPs that have more than 2.5 fold changes were accounted for functional annotation and biological interpretation of proteomics data. To understand the biological roles of the identified HCPs, GO analysis was performed based on relative quantification from fed-batch culture using DAVID (Supplementary figure 1). Most upregulated HCPs in cluster 1 (Figure 4) were associated with transcription (spliceosome) and translation (RNA transport, ribosome, lysosome). Increased level of transcription and translation correlate to increase in the final titer produced in CHO cells. For example, transcription factor BTF3, cellular nucleic acid binding protein (CNBP) and 60s ribosomal protein L36a (RPL36A) were highly upregulated (more than 10 fold) in both perfusion processes. In perfusion culture (cluster 3), HCPs that associated with metabolic pathway were downregulated with a response to changes in concentrations of key metabolites in the cell. NSS perfusion shows downregulation of phagosome and proteasome, which are responses found in starved and growth arrested cells due to the high production of mAb (Vergara et al., 2018). A recent study reported that the primary cellular function of HCPs is an important factor to be considered when preparing feedstocks for ProA purification (Lintern et al., 2016). Cells with a high productivity such as perfusion process expected to produce more mAb per unit of O_2 and nutrient consumed (Pan et al., 2017).

Cellular component analysis shows that compared to the fed-batch, proteins located in both the cytoplasm and nucleus are greatly impacted in both perfusion cultures (Supplementary figure 1). For SS perfusion, the percentage of HCPs associated with the extracellular region, extracellular space was significantly higher due to the high viability. However, in the NSS perfusion, HCPs that are associated with cytosol and nucleoplasm emerged as the most impacted proteins. This result suggests that HCPs in the culture supernatants in SS perfusion culture process include a high percentage of extracellular proteins secreted from viable cells, whereas HCPs in NSS perfusion culture process involve a significant portion of intracellular proteins resulting from cell lysis. Those intracellular HCPs included proteins that were not naturally secreted and could only be found if cells lysed. HCPs that are associated with membrane proteins are upregulated in the early days but downregulated in later days of the culture. In earlier days, a previous study suggested that membrane proteins are secreted by exocytosis after being sorted in the trans-Golgi network into transport vehicles(Le Borgne and Hoflack, 1998). In our case, we believe in both perfusion processes, the active secretion is decreased due to cell lysis as the number of culture days increase. Furthermore, the concentration of lactate dehydrogenase (LDH), which is most widely used to quantify the degree of cell lysis, increased toward the end of the cultures. For the NSS, LDH level is significantly increased that demonstrates shear sensitivity on cell. At the end of the culture, LDH level was $1.1x10^5$ pg/cell/day for NSS and $3.6x10^3$ pg/cell/day for SS. Naturally secreted HCPs include proteins present in extracellular matrix and proteins excreted through exosomes. Overall, the numbers of secreted proteins were upregulated in SS perfusion process while intracellular HCPs are the main contributor to the HCP compositions of NSS perfusion process.

Problematic HCP

The leading cause of particle formation following enzymatic degradation pathway is from a group of host cell proteins, which exhibit polysorbate degradation activity. Lipoprotein lipase (LPL) hydrolyze ester bonds within triglycerides to form alcohols and fatty acid molecules. Knockout mutants of LPL reduce polysorbate degradation by 50% (Chiu et al., 2017). In the development of mAb1, we observed elevated sub visible and visible particles after long term storage at 2-8°C and also showed high PS20 degradation over 4 weeks storage at ambient temperature. LPL was detected in the bulk drug substance in parts per billion (ppb) levels (data not shown). In our proteomic studies, LPL, was found in all three culture processes (FB, SS, NSS). After the ProA purification, LPL was only detected in the fed-batch process, suggesting fed-batch culture process may be harboring higher LPL levels (Table 2).

The DDA method was not adequately sensitive to detect the lipases in purified samples, such as ProA pool. Instead, the PRM method was used to further detect low ppm levels of lipases in mAb1 process sample. The data-acquisition method for PRM contains a list of peptides, which are exclusively present only in proteins that are of interest to the researcher, and their elution time window. After the data is acquired and analyzed, the information on the abundance of peptides observed by PRM is then used to estimate the

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abundance level of their corresponding proteins. PS degradation was also reported during the long-term storage of CHO-derived purified mAbs with detectable quantities of endogenous lysosomal phospholipase A2 isomer X1 (LPLA2) (Hall et al., 2016) and PLBL2 (Dixit et al., 2016). We used PRM method for targeted quantitative analysis of peptide pairs to identify LPL, LPLA2 and PLBL2 (Table 2). Compared from the FB process, both perfusion processes shows less overall lipases and later removed or greatly reduced the quantity after the ProA purification.

Peroxiredoxin 6 (Prdx6) is the uniquely 1-Cys member of the peroxiredoxin family with calcium-independent phospholipase A2 activities (Shanshan et al., 2017). Phospholipases such as LPLA2, Prdx6 and phospholipase A-2 activating protein (PLAA) were detected in fed-batch and NSS perfusion processes but not in SS perfusion process. LPLA2, Prdx6 and PLAA were also detected in fed-batch intermediate purification pool after ProA purification but not in the both perfusion processes.

For long-term storage of mAbs formulated with PS, difficult to remove lipases that were identified need to be further cleared during the downstream purification. In order to evaluate the mAb1 product stability, PS was analyzed using two different methods after 4 weeks of storage at 25°C. Fluorescence micelle assay (FMA) was utilized for PS quantification and 4-methylumbelliferyl decanoate (4-Mud) assay for lipase activity. Table 2 summarized the PS quantification, lipase activity and its relative abundance in different processes samples. In HCCF, PS degraded from 400 ppm at day 0 to approximately 20 ppm after 4 weeks of incubation. This result was expected due to the high HCP concentration in the cell culture fluid. After ProA purification, PS content was measured to above 200 ppm for both perfusion processes but less than 100 ppm for the fed-batch process. Lipase activity also shows great differences between perfusion and fed-batch processes after the ProA purification. Combining all the data collected in this study lead to the fact that fed-batch samples contained more lipases involved in polysorbate degradation and particle formation compared to both perfusion processes. These lipases were not removed by chromatography methods.

Conclusion

In conclusion, clear differences in HCP profile were observed between steady state and non-steady state cultures, as well perfusion and fed-batch culture. Fewer HCPs were detected in SS perfusion process. This is likely due to the high viability and lower cell counts in steady state. Even though the level of HCPs in the final drug product would be very low (1-100 ppm), those trace amounts of difficult or problematic HCPs may effect on the quality attributes of mAb1. To characterize the HCPs potentially affecting mAb quality, the detailed analysis of HCPs in the culture supernatant of culture processing of mAb1 producing CHO cells was performed in this study.

HCPs that were present in HCCF from fed-batch and perfusion processes of mAb1 were identified and quantified using LC-MS approaches including lipases that cause mAb1 stability. Different profiles and levels of lipases were detected in the different cell culture processes. In perfusion process of mAb1, altered distribution of charged variant species may provide an opportunity to clear the lipases by downstream process. Our work demonstrates that future bioprocessing can be shaped by a deep understanding of the impact of cell culture process on specific product quality when we move from traditional fed-batch to next generation high productivity perfusion cell culture. A process specific ELISA assay is likely needed for perfusion process. Combining advanced downstream and analytical techniques in addition to tailored approaches to perfusion cultures can offer greater control over product quality, stability and ultimately the safety of biotherapeutics.

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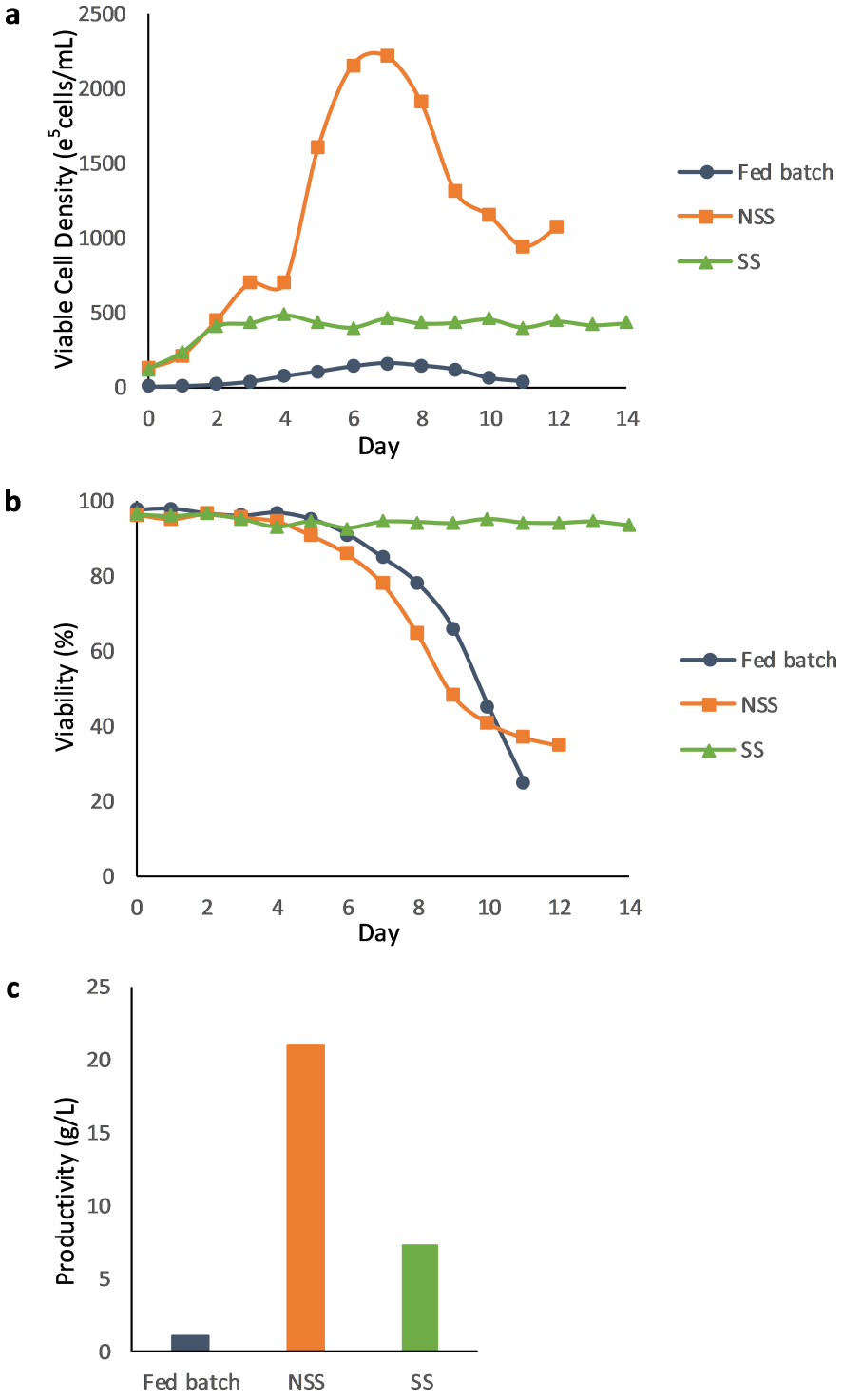
Figure 1. Profiles of (a) viable cell density (b) viability, and (c) mAb1 productivity during fed-batch and perfusion processes.

Figure 2. 2D-gel image for HCP profile comparisons between culture processes from mAb1-producing CHO HCCF. (a) fed-batch culture process on day 11 (b) SS perfusion culture process on day 5 (c) SS perfusion culture process on day 14 (c) NSS perfusion culture process on day 5 (d) NSS perfusion culture process on day 14

Figure 3. Profiles of charge variation of mAb1 during fed-batch (day11), SS perfusion (day12) and NSS perfusion (day12).

Figure 4. Heatmap showing the fold changes in the relative abundance of HCPs. The y-axis reflects different individual proteins and the x-axis represents the fold change in relative abundance of perfusion processes

respect from fed-batch process. The color scale illustrates the relative abundance level of each protein; red and blue indicate high and low abundance compared to the fed-batch culture process, respectively. The color intensity indicates the degree of fold increase or decrease.



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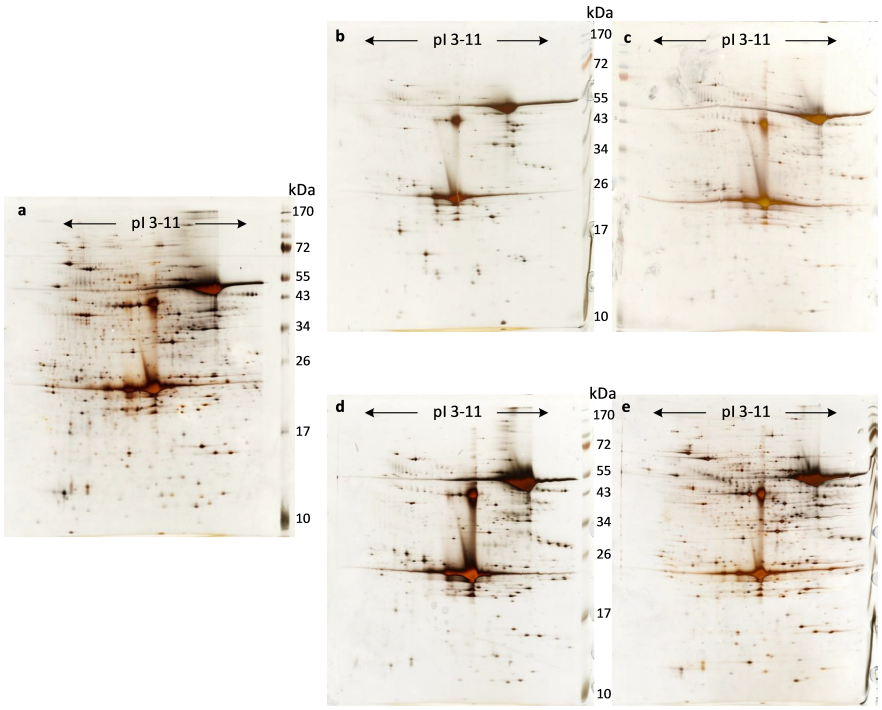

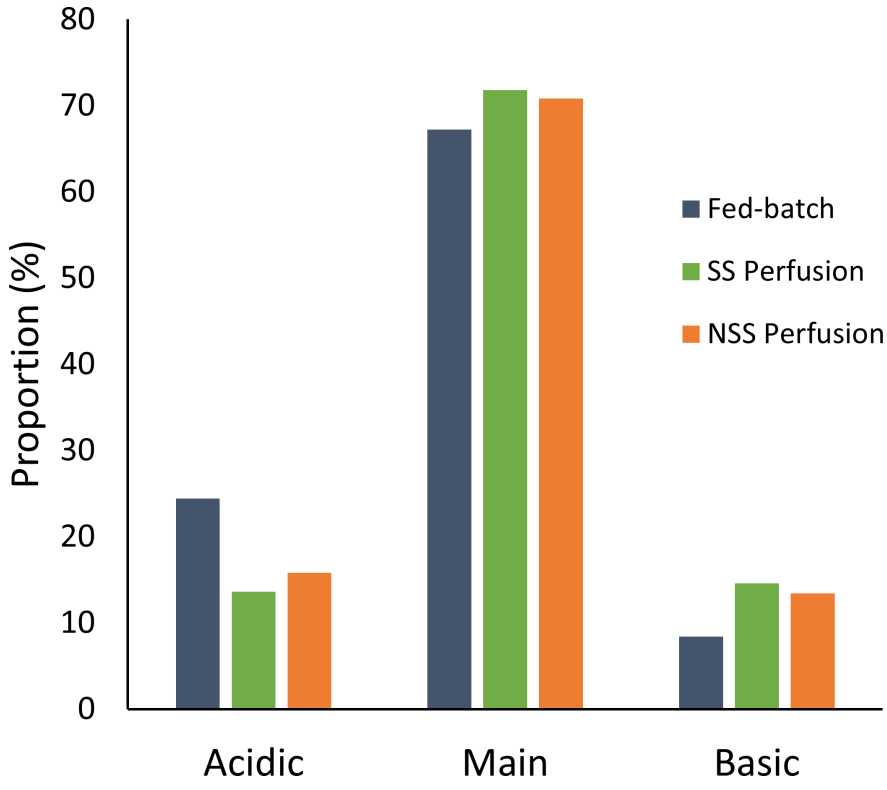

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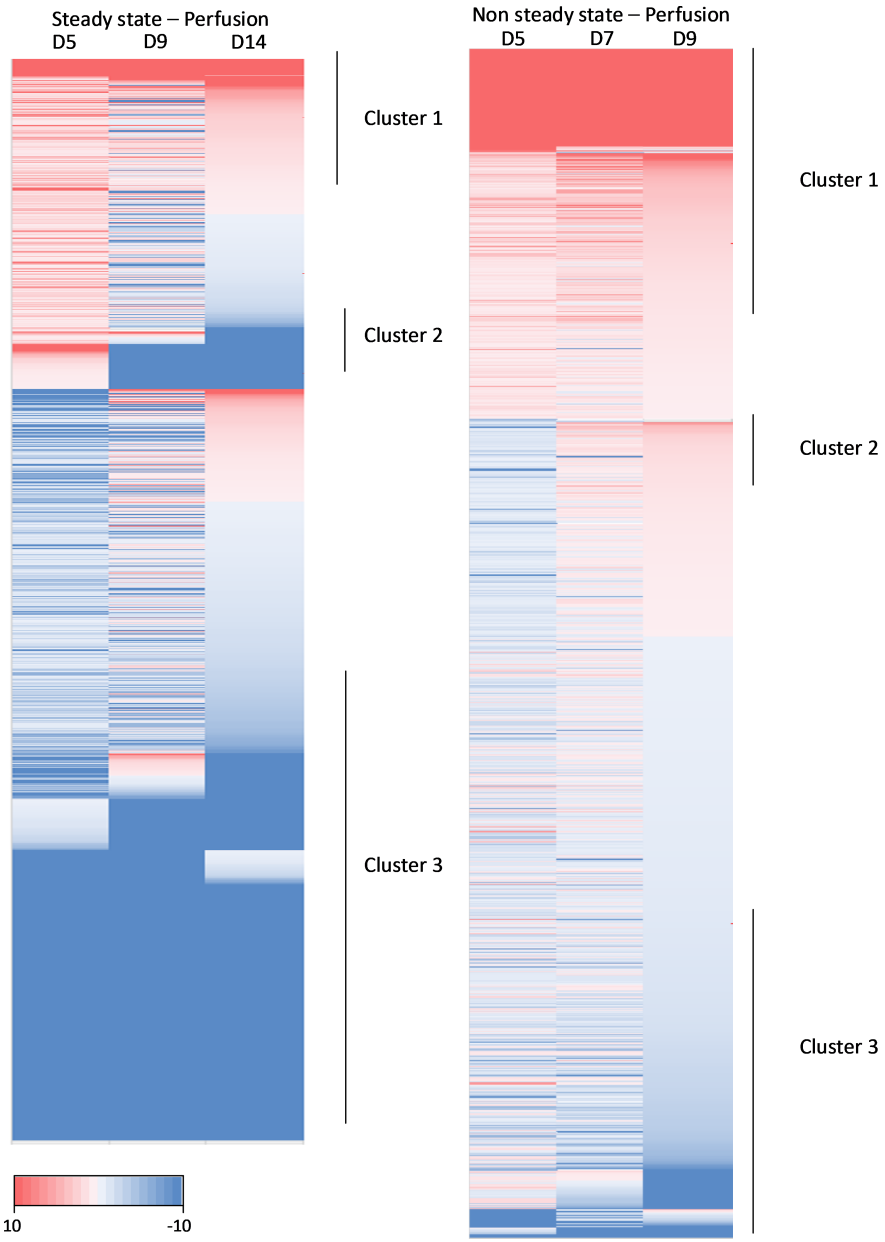

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