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Applications of proteomic methods for CHO host cell protein characterization in biopharmaceutical manufacturing

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Chinese hamster ovary (CHO) cells are the most prevalent host organism for production of recombinant therapeutic proteins, including monoclonal antibodies (mAbs). Regulatory guidance mandates control of the host cell protein (HCP) concentration in the production process, which remains a primary challenge. Although HCP concentrations are typically measured by ELISA, orthogonal proteomic methods are gaining popularity for identification and quantitation of individual HCP species. Recent applications of proteomic techniques to characterize extracellular CHO HCPs include those that have explored the effects of upstream factors (cell line, viability, process conditions), characterized specific HCPs likely to co-purify by mAb interactions, identified HCPs likely to impact drug product quality, and enabled strategies to limit HCP expression (media composition, temperature shift, genetic modification) and maximize clearance (polishing chromatography, wash additives).

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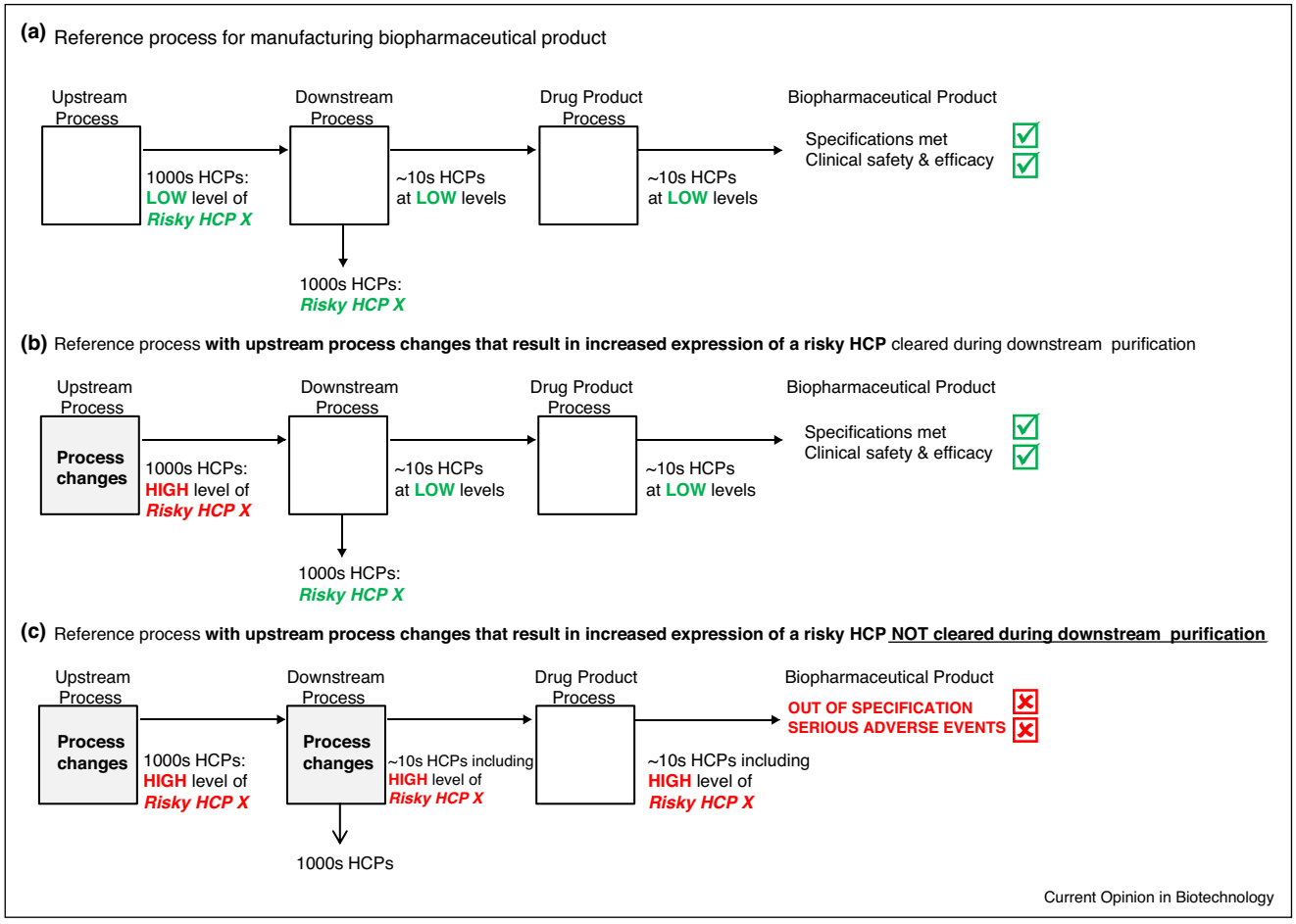
Introduction

Biopharmaceutical manufacturing includes that of therapeutic proteins, of which many are monoclonal antibodies (mAbs), which are typically expressed in Chinese hamster ovary (CHO) cells and secreted into the extracellular medium. Extracellular host cell proteins (HCPs) comprise a heterogeneous mixture of secreted proteins and intracellular proteins released during cell lysis. These HCPs exhibit a range of physicochemical properties and can present clearance challenges during downstream processing. ICH guideline Q6B, the primary reference for biopharmaceutical product specifications, states that HCP levels should be minimized and well controlled but does not provide exact limits [1]; however, less than 100 ppm is a target commonly used across the industry, with lower levels generally preferred for commercial processes. Of the thousands of extracellular HCPs, a small subset pose specific challenges, including: (1) immunogenicity risk [2], (2) activity *in vivo*, which has been reported at concentrations as low as 20 ppm [3], and (3) impact on product quality [4••,5–9].

Enzyme-linked immunosorbent assays (ELISAs) are typically used for in-process and release testing for HCPs. ELISAs provide quantitative measurement of total HCP levels but have several limitations [10], such as preferential detection of highly immunogenic HCPs and dilution-dependent non-linearity of some species. Orthogonal proteomic methods enable identification and quantitation of individual HCP species, with several merits and limitations [11,12]. Additional information regarding different analytical methods used for evaluation of HCP species is available in review articles that address the use of ELISAs [10,11], two-dimensional electrophoresis [11], and mass spectrometry-based methods [11,12]. Recent publications propose application of these proteomic methods for risk-based approaches to HCP content management by considering factors such as HCP identity, experience with the product, and level/route of patient exposure [13], with impact determined by severity, detectability, and abundance [14].

As the composition and concentration of HCPs in a given drug product may be influenced by upstream and downstream processing conditions, process changes or manufacturing drift may impact the HCP profile; examples of upstream conditions that may affect generation of HCP species are discussed in the next section. Figure 1 illustrates the theoretical impact on a biopharmaceutical drug product if upstream and downstream process changes resulted in increased production and co-purification of a generic high-risk HCP. In this review, we

Figure 1



Hypothetical illustration of how modifications to **(a)** a reference manufacturing process can negatively impact product quality by changing the HCP profile via **(b)** upstream process changes and **(c)** downstream process changes.

highlight recent applications of proteomic methods to characterize extracellular CHO HCPs across various upstream processing conditions and identify specific HCPs posing downstream purification challenges through strong interactions with mAbs, especially those HCPs impacting drug product stability. Strategies to limit upstream HCP expression and maximize the effectiveness of downstream purification are also discussed.

Upstream

Sequencing of the CHO-K1 [15] and Chinese hamster [16] genomes has enabled identification of the majority of the extracellular CHO proteome in clarified cell culture fluid. Most recently, around 3000 extracellular proteins have been identified and eight different bioinformatics tools have been applied in the course of determining approximately 1000 proteins that are likely secreted by classical and non-classical pathways [17•]. Isobaric labeling [18,19], spectral counting [20••] and normalized spectral abundance factor (NSAF) [17•,21–23] have enabled relative quantification of extracellular HCPs in culture supernatants, while MS^E (a tandem mass spectrometry technique alternating high and low collision energy) has been applied for absolute quantification [24,25]. Recent efforts have focused on characterization of extracellular proteome changes for different cell lines, culture viability and processing conditions, with relevant studies summarized in Table 1.

Proteome similarity across cell lines enables translatable learnings across different products and laboratories. The demonstration in 2006 that the intracellular proteome from CHO cell lysates was similar across three CHO-K1 DUKX-B11 derived null cell lines (FR4, DP12, DP7) [26] has been complemented by recent findings from the same group. This work demonstrated that extracellular HCP profiles of three strains of null cells derived from different lineage and grown under different upstream conditions were similar [20••]. Each cell line was found to generate approximately 1400 extracellular HCPs, with

Table 1

Summary of recent upstream proteomic studies of differential protein expression and identification. Abbreviations represent two-dimensional electrophoresis (2DE), liquid chromatography (LC) and mass spectrometry (MS)

	CHO cell line	Product	Culture	Detection method	Study explores differences across		
					Cell line	Viability	Conditions
[18]	K1	Null	Shake-flask	2DE, LC–MS/MS			Cell age
[20••]	DUKX, K1	Null	Fed-batch	LC–MS/MS, ELISA	X	X	Varied[a]
[21]	GS	Rituximab	Batch, fed-batch	LC–MS/MS		X	Batch versus fed-batch
[22]	DG, DUKX	Fc-fusion protein	Batch, fed-batch	LC–MS/MS	X	X	Batch versus fed-batch
[23]	3 proprietary	2 proprietary mAbs	Proprietary, varied	LC–MS/MS, ELISA	X	X	Varied[b]
[24]	2 proprietary	2 proprietary IgG1s	Proprietary, varied	2D-LC–MS^E, ELISA	X		
[25]	DP-12	Anti-interleukin 8 IgG1	Shake-flask	2D-LC–MS^E, ELISA		X	
[27•]	8 GS	IgG4	Shake-flask	2DE, ELISA	X		
[29•]	DG44	IgG1	Shake-flask	2DE, ELISA			Media composition
[30]	GS	Chimeric IgG4	Fed-batch	LC–MS/MS, ELISA			Temperature shift

[a] Variable conditions include media composition, nutrient feed and temperature.
[b] Variable conditions range from minor changes in media composition to substantial modifications of upstream and downstream process.

80% of the top 1000 proteins identified common to all three cell lines. Despite qualitative similarities, individual HCP concentrations varied, with phospholipase B-like 2 (PLBL2) changing nearly 5-fold across the cell lines. Similar results have been reported by others [22,23,27•]. For example, although the identities of 92% of the 100 most abundant HCPs were found to be conserved across two different cell lines, many HCPs exhibited greater than a 10-fold difference in abundance [24]. Therefore, similarity in qualitative HCP composition enhances data utility but quantitative changes maintain cell line as an impactful upstream parameter.

Recent proteomic studies have shown that cell culture viability may impact the composition of HCPs in the clarified cell culture fluid through the introduction of intracellular proteins resulting from cell lysis during both batch and fed-batch culture [21,22]. For example, different HCPs were detected in Protein A eluate generated from early-stage and late-stage batch culture, with 80% being secreted proteins at five days (98% viability) and more than 70% being intracellular proteins after seven days (91% viability) [25]. Conversely, another study showed conservation of 86% of extracellular HCPs when the culture viability of a host dropped from over 80% to less than 10% [20••]. Such divergent findings may be attributable to differences across studies in harvest day, cell viability, and clarification methods, which have been shown to impact the HCP profile [27•].

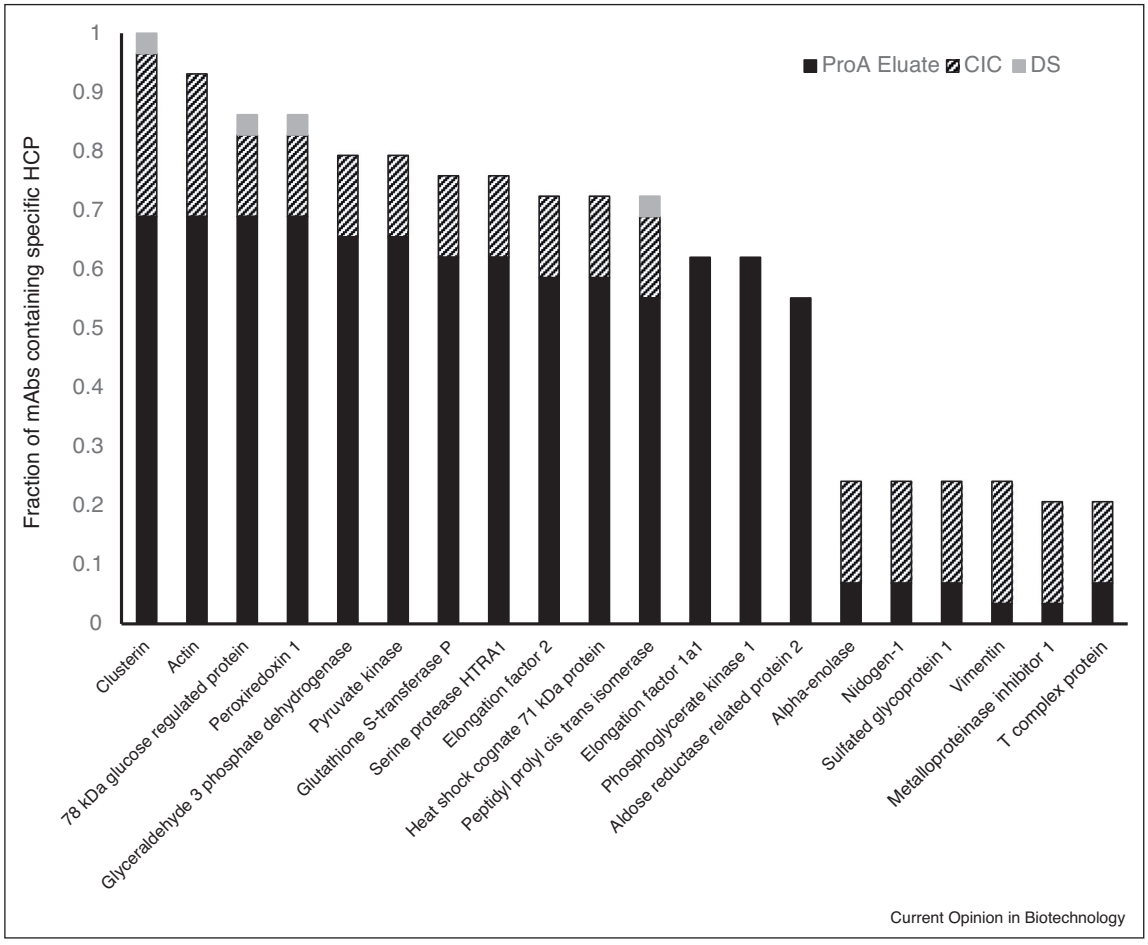
Although upstream processing conditions have historically shown limited influence on the HCP profile [28], these factors have more recently been shown to affect HCP composition and abundance, with the degree of change enhanced by increasingly divergent conditions [23]. Factors such as cultivation duration [18], media composition [29•], and operating temperature [30] have all been shown to have a significant impact on a subset of extracellular HCPs. Additionally, a larger number and higher concentrations of HCPs have been reported in fed-batch culture, when compared to batch culture in three different experiments [21,22]. The authors attributed this increase to the corresponding increase in cell density during extended fed-batch culture. During exponential growth (day 3) both batch and fed-batch cultures generated equivalent cell densities. Consequently, more than 80% of the most abundant proteins were common across different operating modes. Still, a small number of HCPs were relatively more abundant under fed-batch conditions (e.g. serine protease HTRA1). These six articles [18,21–23,29•,30] suggest that, as cell culture platforms continue to evolve and the industry moves towards higher cell densities and perfusion cell culture, additional increases in HCP abundance and changes in composition are possible, particularly if perfusion culture duration extends beyond the current range of 30–60 days.

Downstream

An important subset of persistent HCP impurities in mAb downstream processes are retained due to association with the mAb itself [31,32,33•]. Identification of HCP impurities in downstream process intermediates [19,24,25,34••,35,36] and offline measurements of mAb–HCP interactions via cross-interaction chromatography (CIC) [32,33•] have been used to identify the most difficult-to-remove HCPs. These studies tracked HCPs through downstream processes [19,24], compared HCPs present in Protein A eluate [25,34••,35], and identified HCPs in the final drug substance [36]. HCPs that associate with different mAbs were identified using CIC [32,33•]; the binding was further quantified and improved HCP clearance was achieved by using wash additives to disrupt the mAb–HCP interactions [33•].

A total of 29 mAbs were analyzed for the presence of persistent HCPs in eight recent studies [19,24,25,32,33•,34••,35,36] using different methods, process conditions, and assumptions. 'Persistent' HCPs were

Figure 2



Identification, based on studies using 29 mAbs, of persistent HCP impurities that either associated with the mAb during CIC [32,33•], were identified in Protein A eluate [19,24,25,34••,35] or were present in final drug substance (DS) [36].

defined as ones that either associated with the mAb during CIC [32,33•], were identified in Protein A eluate [19,24,25,34••,35] or were present in final drug substance [36]. The persistent HCPs for each mAb were compiled and ranked based on frequency of identification; the HCPs identified with ≥20% of mAbs are presented in Figure 2. Although the findings are skewed by one data set in which only the HCPs present in all 15 mAb purifications were included [34••], the most frequent persistent HCPs were identified by different groups, process conditions, and analytical methodologies. Clusterin was the only HCP that was identified as 'persistent' with all 29 mAbs, while 13 additional HCPs were found in >50% of mAbs tested; 153 additional HCPs were identified in <20% of mAbs tested. These findings indicate that there is a core group of 'persistent' HCPs that are likely to be challenging to remove in the majority of mAb processes. This analysis also demonstrates that there are many unique HCP impurities that may be difficult to remove from specific mAbs but that are not universally problematic, emphasizing the importance of product-specific studies.

While many of the HCPs discussed above are consistently challenging, the removal of non-consensus HCPs has also required significant investments in downstream process development efforts. The best-publicized example is PLBL2. While identified in only 14% of the mAbs analyzed in Figure 2, PLBL2 was found to bind strongly to IgG4 molecules [37,38], to cause an immunogenic response in patients and to interfere with CHO ELISA due to dilutional non-linearity [37,39]. The starting PLBL2 concentration, the Protein A load ratio and Protein A wash conditions can influence PLBL2 binding and clearance from IgG4 molecules [38]; surface plasmon resonance (SPR) analysis indicated a multivalent binding mechanism. As discussed in the Drug Substance and Drug Product section, PLBL2 also negatively impacts drug product stability [5].

Drug substance and drug product

In addition to potential immunogenicity risks to patients [2,39–41], HCP impurities can have direct or indirect negative effects on drug product stability. Specifically, proteolytic HCPs such as cathepsins have been found to cause fragmentation of the mAb itself [6–9] and HCPs with lipase activity, such as lipoprotein lipase and PLBL2 [4••,5], can cause degradation of polysorbates commonly used as excipients.

Cathepsin D has been cited by multiple groups as having a negative impact on drug product stability. At low concentrations in formulated bulk it was found to cause mAb fragmentation and particle formation [7], a decrease in recombinant therapeutic protein activity [8], and fragmentation of an Fc-fusion therapeutic molecule [9]; mAb fragmentation due to an unidentified acidic protease has also been found [6]. Although cathepsin D is not frequently identified as 'persistent' — it was identified in only 4% of the mAb processes presented in Figure 2 — such proteolytic HCPs, if present, can be quite challenging; a heat inactivation step was found to mitigate the issue in one case [8]. Another well-known but evidently benign example of proteolysis is C-terminal lysine cleavage of mAbs caused by carboxypeptidase D [42].

Polysorbate 20 or 80 is included in the majority of biological drug product formulations [43] to increase shelf-life stability [44]. Polysorbates have many well-publicized routes of degradation [45], and this list has been expanded to include hydrolysis by lipoprotein lipase and PLBL2 [4••,5]; both HCPs have been identified as difficult to remove from some mAbs [32,35]. The enzymatic hydrolysis of polysorbate releases fatty acids, which can form particles in drug product solutions [5].

Strategies for difficult-to-remove HCPs

Mitigating the effects of problematic HCPs can include action in one or more of upstream, downstream, and formulation operations. While upstream research had traditionally focused on productivity and product attributes, recent work has explored strategies for reducing HCP content in clarified cell culture fluid. Design of experiment methodologies have been used to identify key media additives, including folic acid, thiamine, riboflavin, ascorbic acid, and insulin, to reduce HCP levels [29•]. A temperature shift has been demonstrated to reduce HCP species, with mild hypothermic conditions reducing the number of HCPs detected by 36% [30], although these process parameters may not always be effective [20••]. Recently, gene editing technology has been applied to effectively generate knockout cell lines with improved product quality, for example, by reduced polysorbate degradation in lipoprotein lipase knockout cell lines [4••] or a more than 10-fold increase in the C-terminal lysine level by elimination of carboxypeptidase D [42]. Although both of the aforementioned studies demonstrated comparable cell growth to that of control cells, knockout cell lines may be limited in their viability after reduction of the levels of essential proteins, and elimination of all problematic HCPs from a single cell line is likely biologically infeasible.

Downstream strategies for optimizing HCP clearance would be expected to be effective in a suitably robust process. Non-affinity chromatographic steps can achieve excellent resolution of specific HCP impurities [46–49], and even a process lacking a Protein A capture step can clear many of the difficult HCP impurities discussed above [47]. The chromatographic profiles of persistent HCPs on different stationary phases have been characterized [48] and optimization strategies for non-affinity chromatographic removal of HCPs have been described [46,50–52]. These studies demonstrate that for many challenging HCPs, optimization of polishing steps is a viable solution, and the inclusion of proteomic methods in process monitoring may aid in quality control. However, if the mAb-HCP association is sufficiently strong, non-affinity polishing steps may not provide chromatographic resolution.

Enhancements of downstream processes to improve HCP clearance may require relatively modest additional effort. Protein A wash buffer optimization to remove challenging HCPs has been successful for many groups and does not require development or implementation of an additional chromatographic step. Wash additives and combinations of additives to successfully disrupt mAb–HCP interactions identified using CIC methodologies [33•] include various combinations of NaCl, urea, caprylate, and arginine; these additives improved clearance of persistent HCPs such as clusterin, peroxiredoxin-1, actin, and others. Similar findings have been noted for total HCP removal, rather than specific HCPs [53], and indeed rapid wash screening methodologies using multiplexed SPR and ELISA [54,55] can be effective even without the use of proteomic methods. These high-throughput wash additive screening methods provide a framework for optimizing the clearance of both total HCP content and specific HCP impurities across the Protein A capture step.

Conclusions

While HCP ELISA remains a critical tool for bioprocess development, proteomic methods have significantly increased the understanding of HCP dynamics, including both production and removal. Proteomic analyses have enabled identification of the most problematic HCP impurities, including those that are immunogenic, difficult to purify, and degrade both product molecules and excipients. These studies have begun to show the links among upstream process conditions, downstream HCP clearance efficiency, and drug product quality. This end-to-end view of process development will likely continue

to be a focus, particularly as bioprocessing pushes the limits of process intensification, which may increase the burden of HCP removal. Additional challenges may arise as the industry moves further towards continuous biomanufacturing, where factors known to impact HCP composition, such as age and variability of raw materials, can have an increased effect compared to traditional batch processing. The relationship between process parameters and the thousands of HCPs in bioprocesses remains highly complex, but recent studies have greatly enhanced knowledge of these impurities and will be an important resource for development of future processes.

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