Host cell protein removal from biopharmaceutical preparations: toward the Implementation of Quality by Design

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Abstract

Downstream processing of protein products of mammalian cell culture currently accounts for the largest fraction of the total production cost. A major challenge is the removal of host cell proteins, which are cell-derived impurities. Host cell proteins are potentially immunogenic and can compromise product integrity during processing and hold-up steps. There is an increasing body of evidence that the type of host cell proteins present in recombinant protein preparations is a function of cell culture conditions and handling of the harvest cell culture fluid. This, in turn, can affect the performance of downstream purification steps as certain species are difficult to remove and may require bespoke process solutions. Herein, we review recent research on the interplay between upstream process conditions, host cell protein composition and their downstream removal in antibody production processes, identifying opportunities for increasing process understanding and control. We further highlight advances in analytical and computational techniques that can enable the application of quality by design.

Keywords: Host cell proteins; Quality by Design; Chinese hamster ovary; process-related impurities; monoclonal antibodies

1. Introduction

Biopharmaceuticals are medicinal drugs produced by living cells. Biopharmaceuticals revolutionized the treatment of various illnesses since the first human recombinant protein (human insulin, Humulin), was introduced by Eli Lilly in 1982. To date, over 200 biopharmaceuticals, including hormones, growth factors, blood factors, vaccines and monoclonal antibodies (mAbs), have been licensed, of which mAbs have the highest number of approvals. MAbs are important to treat illnesses in oncology, immunology, and neurology. Nonetheless, such treatments require high doses of the drug over an extended period, and the typical annual treatment cost amounts to $35,000 (Campos-Pinto et al., 2017).

The importance of and demand for mAbs has driven research to focus on scaling up mAb production and increasing the production rate over the past 20 years. Cell line selection and engineering, chemically defined media, optimized cell culture protocols, single-use systems and the employment of ‘omics databases are now well-established technologies (Farrell et al., 2014; Gadgil, 2017; Langer and Rader, 2014). Today, well-designed cell culture platforms produce up to 10 g/L of mAbs in 25,000-litre bioreactors (Butler and Meneses-Acosta, 2012; Datta et al., 2013). Consequently, downstream processing (DSP) has to accommodate harvest of 15-100 kg of mAb per batch (Kelley, 2007). However, DSP facilities are designed to process feeds with considerably lower antibody concentration.

While upstream capacity increased by overcoming biological limits, downstream technologies rely on physical separation processes to scale up at least linearly with the size and number of operational units (Gronemeyer et al., 2014). Purification equipment have reached a limit for

throughput and scalability (Chon and Zarbis-Papastoitsis, 2011; Gottschalk, 2008). For instance, the operational flow rate of protein A chromatography remained constant or declined after 2011, and further improvements provided only marginal benefits (Bolton and Mehta, 2016). Processing time, material consumption and operating cost shifted from upstream processing (USP) towards DSP (Chon and Zarbis-Papastoitsis, 2011; Kelley, 2009; Low et al., 2007; Strube et al., 2011), which currently accounts for up to 80% of the total mAb production cost (Farid et al., 2007; Vermasvuori and Hurme, 2011). Affinity chromatography steps, especially protein A antibody capture, are expensive as the operation is driven by mass than volume (Gottschalk, 2008).

Understanding the current challenges in the DSP train is crucial to overcoming the bottleneck in process development. The two principal challenges are protein aggregation and host cell protein (HCP) removal (Bracewell and Smales, 2013), and this review provides a research overview on four aspects of HCP impurities:

(1) Recent reports of the problems brought about by HCPs with respect to mAb production and patient safety;
(2) Challenges in HCP removal and reasons behind HCP-mAb co-elution;
(3) Input variables of the bioprocess train that affect the HCP profile; and
(4) How the concept of Quality by Design (QbD) can help design appropriate strategies for HCP removal.

2. The importance of and problems with HCPs

HCPs are proteins of the host cells and are involved in cell maintenance and growth, and protein synthesis and processing (Baycin-Hizal et al., 2012). Nonetheless, HCPs can threaten patient safety and product quality in three main ways: (1) potential immunogenicity; (2) catalytic activity for product fragmentation and (3) involvement in product aggregation. Hence, HCPs are identified as a critical quality attribute (CQA) of mAb formulations (W. Wang et al., 2014).

2.1. Immunogenicity of HCPs

'Any protein is potentially immunogenic' (Worobec and Rosenberg, 2004) because HCPs are foreign to the human body. Any HCP, even if the HCP is at a minimal concentration, may trigger a detrimental immune response in patients (Champion et al., 2005; Gutiérrez et al., 2012; Janeway et al., 2001). This is the case for cytokines, for example, such as latent transforming growth factor-β1, which is known to be secreted by Chinese hamster ovary (CHO) cells and has been shown to be functional in human cells (Beatson et al., 2011). Concern for patient safety leads to a regulatory guideline of fewer than 100 parts per million (ppm) HCPs in the final drug formulation (Champion et al., 2005; Eaton, 1995; X. Wang et al., 2009b; Wolter and Richter, 2005). However, this requirement cannot be met consistently by protein A chromatography alone without post-capture polishing despite the high selectivity of this capture step (Nogal et al., 2012; Valente et al., 2015).

Recently, advanced clinical trials for two recombinant proteins were halted due to issues of HCP immunogenicity (Gutiérrez et al., 2012). In one case, patients in a Phase III clinical study of a mAb mounted an immune response against CHO protein phospholipase B-like 2 (PLBL2) (Hanania et al., 2015). Although the amino acid sequence of CHO PLBL2 is 80% similar to human PLBL2, many surface exposed residues are different. Consequently, clinical protocols were amended to Phase IIb, and submission of marketing application was suspended. In another case, IB1001-treated patients developed an immune response against CHO HCPs during the antibody testing (Ipsen, 2012). IB1001 is an intravenous therapy to treat and prevent bleeding episodes in adults with haemophilia B. Cases of patients with an immune response triggered by HCPs led the FDA to place the Phase III clinical trials on hold. These reports highlight the importance of understanding and quantifying the immunogenicity of residual HCPs.

Immunogenicity of a therapeutic protein is affected by factors from mainly four categories: product- and process-related factors, such as the final drug formulation and HCP impurities, and

treatment- and patient-related factors, such as dose frequency and the personal conditions of the patients. *In vivo* animal model, e.g. the human leukocyte antigen (HLA) transgenic mice and the human-severe combined immunodeficiency (HuSCID) mice, are typically used to assess antigenicity and anti-therapeutic immunogenicity of a developing therapeutic protein (Wullner et al., 2010).

In vitro assays, e.g. the *in vitro* comparative immunogenicity assessment assay (IVCIA) that uses peripheral blood mononuclear cell (PBMC), have been used to evaluate the immune response in the transplantation field. Additionally, PBMC can predict the immunogenicity of a therapeutic protein candidate in the early stage of drug development. When IVCIA was used to examine the immunogenicity of two therapeutics with reported clinical immunogenicity, the result was consistent with the observations from the clinical trials (Wullner et al., 2010). PBMC model can also evaluate immune response elicited by CQAs like aggregation and glycosylation (Joubert et al., 2016).

Immunogenicity prediction tools have been recently developed following the advancements of 'omics technologies (Bailey-Kellogg et al., 2014). Most 'omics works are publicly available (Tables

Table 1), serving as important reference points for genetically modified or adapted CHO cell lines. This provision provides a unique prospect for HCP identification in cell culture, harvest and downstream purification. A framework to facilitate the application of the 'omics technology in industrial bioprocessing has been proposed by Lewis et al. (2016). Figure 1 **Error! Reference source not found.**illustrates how this framework can help to generate HCP clearance strategies. The system of study may be defined as 'the removal of a subset of problematic HCPs causing aggregation or product fragmentation', as summarized in Tables 2-3. Study methods, including cell culture, harvest and purification technologies, can be chosen from Figure 2. Various HCP analytical methods to obtain the qualitative and quantitative HCP data can be employed (**Table 4**). Multiple genomic databases can be used to identify and analyses the HCP data (**Table 1**). Nonetheless, this step is the bottleneck of the workflow, after which host cell engineering, cell culture process conditions and purification techniques can be optimized. This rational improvement strategy will hopefully lead to full control over and reduction of this subset of HCPs in the bioprocess train.

Although *in vivo* and *in vitro* models can measure the overall immunogenic effect of a drug formulation, they cannot indicate immunogenicity of an HCP down to a single protein level. Nonetheless, the CHO genome and proteome databases enabled the development of algorithm-based tools like the CHO Protein Predicted Immunogenicity (CHOPPI) and the Immune Epitope Data Base (IEDB). CHOPPI calculates the likelihood of an HCP eliciting human immune response according to the number of protein epitopes and their similarity with human proteins (Bailey-Kellogg et al., 2014). This relative risk assessment of HCP immunogenicity can guide future bioprocess design, hence, shift the focus from clearing all HCPs to monitoring and removing a subset of critical HCPs. However, these prediction algorithms cannot yet determine the threshold above which an HCP elicits an immune response (Wullner et al., 2010).

In a recent study, the immunogenicity of HCPs in four different mAbs pre- and post-purification was evaluated with the *in vitro* PBMC assay, and the results were complemented with immunogenicity scores from the CHOPPI and IEDB databases (Jawa et al., 2016). The *in vitro* assays measured the threshold of HCP concentration above which an immune response would be triggered, and the two *in silico* tools evaluated the potential immunogenicity risk associated with 27 most common HCP detected by mass spectrometry. Some mAb samples containing high HCP concentration elicited early phase response in the PBMC assay. These potential adjuvant-like attributes agreed with the presence of high-risk immunogenic HCPs predicted by the *in silico* tools. Interestingly, some mAb samples containing HCPs as high as 4,000 ppm produced a similarly weak immune response as the purified samples in the PBMC assay. Results from CHOPPI and IEDB

reinforced this observation by assessing that HCPs with high immunogenicity scores, i.e. glutathione-S-transferase P, peroxiredoxin-1, procollagen-lysine, 2-oxoglutarate 5-dioxygenase 1 and PLBL2, were in low concentrations in those particular samples, hence, the overall formulation could be considered low-risk. While algorithm-based prediction tools calculate the risk of an HCP, the *in vitro* assays evaluate the thresholds above which these proteins trigger the immune system. Therefore, both *in silico* and *in vitro* analyses are required to achieve a holistic and comprehensive HCP risk assessment.

2.2. mAb fragmentation

mAb fragmentation, which typically occurs through proteolytic activity, reduces the overall product yield (Clark et al., 2004; S. Elliott et al., 2003; Gao et al., 2011; Sandberg et al., 2006; Satoh et al., 1990). Host cell proteases are essential in catalytic and metabolic pathways and extracellular waste turnover (Birkedal-Hansen et al., 1993), but when present in cell culture media can break down the product. This has been observed in serum-free media (Sandberg et al., 2006), where cleavage of peptide bonds produces Fc and Fc-Fab fragments. This proteolytic degradation route is due to the lack of serum protease inhibitors in chemically-defined media that would otherwise block the activity of cellular proteases extracellularly. Proteases can be secreted into media or released during cell lysis. Exposure of the hinge region of a mAb, which is the weakest link on the molecules in solution, accelerates mAb fragmentation (Cordoba et al., 2005; Gearing et al., 2002). Additionally, glycosidases can trim the oligosaccharide chain attached to the antibody's Fc or Fab region, resulting in less mature glycan structures. In turn, they may affect the pharmacokinetics and pharmacodynamics of the glycoprotein (Dorai and Ganguly, 2014). **Table 2** presents a summary of the problematic proteases and glycosidases in bioprocessing.

2.3. Protein aggregation

HCPs bring the second potential problem to bioprocessing – aggregation. The protein structure is altered (Bracewell and Smales, 2013), forming soluble or insoluble aggregates of various sizes from small dimers to visible particles (Eon-Duval et al., 2012a). Aggregation is undesirable in biotherapeutics production. Small aggregates may trigger immunogenic reactions, and administration of large particles may cause various adverse effects (Cromwell et al., 2006; Ratanji et al., 2014; Rosenberg, 2006). As both HCPs and large aggregates can be immunogenic, the formation of large HCP-mAb aggregates may elicit an immune response through an adjuvant-like mechanism (Bracewell et al., 2015).

In large-scale manufacturing, initially native and folded proteins may interact with binding HCPs to form non-native aggregates (Chi et al., 2003). Binding HCPs are among the most abundant proteins in the CHO proteome. Expression of binding HCPs is crucial for proper protein folding, but upregulation of these proteins during high expression of recombinant product promotes intracellular aggregation especially if the recombinant product is not fully or correctly folded (Kim et al., 2010; Y.-B. Zhang et al., 2004). Binding HCPs released during cell lysis interact with partially or misfolded mAbs in the cell culture media to form aggregates through hydrophobic interactions and disulfide linkages (Bukau and Horwich, 1998; Doyle et al., 2013; Giese et al., 2005; Schokker et al., 2000). The beta-sheet folding structure of mAbs further promotes non-specific interactions with HCPs and accelerates the aggregation process (X. Wang et al., 2009a). **Table 3** presents HCPs involved in protein aggregation.

Protein aggregation in bioprocessing can be a multi-stage event that involves HCPs of different nature. Apart from binding HCPs, protein aggregation may follow a series of proteolysis. mAb fragments cleaved from intact mAb molecules by cathepsin D can form visible particles (Bee et al., 2015). Similarly, carboxypeptidase has been shown to clip the C-terminal lysine of mAbs to produce fragmented mAbs with different charge distributions. Uneven charge distribution on the protein surface, in turn, compromised the stability of the molecules and led to aggregation (Lauer et al., 2012).

Additives are used to stabilize a drug formulation, however fragmented additive molecules can destabilize mAb preparations and lead to aggregation. In a recent study, a small amount of triacylglycerol lipase, a ubiquitous enzyme, was found to hydrolyze ester bonds of Polysorbate 80 (PS80), a surfactant, in a concentrated drug formulation (Labrenz, 2014). PS80 was degraded into fatty acids and PS85, which is an emulsifier. These unintended contaminants bound to mAbs through hydrophobic interactions and formed visible protein particles. In another case, a difficult-to-remove CHO HCP, lipoprotein lipase (LPL), degraded polysorbate and reduced mAb stability (Chiu et al., 2017). In nature, LPL hydrolyzes ester bonds within triglycerides to form alcohol and fatty acids (Nilsson-Ehle et al., 1980), but LPL may enzymatically degrade polysorbates that have a similar structure to the triglycerides, and hence, destabilize the drug formulation (Dixit et al., 2016).

2.4. Leaching of immunogenic protein A molecules and column fouling

Catalytic HCPs, e.g., metalloproteinases, in the feedstock of protein A chromatography may cause protein A leaching (Carter-Franklin et al., 2007). Leached protein A fragments are immunogenic and require close monitoring and clearance to less than 10 ppm in the final drug formulation (Fahrner et al., 2001). Removal of protein A fragments is challenging and often involves additional purification steps, adding to the downstream burden and costs (Carter-Franklin et al., 2007). Additionally, HCPs can foul chromatographic resins and reduce the performance and lifetime of the column. In one case, HCPs accumulated on the surface of protein A resins as purification cycles increased (Lintern et al., 2016). Proteomic analysis showed these foulant HCPs to be the most abundant proteins in CHO cells mainly involved in cellular metabolism and protein synthesis. Many cytoskeletal proteins and chaperones were also found.

3. Regulatory requirements

The profile of HCPs eluted from primary clarification and capture steps varies widely depending on the purification unit, the culture methods and harvest conditions (Shukla et al., 2007; Sisodiya et al., 2012). The final drug formulation should achieve a typical purity target of <100 ppm HCPs (i.e., 100 ng HCP/mg mAb), but regulatory agencies like the European Medicines Agency (EMA) and the Food and Drug Administration (FDA) have several guidelines on this matter. Nonetheless, previous regulatory requirements on the final HCP level are expected to be revised to consider standardization of HCP quantification methods and improvements in HCP detection techniques. For example, we can start answering the question ‘should all HCP species be treated as equally dangerous regardless of immunogenicity of individual HCP?’ (Bracewell et al., 2015; Jawa et al., 2016).

The other debate on HCP clearance relates to the comparability of HCP impurities between biosimilars and reference products. Biosimilars are follow-on biologics manufactured by a different company when the patent of the original product expires. From 2013 to 2022, the patents of at least six high-profile mAb products are expiring, with over ten biosimilars of each product currently under development (Udpa and Million, 2016). The ultimate target of process design for a biosimilar is to achieve a ‘similar’ product. Hence, extensive characterization of the structure and function of the protein is necessary to demonstrate the biosimilarity. However, production of biosimilar candidates cannot be comprehensively compared to that of the reference product due to confidentiality in manufacturing processes (European Medicines Agency, 2014; FDA, Center for Drug Evaluation, 2015).

HCPs co-eluting with biosimilar candidates can be significantly different from those for the reference product (Mihara et al., 2015). In one case, even though the total HCP concentration was comparable, no HCPs in the biosimilar candidate were the same as those in the reference product except peroxiredoxin-1 (Mihara et al., 2015). Moreover, an immunogenic HCP, PLBL2 (Vanderlaan et al., 2015), was present in the biosimilar candidate but not the reference product, and this HCP

could only be removed by an additional chromatographic step (Mihara et al., 2015). This report challenges the definition of biosimilarity – how 'similar' should biosimilars be in comparison to the reference products in terms of HCP impurities? This ambiguity further shows the importance of independent and complete characterization of HCP profile of a biosimilar product. Qualitative differences between biosimilar candidates and original products should be well understood even if the concentration of HCPs may be similar.

Regulatory agencies specifically and cautiously recommend that industry quantify process-related impurities with orthogonal methodologies (Section 7.3) during a biosimilarity assessment. With the FDA, process-related impurities in a biosimilar are not expected to match that of the reference product. Nonetheless, a proper record of the potential impact of different impurity profiles, particularly concerning patient safety, must be supported with appropriate data (FDA, Center for Drug Evaluation, 2015). The EMA has a similar approach stating in that if 'qualitative and/or quantitative differences are detected, such differences should be justified and, where relevant, demonstrated to have no impact on the clinical performance of the product' (European Medicines Agency, 2014).

4. The challenges in HCP clearance

HCPs can compromise patient safety as well as product integrity and titer. However, HCP removal remains a major challenge in bioprocessing due to three main reasons: (a) the complexity of HCP impurities; (b) the under-explored characteristics of HCP-mAb interactions and resulting co-elution pattern through the purification train; and (c) the lack of understanding over whether HCP abundance at harvest plays a role in co-elution. HCPs are complex and heterogeneous compared to other impurities like host cell DNA, as demonstrated by the fact that more than 6,000 HCPs have been identified in the CHO proteome (Baycin-Hizal et al., 2012; N. E. Lewis et al., 2013). These HCPs differ in physical properties, e.g. molecular weight (MW), isoelectric point (pI) and hydrophobicity (Champion et al., 1999; Jin et al., 2010), while one protein can have over 20 different post-translational modifications, such as glycosylation, phosphorylation and truncations (Godovac-Zimmermann and Brown, 2001).

This wide range of physicochemical properties of HCPs translates into bottlenecks in DSP (X. Wang et al., 2009b).Despite the high selectivity of protein A chromatography, HCPs that persist after this step can still pose a significant clearance burden on subsequent polishing steps. HCPs co-eluting with the mAb product in the capture step typically range from 200 ppm to 3,000 ppm (Fahrner et al., 2001), but values as high as 70,000 ppm have been reported (Yigzaw et al., 2006). The presence of HCPs in post-protein A samples is mainly due to interactions between HCPs and mAbs (Shukla et al., 2008; Sisodiya et al., 2012; Q. Zhang et al., 2016). Although HCPs can interact with protein A resins, such interactions are not proposed as the main reason for HCP co-elution (Fahrner et al., 2001; Shukla et al., 2008; Tarrant et al., 2012; Q. Zhang et al., 2016).

Interactions between HCPs and mAbs have not been fully characterized yet and are often described as non-specific (Aboulaich et al., 2014; Hogwood et al., 2013; Q. Zhang et al., 2016; 2014). It is believed that HCPs can bind to either the Fc or Fab regions of mAb molecules to form HCP-mAb complexes in the harvested cell culture fluid (HCCF). Aboulaich et al. (2014) identified a subset of HCPs that co-elutes with all four mAbs tested during protein A chromatography and suggested that this HCP subpopulation binds to the shared constant region of the mAbs. A more recent study, however, conducted an in-depth analysis of HCP subpopulations that co-purified with 15 different mAbs (Q. Zhang et al., 2016). They found that ~90% of co-eluting HCPs were common among the mAbs tested and that co-elution was determined by HCP abundance and their ability to interact with mAbs. The group went one step further to study the propensity of HCPs to bind to Fc and $(Fab')_2$ antibody fragments. Interestingly, they found that most HCPs interacted with both types of fragments, which led to the conclusion that most interactions are non-specific. This observation is supported by a previous example of clusterin binding to IgGs at both Fc and Fab regions through

multivalent mechanisms (Wilson and Easterbrook-Smith, 1992). Nonetheless, both Aboulaich et al. (2014) and Zhang et al. (2014 and 2016) agreed that mAb-specific HCPs only interact with the Fab domains, which are the 'sticky' complementarity-determining regions unique to each mAb. Several physicochemical interactions between HCPs and mAbs, involving charged and non-charged associations, are summarized in **Table 5**. Under physiological conditions of pH between 6.5 and 7.0, mAbs are neutral or cationic, and many HCPs are either neutral or anionic (Li, 2017). Different charges between the HCPs and mAbs produce strong electrostatic and hydrophobic interactions between these molecules.

In contrast, Gagnon et al. (2014a and 2015) hypothesized that HCP-mAb co-elution could not be caused by direct protein-protein interaction. They reasoned that IgGs are unlikely to associate with HCPs in a non-specific manner under normal physiological conditions because mAbs are specific to their target antigens. Instead, the multiple interactions between protein A, chromatin-HCP heteroaggregates and mAbs were put forward as the primary reason of the co-elution. Chromatin is considered a vehicle for 'smuggling' a range of HCPs through protein A chromatography (Gagnon et al., 2014a; 2015). Since chromatin is semi-stable in cell culture harvest, i.e., the DNA component of chromatin is electronegative (pKa of ±2.6), and the histone component is hydrophobic and electropositive (pI ±11.5), this chemical surface becomes a prime nucleation center for non-specific HCP binding. Two possible co-elution mechanisms are:

1) Chromatin heteroaggregates, which consist of HCPs accreted onto nucleosomes, bind to protein A more strongly than IgG. HCPs are leached from the heteroaggregates during the elution step.
2) IgG and chromatin form strong electrostatic interactions under the elution condition that destabilize the heteroaggregates-protein A association and cause HCPs to be eluted from the column.

In both cases, removing the chromatin heteroaggregates before protein A chromatography reduced the level of residual HCPs.

Interestingly, Zhang et al. (2016) did not find chromatin to play a major role in HCP-mAb co-elution. Histone proteins were not detected in protein A eluates, and only a minimal level of histones was detected in the HCCF (i.e., 0.67% of the amount of histones in HCCF reported by Gagnon et al.). This observation was consistent for experiments with both reconstituted HCCF from null cell lines and HCCF from mAb-producing cell lines, showing that HCP-mAb co-elution can happen in the absence of histones.

Studies by both Gagnon et al. (2014a and 2015) and Zhang et al. (2014 and 2016) show that HCP-mAb co-elution may be a result of a high level of histones in HCCF or non-specific HCP-mAb interactions. Cell viability was about 20% in the study by Gagnon et al. (2014a and 2015), which is low for a typical industrial mAb production process. The high extent of cell lysis probably increased the level of histones in HCCF, and that became the primary reason of HCP-mAb co-elution. On the other hand, cell viability in the study by Zhang et al. (2014 and 2016) was not reported; therefore, no direct comparison between the two studies can be made. Although mAbs are specific to their target antigens, we cannot neglect the fact that HCP impurities are a complex mixture of proteins with various binding functions (Section 2.3). Therefore, non-specific interactions between mAbs and HCPs are possible, especially if one or both proteins are partially folded, denatured or fragmented, or if the HCPs are native molecular chaperones.

Identifying the different types of HCP-mAb interactions, including characterization of residual HCPs will support a guided design of HCP removal strategy by QbD, and hence, an optimized purification flow. For example, the capture step may be designed to disrupt specific physicochemical bonds between mAb and HCP, especially that of critical and immunogenic HCPs.

Wash modifiers with optimized pH, ionic strength and additive levels of protein A chromatography can be formulated (Gruber et al., 2016). These strategies will be further discussed in Section 7.

Besides interaction with mAb, HCPs can co-elute through chromatography processes if their structures are like that of the antibody (Pezzini et al., 2011). For example, two residual HCPs, peroxiredoxin-1 and cathepsin Z, have a similar distribution of hydrophobic and charged residues on the molecular surface as that of the antibody. A high number of hydrophobic residues exposed on the surface of these two HCPs allowed good adsorption on the resins of mixed-mode chromatography. Two other residual HCPs, HSPG (basement membrane-specific heparin sulfate proteoglycan core proteins) and beta-2 microglobulin, have structural homology as the antibody, which is believed to be the cause of the co-elution (Pezzini et al., 2011). They contain immunoglobulin-like (Ig-like) domains with a specific fold, where two beta sheets form a 'sandwich' stabilized by the interactions between conserved cysteines and other charged amino acids.

Are co-eluting HCPs the most abundant ones in the culture supernatant?

Researchers are questioning if co-elution of a specific HCP can be directly related to the abundance of that HCP at harvest. In other words, are HCPs that are more abundant in cells more likely to co-elute with mAbs through the capture step? To date, two different observations have been made on this topic. On one hand, research by Zhang et al. (2014 and 2016) showed that the HCPs that co-eluted through protein A chromatography were some of the most abundant in the cell culture supernatant. The co-elution mechanism depended on the concentration of the HCP species and mAb titer in HCCF. On the other hand, Pezzini et al. (2011) observed that the level of residual HCPs post-ionic exchange did not correlate with their abundance in cell culture supernatant but primarily depended on their specific physicochemical characteristics. They concluded that the effect of specific hydrophobic zones and charge distribution on the surface of the HCPs is the key to HCP co-elution.

Both Zhang et al. (2014) and Pezzini et al. (2011) used CHO cell lines. Nonetheless, the contradicting observations cannot be fairly compared. Zhang et al. (2014) analyzed the HCP composition of a harvest supernatant from a large-scale mAb production run. Cell viability at harvest was not reported. Pezzini et al. (2011), on the other hand, used a null cell line (CHO K1) and harvested the cell culture when cell viability was above 95%. Additionally, their different conclusions might be due to the different purification techniques employed. Zhang et al. (2014) purified the mAb with protein A chromatography, while Pezzini et al. (2011) used four mixed-mode chromatography columns of different resins. Protein A chromatography and mixed-mode chromatography are orthogonal separation techniques, and the population of residual HCPs could have varied according to the purification method.

Two years after their first study, Zhang et al. (2016) proposed two characteristics of an HCP contributing to HCP-mAb co-elution through protein A chromatography: (1) the relative abundance of the HCP at harvest and (2) the strength of the HCP to associate with the mAb. Twelve of fourteen co-eluting HCPs found in all fifteen purified mAbs were some of the most abundant HCPs at harvest, showing that abundance contributed to HCP presence in purified mAb preparations. Nonetheless, many abundant HCPs at harvest were not detected in the purified samples, suggesting that abundance in HCCF alone is insufficient for an HCP to co-elute. Furthermore, HCPs with relatively low abundance at harvest, including the serine protease HTRA1, were detected in all fifteen mAbs. Their strong association with the antibodies led to their enrichment relative to other HCPs. A relative enrichment factor EF (equation (1)) describes the relative strength and likelihood of an HCP to co-elute through protein A chromatography (Q. Zhang et al., 2016). From Equation (1), the individual HCP α is enriched relative to the overall HCP content through protein A chromatography if the EF is greater than 1, and vice versa.

$$\mathrm{EF}_{\mathrm{HCP}\alpha} = \frac{\mathrm{HCP}\alpha_{\mathrm{ProA}}/\Sigma\,\mathrm{HCP}_{\mathrm{ProA}}}{\mathrm{HCP}\alpha_{\mathrm{HCCF}}/\Sigma\,\mathrm{HCP}_{\mathrm{HCCF}}}, \quad (1)$$

Notable co-eluting HCPs and the transferability of CHO proteome of a null cell line

Previous studies have reported a group of HCP species commonly found in the eluates of protein A chromatography. Notable co-eluting HCPs, including proteases, chaperones and structural proteins that cause protein A fouling, as reported in these studies are summarized in Table 6. Researchers prefer to examine the profile of co-eluting HCPs with a spiking method. In that process, purified mAbs are added into the clarified supernatant of null cell culture to prepare a spiked mixture. This approach can normalize the impact of upstream cell culture parameters on HCP composition. However, this also means that the impact of the cell line and upstream process parameters on HCP profile is not considered. As shown in Table 6, key process parameters that affect the HCP profile at harvest, like cell viability and harvest day, were not discussed during such studies, and the differences between null and producer cell lines were not identified.

Proteomic studies with 2D-gels concluded that the HCP composition of the HCCF from null and producer cell lines were comparable (Grzeskowiak et al., 2009; Jin et al., 2010). Nonetheless, many low abundant HCP species can be masked by the abundant mAb molecules on the gel images (Jin et al., 2010; Tait et al., 2011). Consequently, differences in the proteomic profile of the two cell lines cannot be fully observed. Analysis with mass spectrometry (MS) showed that HCPs from null and mAb-producing cell lines are significantly different (Tait et al., 2011). Therefore, the conclusion regarding the equivalence of HCP composition between null and mAb-producing cell lines should be revised. Current MS technology (discussed in Section 7.3.3) can detect low abundant HCP as little as 1 ppm in highly purified mAb formulations (Doneanu et al., 2015; Reisinger et al., 2014), providing deeper insight into the differences in HCP profile of null and mAb-producing cell lines. Furthermore, research on residual HCP composition should be carried out using mAb-producing cell lines when possible with the main harvest criteria, including product titer, taken into consideration.

5. The impact of bioprocessing conditions on HCPs

Over the past years, researchers began to appreciate the importance of understanding the interconnection between upstream and downstream processes. Research focus has shifted from examining the impact of individual purification units on product purity towards embracing a more holistic approach to the problem. Researchers have begun to acknowledge that improvements in mAb production cannot rely on a higher upstream productivity alone, and that downstream cannot be developed further by tinkering with either a single unit operation or even with the entire DSP train alone; the whole integrated bioprocess must be considered. However, given the multi-stage nature of mammalian cell-based mAb production, characterization of the manufacturing train requires a thorough understanding of the entire bioproduction flow sheet as well as well in-depth knowledge of HCP composition at each step from cell culture operation to final drug formulation, which can be time- and resource-intensive.

In 2010, Jin et al. hypothesized that changes in upstream process parameters impact HCP content at harvest. To explore this hypothesis, they conducted a series of experiments in which cell culture conditions including temperature, media composition, cell line, aeration, and agitation were manipulated. Then, HCP composition in the HCCF was investigated with HCP ELISA and 2D-DIGE. In contrast to their original hypothesis, they did not find significant changes in HCP composition under different culture conditions except for cell viability. Due to these results, research focus shifted from examining the upstream-downstream interplay to understanding HCP-mAb interactions and tracking HCPs throughout DSP (Chiverton et al., 2016; Sisodiya et al., 2012; Q. Zhang et al., 2014). Nonetheless, several research groups revisited the up- and downstream interplay with the emerging ‘omics approach and MS techniques. Recent studies include HCP tracking from harvest to the end of polishing steps and investigation of the impact of upstream culture parameters on HCP composition at harvest and that of purified samples (Chiverton et al., 2016; Goey et al., 2017; Jin et al., 2010; Pezzini et al., 2011; Tait et al., 2013; Q. Zhang et al., 2014), as discussed in the following sections.

5.1. Upstream process conditions

Recent studies on the impact of upstream parameters on HCP clearance are summarized in Table 7. They center on five main themes: the recombinant product type, the cell line and the age of the cells used, the duration of the culture and viability at harvest, the mode of culture operation and the culture temperature.

5.1.1. Recombinant product type

Studies conducted so far indicate that the HCP population that co-elutes with the mAb product through protein A chromatography is mAb sequence-dependent (Aboulaich et al., 2014; Levy et al., 2014; Shukla et al., 2007; Sisodiya et al., 2012). The total concentration and the HCP species present post-protein A purification are different from one type of mAb to another (Aboulaich et al., 2014; Levy et al., 2014). Modifications of a few amino acids on the IgG surface changed have been shown to change the molecule's aggregation propensity and, hence, to affect the interaction between the IgG and HCPs (Levy et al., 2014). However, a baseline set of HCPs has been found to bind to multiple types of mAbs and is believed to interact with one or more domains (Fab and Fc). In contrast, HCPs that bind specifically to one type of mAb may have high affinity towards its variable regions (Levy et al., 2014). Similar conclusions regarding interactions between co-eluting HCPs and mAb domain-specificity were drawn by Aboulaich et al. (2014) and by Zhang et al. (2016) in the aforementioned study of 15 mAbs with different isotype and light chain type.

5.1.2. Cell line, culture viability and duration

Yuk et al. (2015) studied the HCP profile of three null cell lines derived from the original CHO K1 host under entirely different process conditions, i.e., culture temperature, medium and feed formulation. The HCCF of the three null cell lines collected on day 14 contained HCP population similar to each other, which agrees with Jin et al. (2010). Approximately 80% of the 1,000 HCP species were detected in the HCCF of all three cell lines. However, further studies on producer cell lines are required to reach a meaningful conclusion.

Yuk et al. (2015) also observed that cell viability did not significantly impact the predominant HCP population at harvest, which is different from the earlier work of Tait et al. (2011). The latter study reported that the impact of cell viability on HCP profile is especially prominent during the transition period from early decline phase to a steady reduction in viable cell density. The different cell culture methods employed in the two studies might explain the contradicting conclusions. Cells were cultured under mild hypothermia from day 3 in the study by Yuk et al. (2015) but at the standard physiological temperature in the study by Tait et al. (2011). Mild hypothermic culture produces cell lysate and supernatant with a lower number of differentially expressed proteins (Kumar et al., 2008), which is related to a larger population of healthy cells under such conditions (Goey et al., 2017). The impact of culture temperature on HCP profile is further discussed in Section 5.1.4.

The impact of cell culture duration on HCP profile was studied by Tait et al. (2011) and Goey et al. (2017). In the study by Tait et al. (2011), the HCP composition on day 10, 12 and 14 of fed-batch cultures was investigated. The relative abundance of several HCPs, e.g., heat shock protein and protein disulfide-isomerase, across the cell culture decline phase was found to be statistically different. Goey et al. (2017) analyzed the HCP profile of supernatant sampled from stationary phase (day 8) to late decline phase (day 14) of a mAb producer and reported that HCPs found in the cell outer membrane change more dynamically than intracellular or secreted HCPs. They reasoned that increase in blebbing of the apoptotic cell membrane, as observed by Ndozangue-Touriguine et al. (2008) and Stricker et al. (2010), as cell culture progresses might contribute to the release of various cell membrane proteins.

Besides the general HCP profile, cell culture duration was found to impact IgG fragmentation (Karl et al., 1990). Two types of IgG fragments accumulated in hybridoma cell culture supernatant as the culture progressed and the degree of cleavage increased with cell culture time. Proteolytic activity of cathepsin D or E in the supernatant was proposed to be the reason for IgG cleavage (Karl et al., 1990), which was confirmed by Robert et al. (2009) who identified metalloproteinase and cathepsin D as the proteases that clip the Fc region of mAbs. Proteolytic activity in cell culture supernatant was linearly proportional to the integral of viable cell density (IVCD) and reached a plateau at late stage culture (Robert et al., 2009). Additionally, the final glycoform of a protein product may be affected by cell culture time (Gramer and Goochee, 1993; Munzert et al., 1996). The activity of soluble sialidases in cell culture supernatant of a producer CHO cell line remained low when cell viability was still high but increased concomitantly with the increase in the number of dead cells from day 12 (Munzert et al., 1996).

5.1.3. Cell age

Valente et al. (2015) investigated the impact of cell age on the presence of difficult-to-remove HCP species. A CHO K1 cell line was cultured for 136, 251, 366 and 500 days with passages performed every three to five days. At those time points, cells were cryopreserved, revived and cultured until day 11. They found that certain HCPs in the culture supernatant of cells aged 251, 366 and 500 days were differentially expressed compared to that of 136 days. A total of 92 unique HCPs exhibited variable expression, of which 34 were difficult to remove as reported in different proteomic studies (Doneanu et al., 2012; Hogwood et al., 2013; Joucla et al., 2013; Levy et al., 2014; Pezzini et al., 2011). Particularly, 17 HCPs were known to interact with mAbs strongly through protein A purification (Levy et al., 2014), of which 15 had been detected in protein A eluates (Doneanu et al., 2012; Hogwood et al., 2013). This study shows that cell age affects not only the overall HCP composition at harvest but also the difficult-to-remove HCP species.

5.1.4. Culture temperature

Jin et al. (2010) and Tait et al. (2013) studied the impact of cell culture temperature on HCP profile but reached different conclusions. Both experiments were performed with producer cell lines with culture temperature downshifted on day 6. 2D-DIGE proteomic analysis on the HCCF conducted by Jin et al. (2010) showed that changes in protein spots with temperature downshift were statistically insignificant. Cell viability at harvest was not reported, and no information on the harvest techniques was available. In another case, Tait et al. (2013) harvested the cultures at 80% cell viability. The HCP level in the mild hypothermic supernatants was 50% higher than that of the cultures of standard physiological temperatures, which was probably caused by a higher accumulation of dead cells as culture duration was prolonged for five days under mild hypothermia. Information regarding HCP species was unavailable to be compared to that of Jin et al. (2010). Nonetheless, cells cultured under mild hypothermia were more shear-resistant towards downstream clarification. They believed that cell membrane had become more robust under mild hypothermia due to homeoviscous adaptation, supported by previous studies by Los and Murata (2004) and Roobol et al. (2011).

Recently, Goey et al. (2017) established a positive correlation between extracellular HCP concentration and the percentage of dead cells in bioreactors, suggesting that HCPs were released during the cell culture operation and not due to subsequent centrifugation steps. The variety of intracellular HCPs during the cell culture decline phase was substantially reduced under mild hypothermic conditions, which coincided with lower apoptotic cell density. At 80% cell viability, supernatant samples from both the control (physiological temperature) and mild hypothermic cultures contained comparable HCP concentration and HCP/mAb ratio. Interestingly, mild hypothermic supernatants had 32% lower number of unique HCP species, which was in line with the 37% decrease in apoptotic cell density. This study showed that harvesting at high cell viability ensures a minimum level of HCPs in the supernatant, but cell viability cannot predict the diversity of HCPs. Instead, HCP variety was closely correlated to apoptotic cell density in their system.

Additionally, cells cultured under mild hypothermia showed a reduced diversity of chaperones and proteases in the HCCF by 27% and 44%, respectively.

5.1.5 Cell culture mode

Park et al. studied the effect of culture mode, i.e., batch and fed-batch, on the HCPs concentration in the supernatant (Park et al., 2017). As a result of prolonged culture duration and higher accumulation of cell debris, 11% more HCPs could be identified in the fed-batch culture. They found that 74% of HCPs identified in HCCF were also present in exponential growth phase when culture viability was 95%. This indicates that several HCPs were being secreted from viable cells. They identified eight out of the 30 most abundant HCPs present in the supernatant throughout the culture to be either cytoplasmic or secreted proteins, while another eleven secreted proteins were detected in HCCF samples under both culture modes. Interestingly, the concentration of lactate dehydrogenase (LDH), which is correlated with cell lysis, increased significantly toward the end of the culture but was not one of the most highly abundant HCPs. The authors moved one step further to show that concentration profiles of HCPs affecting mAb integrity correlated with changes in mAb critical quality attributes such as aggregation, charge variants, and N-glycosylation during the cultures. Their findings can help refine process intervention and cell engineering strategies, while an obvious next step would be to perform such studies on perfusion bioreactor systems, which promise to enable integrated and intensified bioprocesses.

5.2. Downstream processes conditions

Studies regarding the impact of downstream process conditions on HCP profile are scarce. The work of Hogwood et al. (2013) demonstrated that the primary clarification techniques significantly impact HCP profile across the DSP chain. Two primary clarification techniques, i.e., disc-stacked centrifugation and depth filters with different pore sizes, produced substantially different HCP profile in the clarified culture supernatant (Hogwood et al., 2013). The same study compared the impact of clarification techniques on the HCP profile of a mAb-producing and a null cell line. Changes in HCP profile, quantified based on greater spot changes on 2D-PAGE gels, of the mAb-producing culture were more sensitive to the type of clarification technique. This observation again shows the importance of including mAb-producing cell lines in such studies.

As the HCP level reduces through the downstream purification train, the number of proteases, and, hence, overall proteolytic activity is expected to decrease. However, Sandberg et al. (2006) and Robert et al. (2009) observed a low level of proteolytic activity in crude cell culture supernatants before any purification, followed by a drastic increase of over 200-fold after the primary capture step (Sandberg et al., 2006). Proteolytic activity by cathepsin D was higher in the protein A eluates than in both flow-through and HCCF (Robert et al., 2009). A recent study showed that cathepsin D co-elutes with mAb through protein A chromatography, hence, explaining the increase in proteolytic activity after sample purification (Levy et al., 2014). Since proteolysis is a kinetic-driven process, the activity of co-purified cathepsin D increases in purified samples as other HCPs are removed, and the mAb is concentrated (Robert et al., 2009).

6. The concept of Quality by Design (QbD) and efforts to reduce HCPs in USP and DSP

QbD is an information-driven conceptual framework to develop and approve pharmaceutics (Rathore and Winkle, 2009). Product quality is built into the manufacturing process with an ethos of ‘quality cannot be tested into products; it should be built in by design’ (FDA, 2004). The product quality is monitored and tightly controlled at every stage of the bioprocess (del Val et al., 2010; ICH, 2011; Rathore and Winkle, 2009). From Figure 3, QbD starts with identification and characterization of the critical quality attributes (CQAs) of the product. Then, Design of Experiments (DoE) and *in silico* mathematical modeling are conducted to build relationships between the CQA profile of the product and the critical process parameters (CPPs), forming a design space capable of absorbing inherent variability from input materials by adjusting the operating conditions. Figure 2 shows the range of upstream and downstream parameters that can be considered when creating a bioprocess

design space. The final objective of QbD is achieving reliable product quality through flexible manufacturing approaches. Guiding documents detailing the application of QbD principles in pharmaceutical process developments are provided by the FDA (FDA, 2009; 2004; ICH, 2008; 2005).

Research suggests the presence of inseparable links between cell culture conditions and the HCP composition through the downstream processing stream. In other words, HCP composition of purified samples and HCCF is influenced by and could potentially be reduced by optimizing upstream cell culture conditions via the concept of QbD. Nonetheless, implementing QbD to remove HCPs was challenging as HCPs could not be characterized completely, and information about the nature of HCPs was limited. Low-abundant HCPs in highly purified samples could not be detected by 2D-gels. However, the development of mass spectrometry and the establishment of CHO genome and proteome are significant milestones to identify and quantify HCPs systematically.

Figure 4 depicts the QbD approach to remove HCP and current research gap. The current limitation to anticipate problems in DSP steps is our incomplete understanding of how cellular functions are regulated under different culture environments (Figure 4, red arrows). First, interpretation and organization of HCP data is laborious and requires in-depth knowledge of bioinformatics (Figure 4, Circle 1). Information of each protein species is complicated and should be understood. Second, the relationship between residual HCPs and the equivalent HCP composition in upstream has not been defined fully (Figure 4, Circle 2). We first need to understand if residual HCPs are the most abundant ones at harvest. Third, links between the physiological state of the host cell population and HCPs at harvest are not yet fully characterized (Figure 4, Circle 3). Host cell physiology is complex and cellular molecular machinery changes in different culture environments (Figure 4, Circle 4).

Many interventions to reduce HCPs in upstream and downstream steps have been suggested. From a product integrity perspective, HCP removal should begin as early as possible in the manufacturing process. Host cell engineering to design cell lines that simplify the purification process of a target protein can, therefore, be considered. Previous work on *E. coli* cell engineering to remove challenging HCPs and the relative ease of CHO genetic engineering support this approach (Caparon et al., 2010; Humphreys et al., 2004; Z. Liu et al., 2009). Genetic modifications can be performed with zinc finger (ZFNs), transcription-like effector nucleases (TALENs) or clustered regularly interspaced short palindromic repeats (CRISPR). Nonetheless, potential issues with cell line engineering, e.g., possible side-effects on the stability of the resulting cell lines, should be taken into consideration.

CRISPR and TALENs were recently employed to knock out a difficult-to-remove HCP, lipoprotein lipase (LPL), in order to stabilize polysorbate in mAb formulations (Chiu et al., 2017). Three specific single-guide RNA (sgRNA) expression vectors were designed for the exon targets of (1) an active site of LPL, (2) a heparin-binding site that serves as a bridge between protein and lipoprotein, which if deactivated, would diminish LPL activity and (3) an N-linked glycosylation required for LPL to be catalytically active. Gene knock-out successfully produced frameshift mutations and amino acid deletion at the active sites, which resulted in no expression of LPL or expression of truncated or catalytically inactive LPL. Polysorbate degradation was significantly reduced without substantial impact on cell viability.

In another study, expression of anti-apoptotic genes improved cell robustness, therefore sustaining cell viability for an extended cell culture period (Potty et al., 2014). Less cell debris in the clarified cell culture supernatant was found, and the HCP content was reduced. Other approaches include selecting cell lines with a lower level of endogenous proteases, which hence, exhibit lower proteolytic cleavage potential, during clonal selection (Dorai and Ganguly, 2014). Additionally, Fc candidates with fewer proteolytic-susceptible sites should be prioritized when selecting a recombinant protein. mAb molecules can also be engineered to minimize ‘sticky’ patches on the

Fab structure by changing the amino acid sequence of the unique variable regions, hence reducing the chances of HCP binding on Fab (Q. Zhang et al., 2014). In parallel, they can be engineered to be more robust against proteolysis by eliminating amino acid motifs that are prone to clipping (Dorai et al., 2011).

HCPs in the HCCF mainly come from cell lysis (Jin et al., 2010; Tait et al., 2011). Therefore, improving cell viability or preventing cell lysis would be beneficial. A biphasic cell culture method with a temperature shift to mild hypothermia has been employed to improve cell viability and reduce apoptotic cell population, which decreases HCP levels and reduces the number of HCP species, including proteases and chaperones (Goey et al., 2017; Tait et al., 2013). However, the actual physical state of cells at harvest affects cellular response to shear (Tait et al., 2009). Viable cells have been found to be more shear-sensitive than non-viable ones. This means that they are more likely to break and release their contents into the liquid phase during centrifugation and filtration steps. This finding goes against the principle of harvesting cultures at high cell viability (typically 80%) and merits further investigation to better understand and quantify this potential trade-off between viability and membrane robustness.

In addition to the above efforts to reduce HCPs upstream, several studies have tackled the same challenge in DSP. Positively charged depth filters have been employed to clarify HCCF before protein A chromatography (Yigzaw et al., 2006). Adsorption of HCPs onto the depth filters improved clarification of downstream feedstocks, with the quality of protein A eluates improved with a lower degree of precipitation and turbidity, hence, maximizing loading on chromatography columns (Schreffler et al., 2015; Yigzaw et al., 2006).

Flocculation after harvest may reduce host cell DNA and HCPs. Better clarified protein A feedstock with reduced turbidity may be achieved by manipulating the pH of the medium or by adding polyelectrolytes, polymers or filter aids like diatomaceous earth (Brodsky et al., 2012; Capito et al., 2013a; Minow et al., 2014; Peram et al., 2010; Westoby et al., 2011). For instance, pretreatment of crude supernatant with allantoin and ethacridine removed 98% of the DNA, 99% of histones and 70% of HCPs (Gan et al., 2013). Addition of cationic polymers to flocculate negatively charged HCPs and DNA removed 90% of the DNA with an improved HCP clearance through protein A chromatography (Li, 2017). Nonetheless, flocculation may form product aggregates that need to be removed by subsequent DSP operations (Westoby et al., 2011). Other approaches like aqueous two-phase systems (ATPS) to improve HCP clearance have been reported (Campos-Pinto et al., 2017; Gronemeyer et al., 2016; Oelmeier et al., 2011).

Identifying problematic and immunogenic HCPs in drug substance allows the development of a more targeted purification strategy. Customized buffers that disrupt specific HCP-mAb interactions, as summarized in **Table 8**, may be used as wash modifiers of protein A column. For instance, Bee et al. (2015) developed a salt and caprylate buffer with high pH to disrupt cathepsin D-mAb interaction in protein A chromatography. This strategy removed cathepsin D and successfully reduced proteolytic activity in the final drug formulation. Different DSP strategies for HCP removal have been recently reviewed by Li (2017) and will not be covered herein.

An alternative to conventional affinity chromatography is the use of membranes for capture and polishing steps. Jacquemart et al. (2016) investigated the performance of single-use protein A chromatography membranes and compared them to protein A resign columns in terms of efficiency of HCP clearance. They showed that the performance of these two technologies is comparable for four different mAb products and that protein A membranes supported higher flow rates, thus allowing for a faster elution step. They argued that this is a significant advantage in terms of avoiding aggregation taking place a low pH values. These results are particularly interesting in terms of reducing residence times and enabling flexible manufacturing.

7. Analytical methodologies

Table 4 lists the analytical techniques that can identify and quantify HCPs in complex mixtures, together with the advantages and limitations of each. Bracewell et al. (2015) proposed that the selected analytical techniques should be able to (1) detect protein concentrations across a wide dynamic range, (2) follow the dynamics of HCP concentration and population throughout a bioprocess, (3) monitor or measure a complex protein mixture containing multiple protein analytes and (4) monitor or measure low-abundant HCPs in highly purified samples.

7.1. HCP ELISA

HCP enzyme-linked immunosorbent assay (ELISA) is the most common method to quantify HCPs. It is the 'workhorse' in quality control of bioprocesses and testing of clinical materials since it provides a relatively high throughput and sensitive quantification (Zhu-Shimoni et al., 2014). The greatest challenge with HCP ELISA is that a commercially available kit may not accurately quantify a diverse HCP pool within the same assay. The antisera for HCP ELISA are prepared with polyclonal anti-HCP antibodies produced from animals injected with an HCP pool from a null cell line. Consequently, non-immunoreactive or weakly immunoreactive proteins in the animal cannot be detected by the ELISA assays. Limitations of HCP ELISA have been reviewed by Wang et al. (2009) and Zhu-Shimoni et al. (2014).

7.2. 2D-Gels

2D-polyacrylamide gel electrophoresis (2D-PAGE) is a technique orthogonal to ELISA that identifies and quantifies HCPs. Complex protein mixtures are separated by protein properties, i.e., molecular weight and isoelectric point, stained and visualized (Jin et al., 2010; Krawitz et al., 2006; Tait et al., 2011). However, quantifying HCPs with 2D-PAGE is limited by its narrow dynamic range. Low-abundant HCPs cannot be detected, and the presence of recombinant proteins often masks the visibility, hence, compromise the detectability of these HCPs (Hogwood et al., 2013; Jin et al., 2010). Moreover, HCPs that possess physical properties like that of mAb could not be easily separated with 2D-gel. In one case, a CHO catalase monomer with pI close to the humanized Fc protein fragment could not be detected by 2D-PAGE or Western blot. However, it was the main impurity in the final drug substance with a concentration exceeding 100 ppm when quantified with mass spectrometry (Ahluwalia et al., 2017).

7.3. Mass spectrometry

Mass spectrometry (MS) emerges as the leading proteomic analytical technology to detect and quantify low-abundant HCPs with high confidence in the presence of the recombinant product (Aboulaich et al., 2014; Ahluwalia et al., 2017; Goey et al., 2018; Levy et al., 2014; Q. Zhang et al., 2014). Tandem mass spectrometry (MS/MS) is often coupled with liquid chromatography (LC) to rapidly monitor and identify multiple protein analytes in a high throughput manner (Doneanu et al., 2015). Immunogenic and problematic HCPs can also be targeted in highly purified samples once the MS protocol is set up (Schmidt et al., 2009). Therefore, many previously overlooked HCPs in commercial biologic products may be characterized as this technology continues to improve. Nonetheless, highly skilled personnel are required to operate LC-MS/MS. The equipment is expensive, and absolute quantification of individual HCPs requires the use of synthetic peptides that are cost- and labor-intensive (Reisinger et al., 2014). Subsequent peptide validation work also remains a challenge.

8. Perspectives

The research findings discussed herein make a case for the development of an integrated, cohesive bioprocess design methodology that simultaneously optimizes upstream and downstream operations. The use of mass spectrometry has provided evidence that cell culture conditions play a crucial role in determining not only cell health and HCP concentration but also HCP composition. In turn, these factors significantly affect the performance of DSP steps. Approaches involving quality

by testing (QbT) may only detect problems *a posteriori* and often only look at the overall concentration of HCPs rather than composition. Recent examples of patients developing anti-CHO HCP immune responses during clinical trials clearly demonstrate that QbT is not sufficient. Moving towards the application of QbD, we have at our disposal novel analytical technologies, 'omics databases and computational tools for immunogenicity assessment of individual species, which together can support data- and knowledge-driven process design. Such efforts need to consider upstream and downstream operations together during the design and optimization phases, as changes that may be thought to be beneficial for USP can create bottlenecks downstream with significant time and cost implications. We also have the increasing ability to tune cellular behavior and genetically engineer problematic HCPs out. This can be done on a case-by-case basis or by engineering new host cell lines that are minimal CHO cell chassis. It is likely that successful approaches to designing high-performing, robust and cost-effective bioprocesses will involve both process and cell engineering intervention and will increasingly involve more focus on manufacturability earlier on in the design phases.

Engineering practice can offer tested, reliable tools for design, optimization and control. To this end, statistical and computational approaches to explore the possible process operating range and identify favorable conditions with respect to HCP content offer great potential. An obvious first step would be the use of Design of Experiments (DoE) to quantitatively prove the effect of critical process parameters such as media composition, culture temperature and pH on cell health and product CQAs. Following experimentation, statistical analysis, e.g., principal component analysis (PCA), can be performed to identify clusters of cellular behavior. Gronemeyer et al. (2016) used a DoE approach to investigate the effect of upstream conditions such as medium composition on cell growth, mAb titer and HCP concentration. The presence of three components, folic acid, ascorbic acid, and glycine, were found to reduce HCP concentration in the supernatant. The authors were able to recommend specific concentrations of insulin, thiamine and magnesium chloride to minimize HCP concentration in the system under investigation. Moving towards whole bioprocess design, we can use such approaches to quantify the effect of upstream process parameters on downstream performance. To this end, the impact of dissolved oxygen concentration, temperature and pH at the cell culture step on product CQAs in DSP was investigated by Agarabi et al. (2017) using DoE. Although the results revealed only subtle changes to these outputs for the particular antibody investigated, the methodology put forward in this study by the U.S. Food and Drug Administration showcases how systematic approaches can be used to explore the entire bioprocess design space.

Complete characterization of the design space and optimization of product to impurities ratio through experiments can be prohibitively expensive and time-consuming. Although expensive to develop, deterministic mathematical modeling has a mechanistic basis and could, in theory, be used for extrapolation. In conjunction with Process Analytical Technology (PAT), it can help deepen our understanding of the dynamic cellular behavior and extracellular HCP composition and, hence, assist process optimization with regards to HCP reduction in HCCF and efficient removal downstream. Inherent biological variability poses an impediment to building predictive models of bioprocesses, however. Nonetheless, population heterogeneity can be accounted for using population balance or cell ensemble modeling (Fadda et al., 2012; Y. Liu and Gunawan, 2017). We can envisage that such an approach would be the first step towards the development of mathematical descriptions of the secretion or release of specific HCP species from various cell subpopulations during antibody production and processing and, in the long term, the development of predictive whole bioprocess models that can be used for the simultaneous optimization of multiple unit operations subject to both product yield and quality constraints.

Conflicts of Interest: The authors have no conflict of interest to declare.

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Tables

Table 1. Key references for establishing a CHO 'Omics reference state. Information obtained from Lewis et al. (2016)

Reference	Technique	Institution(s)	Summary
Xu et al. (2011)	Genomics	BGI-Shenzhen, GT Life Sciences, Peking University, University of Delaware, Technical University of Denmark, Stanford University, Johns Hopkins University, University of Copenhagen	First publicly available draft sequence of the CHO-K1 genome.
Lewis et al. (2013)	Genomics	CHOmics, BGI-Shenzhen, BGI Europe, Cytogen Research and Development, Brandeis University, GT Life Sciences, Johns Hopkins University, Technical University of Denmark, University of Copenhagen, King Abdulaziz University	First publicly available draft sequence of the Chinese hamster. Six draft genomes of CHO cell lines derived from CHO-K1, DG44, and CHO-S lineages.
Becker et al. (2011) Rupp et al. (2014)	Transcriptomics	Bielefeld University, Universität für Bodenkultur Wien, Austrian left of Industrial Biotechnology, Justus-Liebig-University	Publicly available CHO cell cDNA libraries. Special emphasis on central sugar metabolism and N-glycosylation.
Bort et al. (2012)	Transcriptomics	University of Natural Resources and Applied Life Sciences, Austrian Center of Industrial Biotechnology	Examined expression of mRNA and miRNA over batch culture time course, including lag, exponential and stationary phases.
Baycin-Hizal et al. (2012)	Proteomics	Johns Hopkins University, Vanderbilt University, University of California San Diego, Technical University of Denmark	First publicly available CHO proteome, identified more than 6,000 expressed proteins.
Slade et al. (2012)	Proteomics	Life Technologies	Identified 352 secreted proteins from CHO-S and DG44 cell lines.
Lim et al. (2013)	Proteomics	Bioprocessing Technology Institute, National University of Singapore	Identified secreted proteins in CHO-K1 fed-batch process.
Levy et al. (2014), Valente et al. (2014, 2015)	Proteomics	The University of Delaware	Quantification and characterization of CHO HCP.
Kumar et al. (2015)	Proteomics	Johns Hopkins University, National Institute of Health, Technical University of Denmark, Brigham Young University, University of California, Johns Hopkins School of Medicine, MedImmune Way	Quantification and characterisation of CHO supernatant-ome (CHO-SO) in CHO-K1 cell line.
North et al. (2010)	Proteomics	Imperial College of London, Albert Einstein College of Medicine	Characterised glycosylation patterns of expressed proteins in nine lectin-resistant CHO cell lines.
Tep et al. (2012)	Proteomics	Biogen Idec, Northeastern University	Developed an MALDI-TOF MS method to quantify glycomic changes in CHO, applied to bioreactor campaign.

825 **Table 2.** Proteases and glycosidases previously reported in the literature

Name	Function	Samples	Reference
Metalloproteinases	Degrade broad range of substrates	HCCF	(P. Elliott et al., 2003)
		Post-Protein A	(Robert et al., 2009)
		Post-ion exchange	(Sandberg et al., 2006)
Cathepsin D	Active aspartyl protease	Post-Protein A	(Robert et al., 2009)
		Final mAb formulation	(Bee et al., 2015)
Cathepsin B	Active aspartyl protease	Post-Protein A	(Aboulaich et al., 2014)
Serine protease (HTRA1)	N-terminal clipping	HCCF	(Dorai et al., 2011)
		Post-Protein A	(Bee et al., 2015; Q. Zhang et al., 2014)
		Post-ion exchange	(Pezzini et al., 2011; Sandberg et al., 2006)
Sialidases	Hydrolyse the oligosaccharide of glycoprotein	HCCF	(Gramer and Goochee, 1993)
Legumain	Lysosomal cysteine protease. Activates other proteases, such as Cathepsin B, H and L	Post-ion exchange	(Joucla et al., 2013)

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827 **Table 3.** Notable HCPs causing aggregation problem

Name	Function	Samples	Reference
PDI	Reduce disulphide bonds	Post-ion exchange	(Maeda et al., 2007)
BiP (78kDa glucose-regulated protein)	Folding and assembling proteins in the endoplasmic reticulum (ER)	Post-ion exchange	(Joucla et al., 2013)
DnaK (Heat shock protein)	Bind hydrophobic regions on unfolded proteins	Post-protein A	(Ratanji et al., 2017)

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829 **Table 4.** Analytical techniques for HCP monitoring and quantification. Information gathered from Hogwood et al. (2014) and Tscheliessnig et al. (2013)

Aim	Analytical technique (Limit of detection)	Application	Limitations (Limit of detection)	Examples
Visualisation of HCPs	2D-PAGE and 2D-DIGE (0.03-52.0ng/protein)	Study the dynamics of HCP profile	Only the most abundant proteins are observed Masking effect of product Labour intensive Concerns of multiproduct assays	(Hogwood et al., 2013; Jin et al., 2010; Tait et al., 2013)
	Western blotting (0.1-0.5ng/protein)	Observe HCP profile using anti-HCP sera	Labour intensive Concerns as to what is used as the immunogen to generate antibodies (e.g. null cell line, host, fractionated pools?) No information on HCP identity	(Beatson et al., 2011; Grzeskowiak et al., 2009)
	SELDI-TOF	Rapid monitor	No information on HCP identity	(Tait et al., 2013)
Quantitation of HCP amount	ELISA (1.0ng/mL)	Measure total HCP levels	No information on HCP identity	(Jin et al., 2010; Tait et al., 2011)
	Western blotting (0.1-0.5ng/protein)	Specific HCP levels relative to other samples	Limited comparability across samples	(Tscheliessnig et al., 2013)
	FT-MIR	Quantitative determination of HCPs in situ	Limit of quantitation unknown	(Capito et al., 2013b)
	LC-MRM	High-throughput quantification		(Doneanu et al., 2012)
Identification of specific HCPs	LC-MS/MS (1-50 fmol)	Can be coupled with 2D-PAGE to identify specific HCPs	Labour intensive if coupled to 2D-PAGE	(Reisinger et al., 2014)
	2D-LC/MS (50 ppm)	Good coverage and identification possible	Time consuming	(Doneanu et al., 2012)
	Western blotting (0.1-0.5ng/protein)	Confirm presence of specific HCPs	Expensive if multiple antibodies required Requires target to be immunogenic and antibodies present in sera to identify a specific HCP	(Tscheliessnig et al., 2013)

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832 **Table 5.** Different types of interaction between HCPs and mAb

HCP-mAb interactions	Reference
Hydrophobic interaction	(Chollangi et al., 2015; Levy et al., 2016; Sisodiya et al., 2012)
Electrostatic repulsion	(Chollangi et al., 2015; Shukla and Hinckley, 2008)
Hydrogen bond	(Chollangi et al., 2015)
Van der Waal's force	(Chollangi et al., 2015)
Ionic interaction	(Pezzini et al., 2011)
Presence of immunoglobulin-like domains	(Pezzini et al., 2011)

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835 **Table 6.** Notable HCPs that co-elute through protein A chromatography.

Function(s)	Protein name	Reference
Proteolysis	Cathepsins	A, C and L
	Matrix metalloproteinase	A
	Serine protease HTRA1	A, Z1 and Z2
Catalytic enzymes	Protein disulphide isomerase (PDI)	A
Structural molecules	Actin	A, L, Z1 and Z2
	Clusterin	A, L, Z1 and Z2
	Vimentin	A
	Nidogen	L
Metabolism	Lipoprotein lipase	L
	Pyruvate kinase	C, Z1 and Z2
	Fructose-bisphosphate aldolase A	C
	Alpha-enolase	Z1
Chaperone	Heat shock protein	C, L, Z1 and Z2
	Peptidyl-prolyl cis-trans isomerase	Z2
Homeostasis	Peroxiredoxin	C, L, Z1 and Z2
	Glutathione S-transferase	Z1 and Z2
Protein synthesis	Elongation factor	Z1 and Z2
Signalling	Thrombospondin 1	Z1 and Z2

836 ***A: Aboulaich et al. (2014)**, Null cell line CHO, harvest day and cell viability at harvest
837 were not reported; **L: Levy et al. (2014),** Null cell line CHO K1; harvested on day 3 or 4
838 at cell viability of 97-99%; **C: Chiverton et al. (2016),** mAb producer CHO S, harvested
839 on day 15 at cell viability of approximately 52%; **Z1: Zhang et al. (2014)**, mAb
840 producer CHO cell line, harvest day and cell viability at harvest were not reported; **Z2:**
841 **Zhang et al. (2016),** Null cell line, harvest day and cell viability at harvest were not
842 reported.

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844 **Table 7.** Summary of major factors that influence the HCP profile of CHO-derived recombinant
845 products during the manufacturing process. Modified from Hogwood et al. (2014)

Parameter influencing HCPs present		References
Upstream processing	Amino acid sequence of target molecule	(Levy et al., 2014; Shukla et al., 2007)
	Cell line selection/selected	(Krawitz et al., 2006; Yuk et al., 2015)
	Fermentation processes (feeding, temperature)	(Goey et al., 2017; Jin et al., 2010; Park et al., 2017; Tait et al., 2013)
	Cell viability at harvest	(Grzeskowiak et al., 2009)
	Cell health	(Goey et al., 2017)
	Culture duration	(Farrell et al., 2015; Tait et al., 2011; Valente et al., 2014)
	Medium formulation	(Gronemeyer et al., 2016)
Downstream processing	Cell robustness/shear sensitivity during centrifugation/collection of cell culture harvest fluid	(Tait et al., 2013)
	Primary and secondary clarification	(Hogwood et al., 2013)

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848 **Table 8.** Different wash modifiers of Protein A chromatography. Typical concentrations of wash
849 modifiers are obtained from Li (2017)

Wash modifiers	Concentration	HCP-mAb disruption	Reference
Arginine	0.1 – 1.0M	Electrostatic and hydrophobic interactions, and hydrogen bonding	(Borders et al., 1994; Chollangi et al., 2015; Sun, 2013; Thomson et al., 2017)
CHAPs	1%	Not available	(Aboulaich et al., 2014)
Guanidine HCl	1M	Not available	(Chollangi et al., 2015)
Isopropanol	5-20%	Reduce hydrophobic interactions. Cons: organic solvent	(Shukla and Hinckley, 2008)
Propylene glycol	5-20%	Hydrophilic, stabilise proteins by altering solvent environment; break down non-specific interactions between mAb and HCPs	(Chollangi et al., 2015)
Tetramethylammonium chloride (TMAC)	0.5M	Not yet established	(Sun, 2013)
Triton X-100	$\leq$ 1%	Similar to the functions of propylene glycol	(Chollangi et al., 2015)
Tween-80	1%	Not available	(Shukla and Hinckley, 2008)
Sodium caprylate	50mM	Compete with HCPs for the binding sites on antibody	(Gruber et al., 2016)
Sodium chloride	0.1-2M	Not available	(Aboulaich et al., 2014)
Urea	0.5-3M	Hydrogen bond breaker, denatures proteins at high concentration of >2M	(Chollangi et al., 2015; Shukla and Hinckley, 2008)

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Figure Legends

Figure 1. Proposed workflow for ‘omics technologies in bioprocessing. Modified from Lewis et al. (2016)

Figure 2. Optimisation areas and parameters in upstream and downstream processing. Modified from Gronemeyer et al. (2014)

Figure 3. QbD development approach. QTPP, quality target product profile; RA, risk assessment; CQA, critical quality attribute; CPP, critical process parameters. Modified from Eon-Duval et al. (2012b).

Figure 4. Quality by Design approach to HCP removal and current research gaps. Green arrows show a systematic and comprehensive study of HCP profile in relation to upstream cell culture condition. Blue arrows show the established research field regarding the impact of upstream process conditions and HCP profile at harvest and after purification. Red arrows depict the missing links between these performance attributes necessary to create a design space with the QbD approach.

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