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Host Cell Proteins During Biomanufacturing

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12.1 Introduction

During biopharmaceutical manufacturing production culture, cells produce and secrete recombinant proteins into the culture medium along with many types of impurities. The International Council for Harmonisation (ICH) quality guideline (Q6B – specification) states that "process-related impurities encompass those that are derived from the manufacturing process, i.e. cell substrates (e.g. host cell proteins (HCPs) and host cell DNA)" and that "product-related impurities (e.g. precursors and certain degradation products) are molecular variants arising during manufacture and/or storage" [1]. Host cell-derived impurities include DNA, proteins, lipids, and metabolites, and they should be removed through downstream purification processes. Of the impurities listed above, HCPs can impose a challenge to downstream purification processes because they may exhibit purification-related properties similar to biopharmaceutical proteins. HCPs include both secreted proteins and intracellular proteins, as intracellular proteins can be released from dead cells during production cultures (e.g. fed-batch and perfusion cultures) or harvesting steps.

The scope of this review will encompass current HCP removal processes, the impact of residual HCPs, as well as HCP detection, quantification, and monitoring methods during biomanufacturing processes. Strategies for effective HCP removal and future directions for HCP risk management are also discussed.

12.2 Removal of HCP Impurities

Current HCP removal processes involve a series of bioseparation methods, such as centrifugation, filtration, chromatography, and precipitation. For antibody products, including monoclonal antibodies (mAbs) and Fc-fusion

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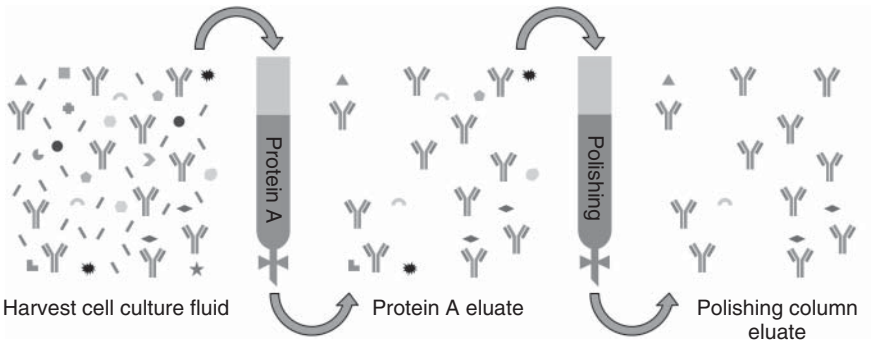

Figure 12.1 Simplified scheme for downstream purification process.

proteins, HCPs are typically removed through a platform purification process (Figure 12.1). For non-antibody products, the process to remove HCPs can vary, depending on the host cell type and product molecular properties. Although the majority of HCPs can be cleared through downstream purification processes, there are still some HCPs that are difficult to remove from the drug substance or drug product.

12.2.1 Antibody Product

As the molecular properties of mAbs and Fc-fusion proteins are highly conserved from product to product and they share more than 95% amino acid sequence homology in their fragment-crystallizable (Fc) regions, a platform process is often employed to remove HCPs and other impurities (Figure 12.1). The three major steps of the process include clarification of the harvested cell culture fluid (HCCF), an initial capture step with protein A chromatography, and a few subsequent polishing steps, often with ion exchange chromatography (IEX) and/or hydrophobic interaction chromatography (HIC) [2, 3].

The clarification of the HCCF consists of a centrifugation step to remove cells and larger cell debris, followed by a depth filtration step to remove small cell debris [3]. The depth filtration step is also effective in HCP clearance through a combination of electrostatic and hydrophobic adsorptive interactions between the depth filter and proteins [4, 5]. However, the majority of HCP removal is realized by the following capture step with protein A chromatography [6]. Protein A, a cell-wall-associated protein on the surface of the bacterium *Staphylococcus aureus*, has a high binding affinity to the Fc region of the mAb or Fc-fusion protein [7]. Additional characteristics, such as stability over a wide pH range [8] and the ability to maintain functional performance after repeated cleanings [9], also add to the functionality and effectiveness of protein A chromatography.

Although the majority of HCPs are removed during protein A capture, additional orthogonal polishing steps are still necessary to further lower the total HCP concentration. There are two types of IEX: anion exchange chromatography (AEX) and cation exchange chromatography (CEX). AEX is usually operated in flow-through mode as mAbs typically have a net positive charge at neutral pH and do not bind to the resin, whereas HCP impurities adsorb to the

resin [10]. CEX is often operated in bind-and-elute mode, where the positively charged mAb binds to the resin and the impurities flow through the column. After loading, the mAb is eluted from the column at higher salt concentrations. It has been reported that CEX can reduce HCP levels from 300–400 ppm to approximately 10 ppm [11]. Besides IEX, HIC has also been adopted as a polishing step to remove HCP impurities. It is usually operated in bind-and-elute mode, and high salt concentration is used to load the protein in HIC to promote hydrophobic binding, whereas low salt concentration is used to elute the protein from the column [12]. Hunter et al. reported that one HIC unit operation could reduce HCP concentrations from 10 000 ppm to approximately 300 ppm [13].

12.2.2 Non-antibody Protein Product

Although the downstream processing of mAbs relies on a relatively rigid platform, the purification of other biopharmaceutical products (e.g. insulin, erythropoietin (EPO), and interleukins (ILs)) varies, depending on the host cells and drug product characteristics.

As a therapeutic drug to treat diabetes, insulin is produced predominantly either in *Escherichia coli* or *Saccharomyces cerevisiae* [14]. In the *E. coli* production platform, insulin is intracellularly overexpressed and then solubilized and renatured to obtain fully functional proteins. Immunoglobulin G (IgG) sepharose affinity chromatography is used as a capture step, followed by preparative reverse-phase chromatography to finally recover the product [15]. In the *S. cerevisiae* production platform, insulin is secreted into the culture medium and CEX is used as a primary capture step, followed by a series of separation methods. Among them, ethanol precipitation selectively targets HCP removal by using an ethanol concentration at which HCPs precipitate while insulin remains soluble [16].

EPO, a growth factor for the treatment of anemia related to kidney disease, is primarily produced in mammalian cells. Several industrial-scale purification processes have been established to remove impurities from the EPO product. All of these methods involve clarification, primary capture chromatography, and subsequent polishing chromatography steps. The various molecular properties of EPO enable multiple choices of capture step. For example, Zanette et al. used phenylboronate agarose (PBA) to capture EPO based on the ability of PBA to form reversible complexes with 1,2-*cis*-diol-containing molecules [17]. On the other hand, blue sepharose affinity chromatography [18] and IEX [19] have also been applied as a capture step for EPO purification.

ILs are a family of proteins that stimulate and regulate the cells involved in immunity and inflammation. Because of the variety of ILs [20], the expression system and purification process are highly specific depending on which IL is being produced. For example, IL-7 has been expressed in *E. coli* cells for a 1000 l fermentation scale and purified with a series of HIC and IEC columns [21]. On the other hand, IL-12 has been produced in mammalian cells and recovered in a single step with heparin sepharose affinity chromatography [22].

12.2.3 Difficult-to-Remove HCPs

Although downstream processing of the product can significantly reduce total HCP levels to meet the criteria required by the Food and Drug Administration (FDA), the persistence of some difficult-to-remove HCPs continues to challenge the entire purification process and jeopardizes drug efficacy and quality.

There are at least three routes by which HCPs can challenge downstream processing. The first route refers to HCPs with variable expression during extended cell culture, as the composition of HCPs generated during upstream processing has been shown to affect downstream purification [23]. A proteomics approach identified 92 extracellular HCPs from Chinese hamster ovary (CHO) cells exhibiting up to 48-fold changes in protein expression over 500 days of cell culture [24]. The second route is HCP association with the product, where "hitchhiker" HCPs bind to the product, especially an antibody product, and are carried along throughout the purification process. This HCP-mAb interaction is considered to be the primary cause of HCPs persisting through protein A chromatography; a batch chromatography binding study exhibited substantial differences in HCP profiles of the protein A eluate between null HCCF and mAb-containing HCCF [25]. Additionally, several groups have identified individual HCP compositions in the protein A eluate [26–29] with proteomics techniques, and the most commonly observed HCPs may be worthy of special consideration. The third route refers to co-elution of HCPs with the product, as some HCPs can bind to the chromatography resin ligands during the loading step and can be eluted together with the product. Co-elution has been shown to occur during polishing chromatography steps, including HIC and IEX [28, 30].

12.3 Impacts of Residual HCPs

Despite all efforts to remove HCPs from drug substance, the complete elimination of HCPs remains a challenge. In addition, HCPs have the potential to negatively impact drug quality and efficacy, thereby establishing HCPs as a critical quality attribute (CQA). According to the ICH guidelines (Q8), "a CQA is a physical, chemical, biological, or microbiological property or characteristic that should be within an appropriate limit, range, or distribution to ensure the desired product quality" [31]. Moreover, residual HCPs in the final drug product can pose safety concerns to patients or reduce product shelf life by degrading the product or other components in the formulation.

12.3.1 Drug Efficacy, Quality, and Shelf Life

Either HCPs in the HCCF before downstream purification processes or residual HCPs in the drug substance or drug product after purification processes can affect drug efficacy, quality, and shelf life. As HCCF samples are collected near the end of a typical fed-batch culture when cell viability is about 70–80%, dead cells release HCPs into the culture medium, allowing various enzymes to catalyze metabolic reactions that can modify biochemical properties of recombinant

proteins. For example, the amount of sialidase, an enzyme that removes terminal sialic acid residues from *N*-glycans, is known to increase in the culture medium during later days of batch or fed-batch cultures [32]. Although sialidases appear to be removed during the purification process, and no detectable amount of sialidase in the drug substance or product has been reported, they can catalyze the removal of terminal sialic residues from the *N*-glycans of product proteins (e.g. antithrombin) and decrease sialic acid levels before their removal through downstream purification [32]. *N*-glycosylated proteins without sialic acid residues expose terminal galactose residues, which facilitates binding to the asialoglycoprotein receptors expressed in the liver, causing faster protein clearance [33]. Another example is the cleavage of C-terminal lysine residues of IgG1 by carboxypeptidase D [34]. Charge variants of mAbs, resulting from lysine removal, may affect stability and biological activity [35].

Residual HCPs in the drug substance or product can also have adverse effects on drug efficacy and shelf life. Many studies have reported degradation of antibody products over time, resulting in a decrease in drug efficacy as well as an increase in risk due to potential immunogenicity against cleaved antibody fragments [36, 37]. Proteases, such as cathepsin D, have been identified and shown to contribute to antibody cleavage and degradation [36, 38]. Additionally, studies have reported that lipase classes, such as lipoprotein lipase and phospholipase B-like2, degrade lipid components, such as polysorbate 20/80, in the final drug formulation [39, 40]. As these lipid additives are used as a stabilizer, a decrease in the concentration of these components can lead to a shorter drug shelf life.

12.3.2 Immunogenicity

The foremost problem with residual HCPs as foreign (exogenous) proteins is that they can trigger an immune response in patients when the drug is administered. Immune responses to foreign proteins occur via T-cell-dependent pathways, whereby proteins are taken up, digested, and presented by antigen-presenting cells, then recognized by T-cells, followed by further activation and maturation of B cells expressing complementary antibodies. Although the scale of CHO HCP immunogenicity is smaller than that elicited from the HCPs of nonmammalian organisms such as *E. coli* or yeast [41], sequence differences between CHO and human proteins are substantial. A recent study reported that only 20% of the CHO proteome has higher than 90% sequence homology to human, whereas over 60% of the proteome has less than 50% sequence homology to human [42]. Indeed, two clinical trials have been canceled because of the adverse CHO HCP-associated immune responses in patients [43, 44]. Besides direct immunogenicity, HCPs have the potential to induce and augment antidrug antibodies or induce an immune response to an endogenous protein [37].

12.3.3 Biological Activity

Beyond immunogenic issues, residual HCPs can have negative biological impacts on patients [45]. Although many HCPs are inactive in the drug substance or product, some HCPs can maintain their biological function and induce unintended

activities. For example, Beatson et al. reported that transforming growth factor β1 (TGFβ1) proteins expressed in CHO are functional, carried through purification steps, and can act on human cells [46]. As TGFβ1 is a multifunctional cytokine with a highly conserved protein sequence, active TGFβ1 can impact a variety of cellular processes in patients, including cell growth, wound healing, apoptosis, and immunosuppression [47–49]. Moreover, it is also possible that other biologically active HCPs, such as cytokines and autocrine signaling factors, can be expressed in CHO cells under certain conditions [50], thereby causing other unintended clinical effects, such as hypersensitivity, toxicity, and cell signaling.

12.4 HCP Detection and Monitoring Methods

There is no well-established pharmacological evaluation for an acceptable, or safe, range of HCPs, mainly because of the heterogeneity and variety of HCPs. For this reason, robust purification processes that can achieve the lowest or "undetectable" residual HCP amount are desirable whenever possible; therefore, the use of sensitive and appropriate HCP detection and monitoring methods is critical. According to the ICH guideline Q6B, "a sensitive assay, e.g. immunoassay, capable of detecting a wide range of protein impurities is generally utilized" for the detection of HCPs [1]. However, immunoassays have their own limitations, requiring orthogonal approaches to fully characterize and monitor HCPs in the drug substance and product (Table 12.1).

12.4.1 Anti-HCP Antiserum and Enzyme-Linked Immunosorbent Assay (ELISA)

Currently, enzyme-linked immunosorbent assay (ELISA) is the industry "gold standard" for the detection of HCPs because of its high sensitivity (0.5–1 ng/ml, [51]), coverage, and throughput [37, 52]. The key component of this immunoassay are polyclonal antibodies against HCPs generated by "immunization with a preparation of production cells minus the product-coding gene, fusion partners, or other appropriate cell lines" [1]. Polyclonal antibodies are typically raised in animals such as rabbit, goat, or chicken. Although individual HCPs are not identified, polyclonal anti-HCP antibodies can capture most, and theoretically all, proteins in a given HCP pool. In brief, a typical ELISA follows a series of steps as described next (Figure 12.2). (1) An assay plate is coated with polyclonal anti-HCP antibodies. (2) Samples potentially containing HCPs are loaded into coated wells. (3) The bound HCPs are recognized by anti-HCP antibodies (primary antibody). (4) HCP–antibody detection is amplified with a biotin–avidin complex on primary antibodies or a secondary antibody conjugated with an enzyme, such as alkaline phosphatase or horseradish peroxidase, that catalyzes substrates into fluorescent signals. Although the immunoassay is a widely accepted HCP detection method and generic HCP detection ELISA kits are available, they do have certain limitations. First, because of the heterogeneous expression levels of individual HCPs and various binding affinities between

Table 12.1 Methods for HCP detection and quantification.

Technique	Application	Strengths	Limitations	References
ELISA	Total HCP level quantitation	High sensitivity Simple procedures	No information about individual HCP identity No detection or biased binding to some HCPs	[52, 53]
1D- or 2D-PAGE	Study changes in HCP expression patterns	Visual investigation of protein isoforms and modifications	Low sensitivity	[4, 13, 54, 55]
DIGE (2D-PAGE with labeling)	Study changes in HCP expression patterns	Visual investigation of protein isoforms and modifications Better normalization than 2D PAGE	Low sensitivity	[56, 57]
LC–MS	Identification of individual HCPs	High sensitivity Identification of HCPs	No information about protein isoforms and modifications	[6, 55, 60, 61]
iTRAQ (LC–MS with labeling)	Identification and quantitation of individual HCPs	High sensitivity Identification of HCPs Quantitative comparison of the entire proteome between samples	No information about protein isoforms and modifications Limited number of samples per run (4-plex or 8-plex)	[28]
MRM	Quantitation of targeted individual HCPs	High sensitivity Quantitative comparison of multiple HCPs across multiple samples	No information about protein isoforms and modifications Prior knowledge about target is necessary	[40]

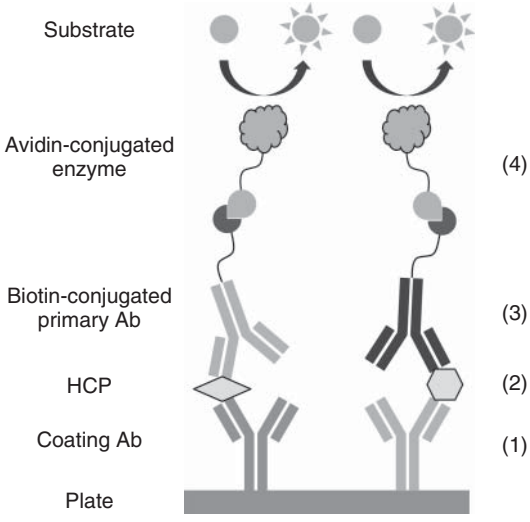

Figure 12.2 Schematic of typical ELISA for HCP quantitation. Numbers in the parentheses represent the ELISA procedures described in Section 12.4.1.

individual proteins and antibodies, the overall HCP measurement can be biased (i.e. more antibodies against the most abundant HCPs), rather than reflecting the true HCP amount [37]. Secondly, some HCPs are not detected because antibodies are not necessarily made against all HCPs. For example, one HCP, glutathione S transferase-α (GST-α), in the drug substance was detected by capillary electrophoresis-sodium dodecyl sulfate (CE-SDS) but not by HCP ELISA [53]. Finally, variations in cell line, product, or bioprocessing can affect HCP profiles, leading to biased immunoassay measurements [37]. Therefore, the development of manufacturing process-specific assays should be initiated for products in the later phases (phase III or commercial) of the pipeline; however, they require a substantial amount of time and effort. These limitations emphasize the importance of orthogonal approaches, such as protein identification by electrophoresis and/or mass spectrometry.

12.4.2 Proteomics Approaches as Orthogonal Methods

As complementary methods to immunoassay, protein separation and visualization by one-dimensional (1D) and two-dimensional (2D) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) have been utilized [4, 13, 54, 55]. HCP-containing samples are separated by molecular weight (1D) or by both molecular weight and isoelectric point (2D) and subsequently visualized by gel staining or by immunoblotting. In addition, 2D-differential in-gel electrophoresis (DIGE) allows comparison of individual protein amounts and reduces gel-to-gel variations by running multiple samples labeled with different fluorescent dyes in one gel [56, 57]. Although these methods can be combined with mass spectrometry for spot identification, they have relatively poor sensitivity (8–52 ng protein by Coomassie staining and 0.3–1 ng protein by SYPRO Ruby staining) and therefore are only effective for the characterization of abundant proteins [51, 58].

Non-gel-based proteomics approaches, such as liquid chromatography–tandem mass spectrometry (LC–MS/MS), have much better sensitivity (0.92–46.2 pg, converted from 1 to 50 fmol [51], based on a report that the average length of trypsin-digested peptide is 8.4 amino acids [59]) and throughput [6, 51, 55, 60, 61]. Notably, isobaric tags for relative and absolute quantitation (iTRAQ) and multiple reaction monitoring (MRM) have recently been adopted to detect and monitor HCPs. In these methods, enzyme-digested peptide samples (either labeled or unlabeled) are separated by liquid chromatography, followed by MS/MS analysis [62]. iTRAQ allows comparison of multiple samples (4-plex or 8-plex) in a single run by using different isobaric labeling tags, while MRM enables quantification of multiple target proteins across multiple samples by selecting precursor ions of interest during the first MS stage and by identifying the selected precursor ions during the second MS stage [28, 40].

12.5 Efforts for HCP Control

Given that the impact of individual residual HCPs is not fully understood at present and that it is uncertain whether a 100 ppm range is acceptable, robust and

Table 12.2 Approaches for HCP removal.

	Stages	Approaches	References
Upstream processing	Cell line development	Knockout of critical HCPs	[34, 40]
	Cell culture	Sustaining high cell viability (e.g. adjustment of harvest time)	[64, 65]
Downstream processing	Harvest clarification	Selecting depth filter with high HCP removal capacity	[4]
	Protein A chromatography	Column wash to disrupt HCP–product interactions	[68, 69]
	Polishing steps	Alternative operating conditions (e.g. pH gradient elution)	[70]
		New modes of chromatography (e.g. mixed mode chromatography)	[71, 72]

effective removal of HCPs from the drug substance and drug product is imperative. In addition, to achieve effective removal or control of HCPs, it is of great importance to identify which factors affect HCP profiles during manufacturing processes. Many studies have implicated a variety of factors, such as product type, cell viability, culture conditions, and chromatography resins, in impacting the HCP profile in both upstream and downstream processes (Table 12.2). Additionally, approaches to assess and predict HCP-associated risks that can adversely affect product efficacy and quality are currently in development.

12.5.1 Upstream Efforts

Studies have shown that many variables in the upstream process, such as host cell line selection, cell age, products (amino acid variations between biopharmaceutical proteins), culture conditions, media and feeding supplements, and harvest time, can affect HCP profiles in the HCCF [2, 24, 56, 63]. Yet the most important culture parameter impacting the HCP profile is cell viability at harvest [55, 56]; the relative abundance of intracellular HCPs in the HCCF shows a substantial increase at the later days of a production culture (i.e. at low cell viability) because dead cells lyse and release intracellular components including proteins. Indeed, 2D-DIGE studies confirmed that cell viability exhibited greater impact on the HCP expression pattern than other culture variables, such as media components, feeding strategy, temperature shift, and different clones expressing the same product [56, 57]. Considering the impact of cell viability on HCP profiles, efforts have been made to sustain high viability during the culture until harvest, including overexpression of antiapoptotic genes to prevent programmed cell death, shift to a lower temperature, and adjustment of harvest time [64, 65]. However, it has also been shown that the amount of extracellular HCPs accumulates substantially (1–2 g/l on day 14) throughout the culture with high (>70%) cell viabilities [63], suggesting that sustaining high cell viability

may not be sufficient to control HCPs. Moreover, as cell age-dependent changes in HCP expression patterns eventually challenge downstream purification processes, the ideal solution is to completely remove HCPs that are detrimental to the drug efficacy and/or difficult to remove.

With the recent development of genome editing tools, knockout of critical HCPs has been reported. For example, carboxypeptidase D, an HCP responsible for C-terminal lysine cleavage of antibodies, was knocked out using clustered regularly interspaced short palindromic repeats (CRISPRs)/CRISPR-associated 9 (Cas9) technology, leading to the complete removal of C-terminal lysine heterogeneity [34]. In addition, lipoprotein lipase, which persisted through downstream purification processes and caused cleavage of lipid components in the final formulation, was knocked out using both transcription activator-like effector nucleases (TALENs) and CRISPR/Cas9 technology [40]. Although these examples demonstrate the applicability of genome editing tools for HCP removal, there are considerations when performing gene knockouts. Studies have reported that the knockout of one gene can result in the activation of other genes to restore phenotype (gene function), a phenomenon referred to as genetic compensation [66]. Therefore, if one attempts to knockout a gene encoding a particular enzyme, the gene expression level of other enzyme genes in the same family should be carefully examined. Additionally, it is important to choose target HCPs that are not essential to cell growth and survival, or protein production.

12.5.2 Downstream Efforts

Given the persistence of some difficult-to-remove HCPs during protein A and polishing chromatography steps, various downstream strategies for effective HCP removal have been proposed [58, 67]. For the protein A capture step, as mAb-HCP interactions were proven to be the primary cause of HCPs entering the protein A elution pool, disrupting these interactions allows HCPs to flow through the column and achieves better separation. Post-load washing, as an intermediate step between loading and elution, is currently a principal way to dissociate HCPs from the product. As HCPs can bind to the mAb through various mechanisms, including electrostatic interaction, hydrophobic interaction, and hydrogen bonding, different wash buffers have been used to disrupt this binding. For example, the work by Chollangi et al. [68] showed that a column wash with basic buffer ($pH \geq 8$) can be effective in improving HCP removal by anionizing both mAbs and the majority of HCPs so that they exhibit repulsive interactions. Additionally, additives such as arginine, isopropanol, and sodium chloride were also shown to significantly reduce HCP levels in a protein A elution pool by disrupting one or several interaction mechanisms between HCPs and mAbs [68, 69]. For polishing steps, strategies were focused on optimizing the conventional chromatography methods (IEX and HIC), as well as developing new modes of chromatography. For example, during CEX, as an alternative to salt gradient elution, pH gradient elution has been applied to mAbs and was shown to remove substantial amounts of HCPs [70]. Recently, mixed mode chromatography is gaining popularity as a polishing step to clear HCPs [71]. The mixed mode resin can adsorb the proteins through more than one mode of

interaction, resulting in higher selectivity and specificity. For example, Capto Adhere, a particular mixed mode resin, has been reported to achieve 99% HCP clearance to achieve a final level below 10 ppm for a mAb product [72].

12.5.3 HCP Risk Assessment in CHO Cells

Despite all the aforementioned efforts, it is impractical to evaluate the impact of each individual HCP and to fully control them because of insufficient knowledge regarding the CHO proteome. Although about 24 000 genes have been identified from the current Chinese hamster and CHO cell annotations [73], biological function and protein expression level of these genes remain largely unknown. Moreover, a single gene can be translated into multiple protein isoforms, and a single protein can be posttranslationally modified in many ways (e.g. glycosylation, phosphorylation, acetylation, sumoylation, and truncations), further expanding the already overwhelming number of potential HCPs [74]. Another challenge is that knowledge about the characteristics of individual HCPs is lacking; an abundant HCP is not necessarily a persistent HCP, nor a critical one (i.e. immunogenic). Therefore, risk assessment of HCPs is necessary to predict, evaluate, and control critical HCPs throughout bioprocessing. Proposed elements that determine the risk surrounding a particular HCP include (i) severity, (ii) detectability, and (iii) abundance of the HCP [41]. Severity refers to the potential of a HCP to impact patient health, such as immunogenicity and biological activity, and can be determined experimentally or computationally. For example, CHOPPI (CHO protein-predicted immunogenicity), an immunogenic risk prediction tool [42], has been developed to provide information about the potential presence and immunogenicity of CHO HCPs using the CHO proteome database and EpiMatrix, an *in silico* platform for epitope identification and prediction [75]. Detectability refers to how easily a particular HCP can be identified and quantified; while abundance refers to the amount of an HCP. These elements depend, in part, on the detection method employed. Assessment based on these elements would allow a reduction in HCP-associated risks, as well as, development of critical HCP-specific clearance methods.

12.6 Future Directions

With encouragement from regulatory groups, the biopharmaceutical industry is moving toward continuous biomanufacturing paradigms because of the many anticipated advantages over fed-batch, including less lot-to-lot product quality variability, operational flexibility, cost effectiveness, and smaller environmental and operational footprints [76, 77]. Continuous biomanufacturing requires on-line or at-line analysis and monitoring of CQAs; therefore, prompt HCP detection and monitoring methods must be established to replace current off-line methods. Moreover, a major challenge to continuous biomanufacturing is cell line instability, which may result in unexpected changes that can affect CQAs. As changes in HCP expression patterns during long-term cultures (up to

one year) have been reported [24], HCP profiles should also be monitored when assessing cell line instability.

Lastly, new immunoassays must ensure the detection of a broader spectrum of HCPs early in the process development cycle (e.g. HCPs in HCCF rather than HCPs in the final drug product) such that the assays are able to readily identify and quantify changes in HCP expression patterns resulting from any process changes. Although immunoassays are stipulated in the regulatory guideline (ICH Q6B) and are most widely utilized in industry, there are no restrictions on the types of methods that can be used in assaying HCPs and employing orthogonal approaches is encouraged. An ideal HCP quantification method should be able to (i) identify the entire profile in a single run, (ii) detect trace amounts of HCPs, (iii) accommodate a wide quantification range, and (iv) specify and monitor individual HCPs.

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