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REVIEW

Bioseparations and Downstream Processing

BIOTECHNOLOGY PROGRESS

Host cell proteins in monoclonal antibody processing: Control, detection, and removal

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Abstract

Host cell proteins (HCPs) are process-related impurities in a therapeutic protein expressed using cell culture technology. This review presents biopharmaceutical industry trends in terms of both HCPs in the bioprocessing of monoclonal antibodies (mAbs) and the capabilities for HCP clearance by downstream unit operations. A comprehensive assessment of currently implemented and emerging technologies in the manufacturing processes with extensive references was performed. Meta-analyses of published downstream data were conducted to identify trends. Improved analytical methods and understanding of "high-risk" HCPs lead to more robust manufacturing processes and higher-quality therapeutics. The trend of higher cell density cultures leads to both higher mAb expression and higher HCP levels. However, HCP levels can be significantly reduced with improvements in operations, resulting in similar concentrations of approx. 10 ppm HCPs. There are no differences in the performance of HCP clearance between recent enhanced downstream operations and traditional batch processing. This review includes best practices for developing improved processes.

KEYWORDS
cell culture, continuous bioprocessing, downstream process, host cell protein, monoclonal antibody

1 | INTRODUCTION

Monoclonal antibodies (mAbs) have had the highest number of health authority approvals in recent years and currently constitute the most important class of recombinant protein therapeutics. The majority of mAb manufacturing processes use Chinese Hamster Ovary (CHO) cell lines to express the biotherapeutic and recover it from harvested (clarified) cell culture fluid (HCCF). Host cell protein impurities (HCPs, frequently referred to as CHOP) are secreted into the HCCF from viable cells and released through cell lysis over the course of culture and harvest.[1-3] The amount and components of HCPs in HCCF vary depending on the clone selected, mAb expressed, bioreactor types, and culture conditions.

HCPs are process-related impurities that originate from cells expressing mAbs,[4] and can include thousands of unique proteins with widely varied concentrations, molecular weights, isoelectric points (pI), and surface charges.[5-7]

HCPs are classified as a critical quality attribute (CQA) in therapeutic mAb manufacturing. Many HCPs are essential for key metabolic pathways of the cell but can be a risk to patient safety at parts

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per million (ppm) concentrations. They can cause an immune response in immunocompromised patients (immunogenicity) or by being biologically active. They can alter mAb stability or solubility and can enzymatically attack a mAb therapeutic to alter its potency.[8,9] While most HCPs exhibit no enzymatic activity, some HCP components may show lipase,[10] glycosidase, or protease behavior in the enzymatic breakdown of a mAb[1] and possibly promote aggregation in the mAb formulation with a consequent impact on shelf life.[11]

Health authorities (e.g., the U.S. Food and Drug Administration [FDA] and European Medical Agency [EMA]) do not provide written guidance on specific acceptable levels of aggregate HCPs in the final drug product to be injected into patients. However, there is an informal empirical target level of HCPs as <100 ppm, or <100 ng/mg product.[12,13] From the recent development in HCP analysis technologies and immunogenicity assays, the limit of 100 ppm is inadequate to ensure drug stability and patient safety.[14] The accepted level of HCPs in a final product is determined by risk assessment and depends on multiple factors, including the concentration, therapeutic dose, frequency of drug administration, type of drug, and severity of the disease.[7,14] Accordingly, ensuring that the product is safe and efficacious requires a manufacturing process capable of robust HCP control and removal such that the final product has a level of HCPs that neither influences product quality nor compromises patient safety. This may require monitoring and restricting a subset of "high-risk" HCPs.[15]

Many biopharmaceutical manufacturers are exploring new unit operations, and process intensification with high cell density cultures linked with downstream unit operations in series to operate as a continuous process. Although these are often discussed in terms of the advantages of higher productivity and lower cost of goods, managing HCPs is a key quality attribute for patient safety. Upstream developments to increase mAb titers by prolonging production time results in changed cell density and viability, which may lead to a higher HCP level and modified composition. This poses a higher removal challenge to downstream purification[1] and HCP detection.

This review covers the current biopharmaceutical industry trends relating to HCPs as a CQA, as well as innovative technologies for analytical assays and manufacturing processes. Data on in-process HCP levels and clearance were analyzed by way of a comprehensive assessment of the literature on upstream generation, control, and downstream removal of HCPs.

2 | REGULATORY AND QUALITY ASPECTS

The levels and types of HCPs depend on both the host cells and clones used for product expression[9] and the downstream manufacturing processes.[16] The relative levels of individual HCPs determined in the 29 approved mAb and mAb-based therapeutics were generally low, at an average of 20 ppm.[17] Clinical response to HCPs depends on several factors, including the patient population, dosage, route of administration, and frequency, etc.[9] For example, a high-dose mAb administered repeatedly to treat a chronic disease such as rheumatoid arthritis can trigger a patient's immune response against the mAb therapeutic. In such a product, lower levels of HCP are required compared to a low-dose mAb therapeutic that is administered once to treat an acute disease such as cancer.

Global health authorities and the industry have developed a quality management framework using the Quality by Design (QbD) methodology. This framework is intended to design and manage processes for patient safety and product quality and is based on sound science and quality risk management (International Council for Harmonization, ICH Q6B (1999), S6R1 (2011), Q8R2 (2009), Q9R1 (2023), Q10 (2008), and Q11 (2017)). HCPs are a CQA (ICH Q11). The identification of critical process steps relevant to HCP removal in QbD starts with using prior knowledge and HCP removal studies, as well as risk assessments. The design space establishing operating limits of unit operations for HCP clearance in the downstream process has been described in terms of a multivariate statistical model that links process performance with three chromatography steps.[18] Upstream processing conditions can change HCP composition and thereby impact downstream processes for HCP removal.

HCP levels must be monitored and managed to ensure acceptable levels (ICH Q6B). However, it is not possible to provide a universally accepted residual HCP level for different mAbs, as each immunoassay for residual HCPs is unique in its reactivity to the almost infinite combination of HCPs. HCP composition is impacted by both the host cell line and manufacturing process conditions. For this reason, there are no specific limits specified in the regulations, and the acceptable levels of HCPs are reviewed by the regulatory agencies on a case-by-case basis using risk-based approaches. Guidance for testing HCPs emphasizes that levels should be as low as technically possible but does not state specific targets for residual levels (U.S. Pharmacopeia Monograph <1132> 2016, European Pharmacopeia Monograph 2.6.342017). HCP specifications should be determined based on a systematic understanding of the process and product as well as scientific knowledge.

The risk of HCPs can be assessed from a SOD (S, severity; O, occurrence; and D, detectability) framework.[9] From a regulatory standpoint, the factors to be considered in the risk assessment are the identity of the HCPs and their homology with human counterparts, prior experience with the material, the clinical indication/population, the therapeutic mechanism of action, the route of administration, the development phase, the duration of exposure, and the frequency of dosing.[19]

A set of 79 HCPs was observed from the analysis of 29 commercially available biotherapeutic products.[17] From these, several problematic and difficult-to-remove HCPs were identified.[19] These include mAb product-bound species, such as nidogen-1, secreted protein acidic and cysteine-rich proteins (SPARC), and clusterin.[20–22] In addition, species such as histone can co-elute with mAb from Protein A chromatography.[23] Enzymatic HCPs, such as lipoprotein lipase, are problematic for stability during processing and product storage.[10] Immunogenic HCP stimulants, such as phospholipase B-like protein, also require higher levels of clearance.[24] Given the vast number of unique HCPs, testing every single one for its immunogenicity and enzymatic effects is impossible. A combination of in-silico simulations

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and database reviews have been used to predict which residual HCPs are high risk. This should be done during the early stages of downstream process development to enable the design of downstream processes. If HCP species persist through the purification train, knock-out cell lines could be generated. CHO Protein Predicted Immunogenicity (CHOPPI) is an in silico tool that predicts the potential immunogenicity risk of CHO proteins and the potential presence of CHOP expression and secretion.[25] The Immune Epitope Database (IEDB) also has algorithms for assessing the risk of immunogenicity.[26] These considerations may require monitoring of a subset of "high-risk" HCP components. To help establish recommendations for individual HCP thresholds, an industry coalition of 26 companies has developed a searchable database of "high-risk" HCPs which continues to be updated.[15] A comprehensive list of 86 frequently seen HCPs was compiled in this database, with HCPs being categorized as "high-risk" HCPs based on their impact (Table S1 summarizes this information as Supporting Information).[15]

A number of approvals for therapeutics (e.g., Xigris, Aranesp, and Aldurazyme) have been granted subject to the condition that ongoing patient monitoring is performed (Phase IV clinical trials) to identify adverse reactions with possible changes in CMC specifications.[27]

3 | ANALYTICAL METHODS FOR HCP DETECTION AND CHARACTERIZATION

The main concern for HCP assays is their coverage of the whole HCP population and their sensitivity, particularly for "high-risk" components. Among HCP detection methods (Table 1), the enzyme-linked immunosorbent assay (ELISA) is the most common.[29] HCP ELISA assays can be grouped into 3 main categories: generic, platform, and process-specific HCP assays.[9] Antibody-based detection chemistry includes direct and sandwich ELISA assays, colorimetric assays, and fluorescence-based assays. While process-specific HCP assays are customized versions developed specifically for the process and expression system, platform HCP assays imply the use of the same in-house assay developed for similar purification processes and strains (i.e., strains from a common starting cell line). However, care should be taken with the use of platform HCP assays, as HCP patterns could potentially differ with changes in the purification process and biologic molecules. Regulatory agencies usually require a process-specific HCP assay in late clinical phases, unless a generic or platform HCP assay can be validated to cover the range of HCPs present in the process. The effectiveness of the ELISA also depends on antigen generation and purification, as well as how the assay was developed. Antigens used for commercially available generic assays can vary widely, from null cell cultures to peptide constructs and protein fragments. It is critical to test the specificity of the antibody for HCPs, as many antibodies, especially those produced from peptide constructs or protein fragments, might not be able to recognize the actual target HCPs.[15] Even the use of cell culture supernatants (CCS) from null cell lines as the antigen has limitations, as antibodies might not be effectively generated against low immunogenic[34] or low abundance HCPs. In addition, due to the absence of those HCPs secreted only during target mAb expression, antigens against such HCPs will also not be generated.[34]

Besides ELISA, other antibody-based methods include 2D-gel electrophoresis (2D-GE) in combination with Coomassie blue, silver, or fluorescent protein-dye staining to detect proteins, or Western blot (WB) to detect immunogenic response. Our in-house data of a 2D-stained gel using a CHO-expressed clarified model mAb with Coomassie blue and silver staining provides a qualitative picture of the diversity and relative levels of HCPs (Figure 1). These gels are useful for developing HCP removal strategies. In this case, most CHOP components fall into a range of 5–6.5 pI (negatively charged at pH 7), 30–200 kD molecular weight, and have a mainly hydrophilic character.[35] Silver staining is very sensitive and can detect proteins at very low concentrations. Spiking samples with reference proteins at known concentrations can be used to calibrate conditions and aid interpretation.

The diversity of CHOP is shown using two-dimensional fluorescence difference gel electrophoresis (2-D DIGE). Up to 800 different spots were identified by 2-D DIGE.[36] This creates a unique challenge for HCP analysis, as the ideal detection method must be able to measure a wide dynamic range with high sensitivity in the presence of high target mAb concentrations.[14] HCP clearance capabilities can be demonstrated using a spiking study with relevant HCP concentrates and a process run at manufacturing conditions.[37]

Health authorities have requested testing with orthogonal methods, such as liquid chromatography-mass spectrometry (1D- or 2D-LC–MS/MS). To improve the limit of detection (LoD), various sample concatenation strategies involve the pooling of early, middle, and late high pH-RPLC (reversed-phase liquid chromatography) fractions into a single fraction.[31] Pre-concentration using hydrophilic interaction chromatography (HILIC) for HCP enrichment followed by centrifugal vacuum concentration has been employed during or prior to 2D-LC–MS/MS analysis.[38] The obtained LC–MS data are analyzed by data-dependent acquisition (DDA) or data-independent acquisition (DIA). DIA involves creating spectral libraries and fragmentation of all precursor ions and is, therefore, more labor-intensive than DDA. This method can both identify and quantify the HCPs present, regardless of the immunogenicity of the protein. LC–MS/MS can also be used to analyze the coverage of HCP ELISA without bias due to antibody sensitivity or WB transfer efficiency.[34] LC–MS/MS is mostly used for process development and troubleshooting, while ELISA is mainly used for routine batch-release testing.[15] This is because ELISA, once validated, is an easier and faster method than LC–MS/MS. In troubleshooting, unexpected HCP results generated with ELISA were investigated, which led to the identification of a co-purifying HCP by LC–MS/MS and a subsequent risk assessment.[39]

A new development using a high pH–low pH two-dimensional reversed-phase (RP) LC–MS/MS approach can detect a low level of HCPs, at around 10 ppm.[40] 2D-LC coupled to ion mobility mass spectrometry has been shown to reduce the detection limit of HCPs to 1 ppm.[30] The detection of low abundance HCPs can be significantly improved by implementing a single step of molecular weight cut-off filtration followed by a shotgun proteomics analysis prior to LC–MS/MS.[41] Harvested HCP profiles obtained through LC–MS/MS (<200 kDa, and greater acidic pI proportion in a broad 4–11 pI

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TABLE 1 Main characteristics of HCP analytical methods used in mAb manufacturing.

HCP Analytical Methods	Pros	Cons	Detection limit (ppm)	Detection time (h)
ELISA (quantitate immunogenic HCP only)				
Generic/Platform/ Process-specific (Wang et al. 2009)[9]	Generic: less time required for assay development Platform: more time required for 1st time, comparability across processes Process-specific: time consuming	Generic: may not be as specific Platform: may not be as specific for the occasional process-specific HCP (Zhang et al. 2016)[28] Process-specific: improved HCP coverage	Generic (F550-1, Cygnus Technologies): 1	~3 to 4
Direct/Sandwich	Direct: shorter assay time Sandwich: lower background signal	Direct: prone to high background signal Sandwich: longer assay time	–	–
Colorimetric/Fluorescence	Colorimetric: cheaper Fluorescence: higher sensitivity	Colorimetric: lower sensitivity Fluorescence: more expensive	–	–
2D-GE, WB				
2D-GE	Enables visualization of HCP	Unable to detect low abundance HCP (Bracewell et al., 2015),[14] Semi-quantitative method, cannot identify HCP	Coomassie blue: 0.05 to 0.1 µg Silver stain: 1 to 5 ng (Wang et al. 2009)[9]	–
WB	Enables visualization of HCP	Detection dependent on coverage of antibody (Bracewell et al., 2015),[14] Risk of over or under-transfer of protein to the membrane (Pilely et al., 2020)[34]	pg level (Wang et al. 2009)[9]	–
1D- or 2D-LC–MS/MS (identify and quantitate HCP, regardless of immunogenicity)				
1D-LC–MS/MS	Able to both identify and quantitate HCP	Unable to detect low abundance HCP, requires highly skilled personnel to perform analysis	10 to 50 (Doneanu et al. 2015)[30]	-
2D-LC–MS/MS	Able to identify and quantitate low abundance HCP	Time required for the concentration step requires highly skilled personnel to perform analysis	10 (Yang et al. 2018)[31]	6 (Yang et al. 2018)[31]
Others (under development, not currently used in mAb manufacturing)				
Sequence-defined aptamer-based flow cytometry (McGivney et al. 2018)[32]	Able to quantitate non-immunogenic HCP, ease of assay customization by aptamer sequence combination	Cumbersome SELEX process, prone to generating aptamer with affinity to background matrix	16	-
Proximity ligation assay (Liu et al. 2012)[33]	Broad dynamic range	-	1	~3 to 4

Abbreviations: 2D-GE, 2D-gel electrophoresis; ELISA, enzyme-linked immunosorbent assay; LC–MS/MS, liquid chromatography with double mass spectrometry detectors; LC–MS, liquid chromatography with single mass spectrometry detector; SELEX, systematic evolution of ligands by exponential enrichment; WB, Western blot.

distribution) show a similar profile to the 2D gels shown in Figure 1. LC–MS/MS is able to cover the detail of each HCP component.[17]

A publicly available, comprehensive, CHO-specific spectral library with sequential window acquisition of all theoretical mass spectra (SWATH-MS) has identified more than 10,000 proteins, consisting of 199,102 identified peptides. It has the potential to help with more detailed characterization of HCPs through quantitative analyses of peptides as a future process analytical technology.[42]

Other methods under development include a sequence-defined, aptamer-based (SDAB) flow cytometry binding assay[32] and a proximity ligation assay.[33] The SDAB assay has the unique advantage of recognizing non-immunogenic HCPs but the fact that 32 DNA aptamers can recognize a wide range of antibodies shows that each aptamer does not have a specific affinity for the antibodies. This might pose a challenge for the use of aptamers as a platform assay, given that each of the 32 aptamers might also bind to the target protein. The development process is also quite cumbersome, as each round of systematic evolution of ligands by exponential enrichment (SELEX) is prone to enriching the background matrix binding aptamer if the coating of the bead surface is not fully performed.

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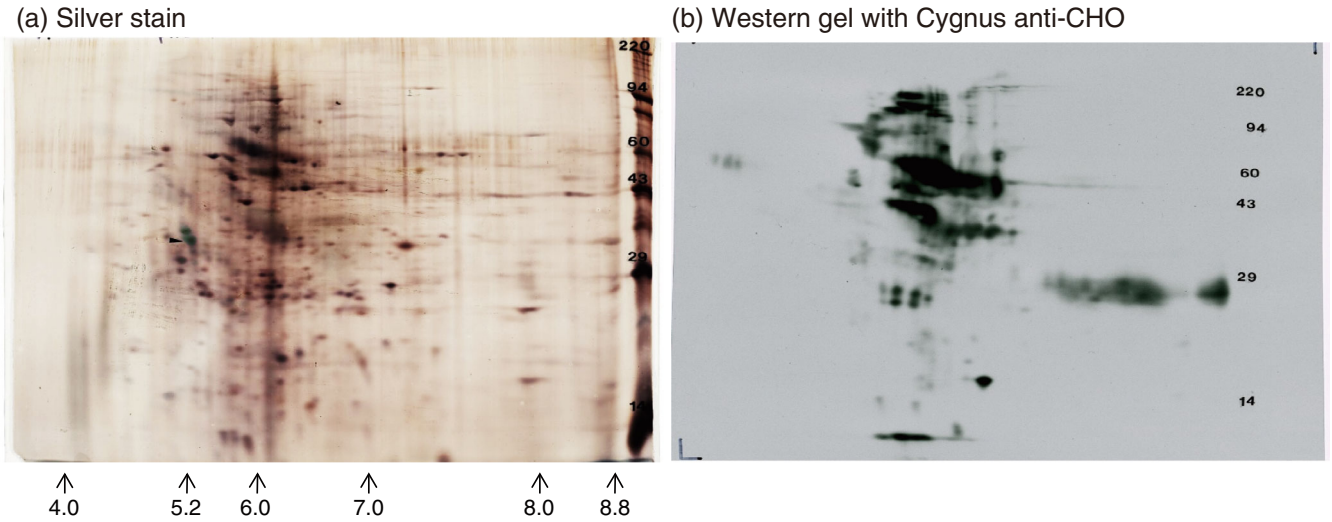

FIGURE 1 2D Polyacgrlamide gel electrophoresis (2D PAGE showing the distribution of molecular weight (vertically) and isoelectric point (horizontally) of HCP with a clarified CHO harvest at 97% viability (Courtesy of MilliporeSigma)). (a) Silver stain darkness corresponds with protein concentration and (b) Cygnus darkness corresponds with immunogenicity to the Cygnus test kit antibodies.

Upstream processes also directly affect the HCP content of CCS. Notably, the development of high-viability cell lines and knockout cell lines might facilitate HCP detection by reducing the complexity of the protein matrix. On the other hand, the use of fed-batch cultures has been reported to increase overall HCP content rather than the batch culture.[2] It would be ideal if a real-time and inline method could be developed to monitor this phenomenon, especially for continuous processing in perfusion mode. Currently, there are no reports of suitable inline methods to rapidly detect HCPs.

The HCP profile for each commercially approved product is unique.[17] Comprehensive tracking of individual HCPs in downstream processes for several mAbs by way of 2D-LC–MS observed that widely differing HCP numbers and levels in purification intermediates originate from a small subset of intracellular HCPs.[43] A comparison of the supernatants produced by two CHO host cell lines with some transfected/un-transfected clones, using UPLC–MS/MS with iTRAQ 8-plex labeling, revealed no significant difference in the several hundred proteins identified. However, there was a slight difference in expression between the protein of the host and the transfected clones.[44] A very similar HCP composition was also found by 2D-DIGE in the 15 CHO null cell culture harvests by five different cell lines. This similarity might demonstrate that HCP impurities were not significantly affected by a wide range of manufacturing processes.[45] For biosimilar mAbs, health authorities require similar mAb product profiles and HCP content compared with the originator mAb. However, it is not clear how similar identities and quantities need to be achieved for so many different HCPs.[46] Clinical trials are therefore necessary when developing biosimilars. The presence of HCPs might either increase or decrease drug efficacy. Although it is generally accepted that a purer mAb, depleted of HCPs, should have better safety and efficacy, further testing is needed to demonstrate whether biosimilar mAbs with specifically lower HCP levels are different in terms of their efficacy or safety.

Through continued advances in analytical technologies, the next aim is to (1) speed up detection time without compromising sensitivity, and (2) improve prediction of HCP risk factors (via a combination of improved quality in in-silico and shared database analyses). Faster detection times will allow more samples to be analyzed, thus allowing HCP distribution under different upstream culture conditions, downstream purification stages, and safety limits to be established. Faster feedback on HCP concentrations can potentially enable the implementation of process analytical technology (PAT)-based feedback control of in-process parameters for higher product quality and efficiency out of the bioreactor or in downstream purification steps. Better predictions of HCP risk factors can help problematic proteins to be studied and generate a shared database.

4 | CONTROL OF HCPs IN UPSTREAM BIOPROCESSING

As described in the introduction, HCPs are produced and released into the HCCF by secretion, cell death, and cell lysis over the course of culture. Among the parameters influencing HCP abundance and species, cell viability at harvest may have the highest impact.[13,47–49] The QbD case study conducted by the CMC Biotech Working group concluded by design of experiments (DoE) with $p < 0.0001$ that the only parameter impacting HCPs in the HCCF was the duration of culture harvest.[17] HCP control to 3–8 $\times$ 10^6 ppm limited the bioreactor design space range of this parameter to 15–19 days. Optimization of the upstream process can reduce the load of HCPs and may increase the efficiency of downstream processes to achieve target HCP levels in the final drug. However, due to other constraints in achieving critical quality attributes, meeting design space for HCP control in upstream processing is a challenge. The comprehensive approach to HCP control involves downstream purification.

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4.1 | Production cell line

Production cell clones generate the target mAb while also producing and secreting HCPs into the culture during the process. Some of these HCPs can degrade the mAbs present in the culture during production and can also trigger an immune reaction in immunocompromised patients, which constitutes a risk to the patient's safety. One approach is to reduce HCP expression by the cell line and create a "clean" production cell line.[50] In a study by Dovgan et al. (2021),[51] the downregulation of cathepsin D, a difficult-to-reduce HCP, greatly reduces the level of co-purifying HCPs. Other cell line engineering targets can be apoptosis-related genes,[11,52–54] whereby HCPs are reduced via the engineering of more robust cell lines that are inhibitory to apoptosis and able to maintain high cell culture viability for an extended culture duration. Another approach is to directly screen candidate CHO cell lines and production clones for reduced expression of problematic HCPs[55] and enzymes promoting product degradation.[56]

4.2 | Cell culture media

Components in cell culture media can alter the composition of HCPs.[57] A higher concentration of insulin, folic acid, glycine, and riboflavin decreased HCP concentrations; conversely, the addition of magnesium chloride ($MgCl_2$) increased them.[58] In the study, the population of HCPs shifted to lower pI values, facilitating their clearance by downstream IEX columns. Reductions in HCP levels without significant compromises in mAb production were achieved by the addition of insulin, folic acid, glycine, and riboflavin. Although an increase in $MgCl_2$ concentrations increased HCP levels, little is known at present regarding the mechanism behind the impact of cell culture media components on HCP production. An understanding of the mechanism behind the reduction in HCPs by cell culture media components can aid in the selective reduction of HCP levels without impacting other critical protein quality attributes.

4.3 | Process parameters in bioreactor

Major upstream bioprocess parameters influencing HCP profiles during manufacturing processes for CHO-derived biotherapeutics, as well as their control strategies, are summarized in Table 2. Bioreactor process parameters such as reactor type, impeller shear, media additives, pH, temperature, osmolality and dissolved oxygen, culture duration, and harvest viability, can contribute to HCP profiles.[11,21,59,60,63] Among these factors, cell viability at harvest was found to have the highest impact on HCP profiles.[48,59] The release of intracellular HCPs into the medium, to which dead cells contribute, led to a substantial increase in HCP levels.

Reducing the cell culture temperature also reduced a variety of HCPs in the HCCF.[62] Chaperones and protease, which are known to contribute to protein aggregation and degradation, respectively, were significantly reduced. The authors hypothesized that lowering the temperature reduced secondary necrosis and improved the homeoviscous adaptation of cells. This contributed to the suppression of apoptotic cells and reduced the release of product-damaging HCPs. However, it is also important to consider the population of HCPs that interact with mAbs. As shown in a follow-up study by Valente et al. (2015),[21] mild hypothermia conditions generated 3.6 times higher residual HCP post-Protein A, possibly due to increased HCPs that interact more strongly with mAbs. HCP levels can vary on a case-by-case basis depending on the number of days before harvest (days are easier to measure, but a more accurate indicator is cell viability). Earlier harvesting with higher cell viability reduces cell breakage and cytoplasmic HCP release. However, harvesting the culture too early could also lead to cells being more shear-sensitive, resulting in increased HCP generation during the downstream clarification step.[3]

Further investigations may be required to integrate upstream and downstream processes and balance the trade-offs between high viability and HCP levels during harvest and post-harvest steps.[13,62] Some biomanufacturers focus primarily on therapeutic titers while others focus more on quality attributes. An improved mechanistic understanding of the cellular processes impacting HCP profiles and concentrations can assist improvements in upstream HCP reduction.[64]

4.4 | HCP clearance through harvest clarification

HCCF production by clarification removes plugging particulate matter (e.g., cells, cell debris, colloids, aggregates, etc.) to enhance the capacity of downstream technologies and potentially reduce the concentration of impurities like HCPs and DNA. Higher-density cells settle slowly over time, so centrifuge technology employing artificial gravity is used to settle smaller-sized particles more quickly (precipitated HCPs, flocculants, etc.). However, high shear forces in the inlet region can lyse cells and lead to increased HCP levels.[63]

MF-TFF (microfilter tangential flow filtration) technology has also been used to clarify the harvest by retaining particles on a membrane surface. Few nanometer-sized HCPs are removed as they pass through the micrometer-sized membrane pores with the mAb. Pumping during the MF-TFF operation may lead to an increase in HCPs due to degassing through flow restrictions, such as air in pumps due to poor seals, or gas entrainment in mixers and tank outlets.[65]

Depth filters utilizing normal flow filtration trap particles and reduce HCP levels in cell culture fluid.[66,67] Depth filters are typically composed of support layers of fibers (e.g., cellulose, polyacrylic) and filter aids (e.g., diatomaceous earth, silica) to increase the adsorptive surface area.[66] A resin binder improves filter media strength and modifies surface properties with charges and hydrophobic regions. Depth filters are available as a lenticular stacked disk with bulky housing or as integrated "pods" for easier assembly and improved sealing.[68,69]

HCP removal with depth filters occurs via a combination of size exclusion (for large particles) and adsorption (electrostatic, hydrophobic, hydrogen bonding), but differences in HCP composition can result in different behaviors. HCPs with a net positive charge exhibited higher retention on a negatively charged depth filter material than

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TABLE 2 Summary of major upstream bioprocess parameters influencing the HCP profiles during the manufacturing process of CHO-derived biotherapeutics and control strategies.

Parameter	Influence	Control strategies
The amino acid sequence of biotherapeutic	Co-elution of HCP with monoclonal antibodies (mAb) through Protein A Chromatography led to variable HCP levels in eluate (Aboulaich et al. (2014),[22] Levy et al. (2014)[20]).	Modification of amino acids on the IgG surface changed the molecule's aggregation and propensity, affecting the interaction of mAb and HCP (Aboulaich et al. (2014),[22] Levy et al. (2014)[20]).
Cell culture media	Cell culture media has been shown to influence HCP abundance and population (Kornecki et al. (2017)[57])	Media optimization to change HCP composition towards a population that is easier to separate and exhibits a lower HCP concentration (Kornecki et al. (2017)[57])
Culture duration	A higher level of HCP was identified and quantified in fed-batch cultures due to extended culture duration and constant shear during culture to viable cells during culture and a decrease in culture viability during harvest, as compared to batch cultures (Gilgunn & Bones (2018),[1] Park et al. (2017),[2] Tait et al. (2012)[59])	Cell line engineering to generate cell lines with decreased HCP i.e., knockdown expression of difficult-to-remove HCP, overexpression of anti-apoptotic gene (Dovgan et al. (2021),[51] Hogwood et al. (2013),[55] Kol et al. (2020)[50])
Cell age Culture days	Cultures with cells of extended age showed Increased expression of difficult-to-purify HCPs. Hence, having to consider the downstream purification process removal along with upsstream generation (Valente et al. (2015)[21])	Inclusion of HCP analysis during the production clone screening step during the cell line development process as one of the selection criteria i.e., exhibiting reduced levels of problematic HCPs (Jin et al. (2010)[48])
Culture vessel type	Bioreactor cultures had a significantly higher number of HCP species which correlated with apoptotic cell density as compared to shake flask cultures (Goey et al. (2019)[60])	
Cell viability	Increased HCP abundance and changes in HCP species were observed with decreased cell viability during culture (Goey et al. (2019),[60] Jin et al. (2010)[48])	
Culture type	Lower HCP levels were observed in perfusion cultures, enabling higher clearance of problematic HCPs as compared to fed-batch cultures (Hong et al. (2020)[61])	NA
Culture temperature	Mild hypothermic cultures at below 80% viability had approximately 44% lesser proteases and 27% lesser chaperones despite having similar HCP concentration to the control cultures at 36.5°C. (Goey et al. (2017)[62])	Biphasic cell culture by temperature shift (for example, from 36.5°C to 32°C on Day 5) (Goey et al. (2017)[62])

negatively charged HCPs.[67] Retention decreased using a higher conductivity buffer, as salt ions can screen between filter and protein charges.[66] Electrostatic removal of HCPs with a pI close to the mAb molecule may be difficult to separate. Hydrophobic adsorption was observed because adsorbed HCPs were released when organic solvents such as ethanol were used to flush the depth filter media.[67] While it has been suggested that hydrophobicity and electrostatic interactions play a contributing role in HCP removal, the density of filter aid and uniformity of the resin binder may also have an influence. Due to denser packing and different adsorption orientations, elongated HCPs were found to be better retained in filter aids.[70] In the same study, a positively charged resin binder that was uniformly distributed across a depth filter allowed mostly negatively charged HCPs to bind. However, if the filter aid was less uniformly distributed on the media, it also allowed pockets of negatively charged regions in the depth filter to interact with positively charged HCPs.[70]

In a meta-analysis by the authors, depth filter HCP binding capacities were evaluated across several studies, and HCP clearance was predicted using an adsorption model (Figure 2). Although predicted HCP log clearance trends fall with increases in feed HCPs, it did not affect the increase in predicted filter-bound HCPs, suggesting that HCP clearance by depth filters is generally limited by available filter area with increasing feed HCP levels. High HCP levels in feed result from high cell density cultures with high rates of mAb expression. This can lead to very large depth filter areas of >1 m^2/100 L feed.

Particle aggregation using flocculation or precipitation can improve settling, reduce filter area, and remove HCPs.[78] Acidification is a classical process in biomanufacturing, including in the precipitation of CHO cell cultures, which utilizes the pI properties of HCPs and mAbs. For HCPs with a pI of 4.0–6.5, lowering the pH of the feed stream using a weak acid can precipitate HCPs while preserving the solubility of the target mAb.[79] Caprylic acid (CA) is a fatty acid containing 8 carbon atoms and hence is also called octanoic acid. CA precipitation has been explored to induce flocculation of impurities and thereby reduce the surface area of the subsequent depth and bioburden reduction filters.[80] CA shows >90% HCP removal by enhancing HCP precipitation.[81] This is due to the weak solubility of CA in water, causing it to precipitate. The hydrophobicity of CA at low pH levels (4.5–5.5) allows HCPs with an acidic pI range to co-precipitate, leading to higher HCP removal.[81,82]

Uncharged polymers such as polyethylene glycol (PEG) are frequently used to precipitate proteins through a volume exclusion

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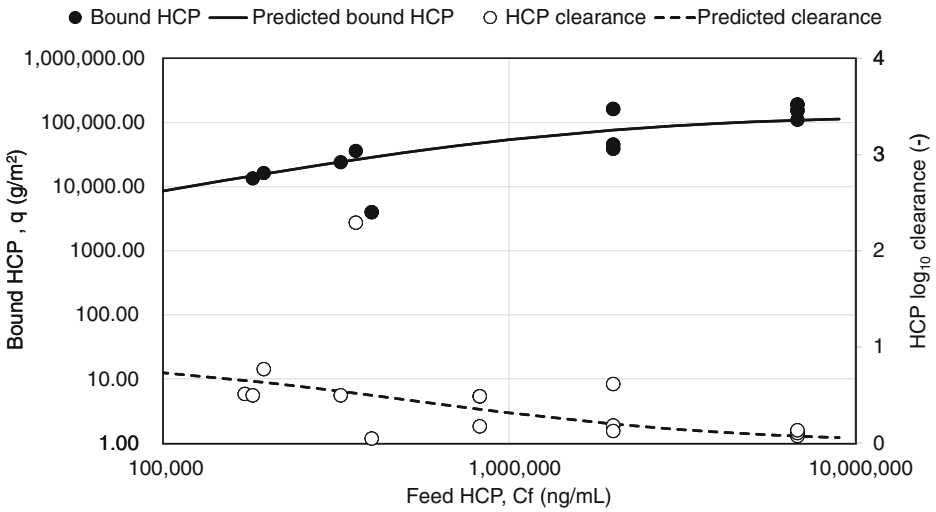

Reported data from reference article were fitted by Langmuir type of Equation: q = AxBxCf/(1+BCf) at A = 215391 and B=4.75x10^{-6}.
Clearance curve prediction was calculated by 105L/m^2 loading.

FIGURE 2 Predicted HCP log clearances and bound HCP levels across different depth filters and initial feed levels. Data were read from references: Amara and Dullen (2016),[71] Yamada et al. (2017),[72] Susienka et al. (2017),[73] Kateja et al. (2018),[74] Ishihara et al. (2019),[75] Kulenko et al. (2020),[76] Marczak et al. (2020).[77]

mechanism; PEG occupies a large portion of the solution, thereby squeezing proteins into a smaller volume where they exceed their solubility limit and precipitate. However, the limited size selectivity of PEG may result in the precipitation of target proteins as well.[83] To overcome this issue, Aqueous Two-Phase System (ATPS) have been applied to various processes. ATPSs are composed of mixtures of uncharged polymers and salt polymerization, resulting in two aqueous phases that contain the target protein and the impurities separately.[84]

Cationic polymers, such as polydiallyldimethylammonium chloride (pDADMAC) and polyamines, are frequently used as flocculation agents in combination with graded depth filters.[85] For HCPs with a net negative charge, pDADMAC can bind to HCPs, causing them to flocculate and leaving the mAb protein of interest soluble, resulting in a harvest recovery of 90% with a reduction in HCPs.[86]

HCP precipitation followed by depth filtration in downstream purification can be less expensive but generally shows reduced selectivity compared to other methods, resulting in poor purification and/or poor mAb yield.[87] Cold ethanol precipitation (e.g., Cohn fractionation) has historically been used in industrial-scale plasma protein purification where cost is important and selectivity less so.[88] Polyethylene glycol (PEG) is commonly used for purifying proteins and is more expensive.[89] With the mAbs eluting from Protein A at an acidic pH, the application of the CA step at this stage has been shown to be effective for HCP removal with a 2 LRV (log_{10} reduction value) clearance at pH range 4.5–5.5.[90] A combination of different precipitants (CEP, PEG, CA, and $CaCl_2$) resulted in HCP removal comparable to that with Protein A affinity chromatography.[87] The addition of PEG to flocculated solutions can aid floc aggregation and speed floc settling such that depth filtration of the supernatant can be reduced or eliminated. Flocculant wash steps improve yield. Due to the variability between different mAb therapeutics and their expression, there does not seem to be a single approach that is ideal for all therapeutics. Rather, it would be useful to develop several methods as tools in a toolbox that can be used on a case-by-case basis where they are most effective.

5 | CONTROL OF HCPs IN DOWNSTREAM BIOPROCESSING

5.1 | Protein A chromatography and HCP clearance

Protein A chromatography is widely used as a capture step in bind-and-elute mode to purify mAbs by removing both process- and product-related impurities.[91] Due to its high degree of specificity for the IgG Fc region, Protein A chromatography provides high HCP removal in a downstream processing step, as most HCPs are removed in the flow-through fraction.[92] However, high HCP levels (ranging from 2000 to >10,000 ppm, Figure 3) may occur in the mAb elution fraction due to non-specific binding and/or mAb-HCP interactions.[67,104,105] The QbD case study working group characterized HCPs in the elution pool as ranging from 5575 to 9893 ppm (~0.9 LRV), depending on critical parameters of mAb load (10–50 mg/mL) and elution pH (3.2–3.9).[17]

Protein A carryover HCPs include those HCPs bound to the resin or the adsorbed mAb which are released along with the mAb during the elution step. The pIs of HCPs in harvest material are widely distributed, with the majority being acidic. The HCPs identified in approved mAb products also reflect a similar broad range of pIs. However, the most frequently observed HCPs across mAb products were basic pIs.[17] Many possible causes can result in co-elution. The presence of chromatin in the harvest strongly affects this HCP co-elution. Prefiltration by anion exchange media for chromatin removal reduces the level of HCPs in the Protein A elute.[106,107] As regards non-specific associations with mAbs, a small subset of mAbs form transient clusters with high charge–charge interactions. This significantly greater

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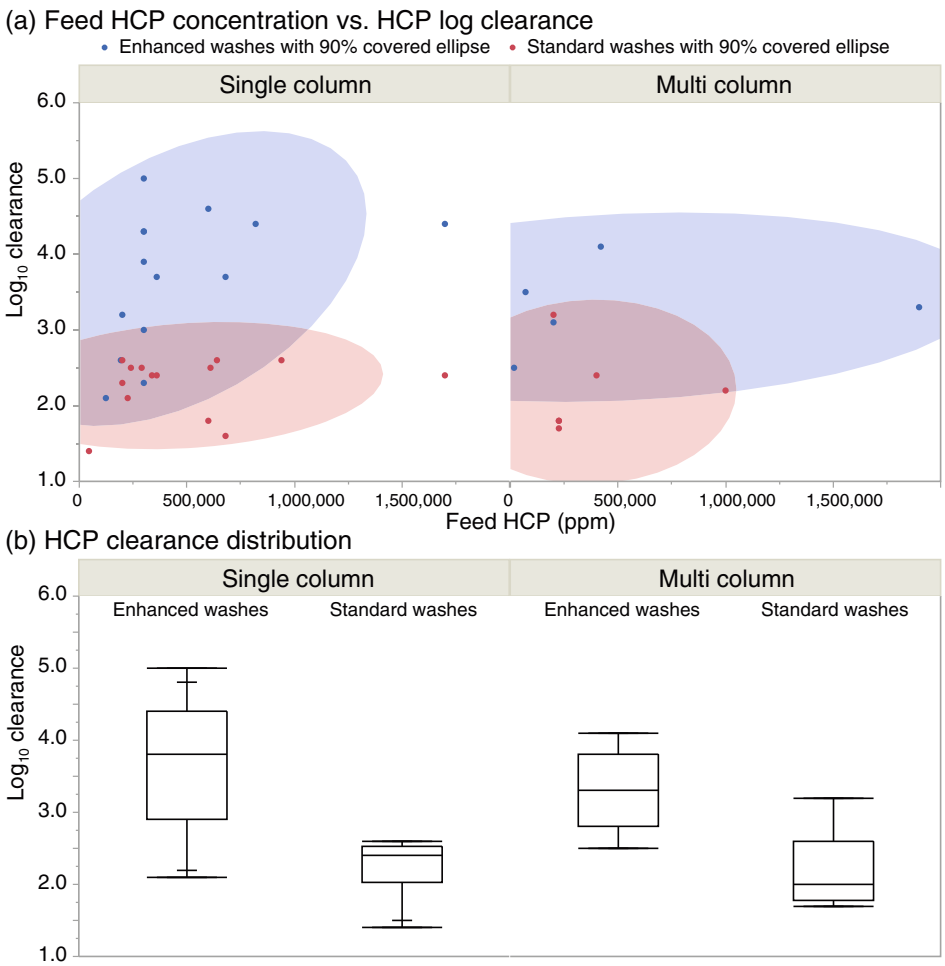

FIGURE 3 Effectiveness of enhanced washes compared to standard washes in terms of HCP removal. (a) Feed HCP concentration vs. HCP log clearance. (b) HCP clearance distribution. Data were read from references: Shukla and Hinckley (2008),[92] Aboulaich et al. (2014),[22] Chollangi et al. (2015),[93] Bee et al. (2015),[94] Amara & Dullen (2016),[71] Mothes et al. (2016),[95] Steinebach et al. (2017),[96] Arunkumar et al. (2017),[97] Utturkar et al. (2019),[98] Gao et al. (2020),[99] Goussen et al. (2020),[100] David et al. (2020),[101] Xu et al. (2020),[102] Coolbaugh et al. (2021).[103]

number of net positive charges is proposed as a reason for the co-elution of negatively charged HCPs.[108] A wash step can be introduced after the binding step with excipients to disrupt HCP binding and control a pH >4.5 to maintain adsorbed mAb.[109] Wash solutions with a pH of 8–10 would be above the pI of both most mAbs and HCPs causing them both to be positively charged and repel each other while leaving the mAbs intact.[93]

Wash buffer excipients (Table 3) can reduce bound HCPs by disrupting attractive forces between HCPs and mAbs or the substrate.[22,92,93,110,112] Salts (e.g., sodium chloride) disrupt electrostatic binding. And detergents (e.g., Triton X-100), and solvents (e.g., propylene glycol) disrupt hydrophobic binding.[92,93] Combining high pH with salt and caprylate improves HCP clearance.[94] Arginine is commonly added to relax electrostatic, hydrophobic, and hydrogen bonds while stabilizing folded proteins to reduce aggregation.[111,113] PEG 4000 addition led to sharper elution peaks, reducing HCP levels and elution pools.[112] Enhanced washes including additives or excipients show improvements in HCP removal (over 5 LRV) compared to standard washes (2 LRV), as shown in Figure 3. Interestingly, almost the same level of HCP clearance was reported in intensified processing, where multiple Protein A columns are used and cycled at high binding capacity. However, additional chromatographic polishing steps are used to provide a robust purification platform for HCP removal.

5.2 | Polishing chromatography and adsorbent for HCP removal

Following the Protein A step, mAbs are held at low pH for retrovirus inactivation and then neutralized for subsequent processing. The neutralization step is frequently associated with HCP precipitation and requires subsequent depth filtration for clarification and HCP removal.[67,114] The HCP removal mechanism is the same as that for the harvest, and the clearance performance is similar to that of chromatography even at low HCP concentrations (Figure 4).

Polishing chromatography steps are then used to remove the remaining HCPs using a variety of chromatographic matrices (resin, membranes) operated in different modes (bind-and-elute, flow-through, weak partitioning) and using a variety of binding ligands. These have been used in various sequences (Table 4), with the most common sequence of steps for commercial mAb production being as follows: Depth–Protein A–virus inactivation–cation exchange chromatography–anion exchange chromatography–virus filtration–ultra

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TABLE 3 Intermediate wash additives for Protein A chromatography proved to be effective for HCP clearances.

Additives	Concentration	Effects	Reference
pH	-	Reducing electrostatic interactions based on pI. Acidic pH 4.4–6.0 Basic pH >8.0	Shukla and Hinckley (2008)[92] Chollangi et al. (2015)[93] Bee al. (2011)[94]
Sodium chloride	0.1–2 M	Shielding the electrostatic interactions between charged residues	Aboulaich et al. (2014)[22] Holstein et al. (2015)[110] Arakawa et al. (2007)[111] Bee et al. (2015)[94]
Arginine	0.1–1 M	Breaking non-specific interactions between proteins and preventing aggregation	Aboulaich et al. (2014)[22] Chollangi et al. (2015)[93] Holstein et al. (2015)[110] Arakawa et al. (2007)[111]
Guanidine hydrochloride	1 M	Binding to protein surfaces disrupting protein–protein interactions	Chollangi et al. (2015)[93] Holstein et al. (2015)[110] Arakawa et al. (2007)[111]
TMAC	0.5 M	Eluting protein from silica resin	Aboulaich et al. (2014)[22] Shukla and Hinckley (2008)[92]
Sodium caprylate	50 mM	Disrupting protein–protein interactions with hydrophobic interactions.	Aboulaich et al. (2014)[22] Bee et al. (2015)[94]
Urea	0.5–3 M	Chaotrope. Stabilizing the protein structure. Hydrogen bond breaker	Aboulaich et al. (2014)[22] Chollangi et al. (2015)[93] Shukla and Hinckley (2008)[92]
Isopropanol	5%–20%	Reduction of hydrophobic interactions.	Shukla and Hinckley (2008)[92]
Propylene glycol	5%–20%	Stabilizing the protein structure by interacting with the polar residues.	Chollangi et al. (2015),[93]
PEG4000	5%	Changing of HCPs elution behavior with an improvement of the monomer/aggregate separation	Stange et al. (2021)[112]
CHAPS	1%	Breaking protein–protein interactions	Aboulaich et al. (2014)[22]
Triton X100	<1%	Non-ionic surfactant	Chollangi et al. (2015)[93]
Polysorbate 80	1%	Non-ionic surfactant	Shukla and Hinckley (2008)[92]

Note: Triton is a trademark of The Dow Chemical Company or an affiliated company of Dow.
Abbreviations: PEG, polyethylene glycol; TMAC, tetramethylammonium chloride.

filtration/diafiltration.[17] The adsorption steps employ different mechanisms to remove HCPs.

For positively charged anion-exchange (AEX) column binding in flow-through mode, operation at a pH below that of the mAb pI (typically >7.5) leaves the mAb positively charged and unbound, while HCPs with lower pIs will be negatively charged and bound.[129] Conductivity levels also need to be kept low to keep the electrostatic binding strength high and avoid charge passivation by salts. The low HCP load in the polishing step feed does not require high binding capacities for the AEX resin or membrane (Figure 4). The wide range of HCP feed concentrations also reflects different positions in the purification train. For a final step, the QbD case study working group characterized HCPs in the flow-through as ranging from 7 to 26 ppm (~0.5 LRV), depending on critical parameters of load conductivity (1.6–5.6 mS/cm) and pH (7.2–7.8).[17] In the final step, HCPs also behave more like a single species, remaining as a single peak without separating into different peaks traveling through the adsorption bed at different speeds.[114] AEX results in an HCP clearance of 2–3 LRV at an HCP feed concentration of around 10^3 ppm; below 10^2 ppm, less than 1 LRV is obtained (Figure 4).[130]

AEX membrane chromatography is particularly well-suited to this application requiring short residence times. This leads to significantly reduced sizing and facilitates single-use operation.[131] A variety of membranes with a high density of binding sites and rapid mass transfer can reduce HCPs by 85–93% with membrane loads of 2–14 kg mAb/L and a residence time of only 2–6 seconds.[119] New formats such as monoliths and packed fibers have not been widely applied for mAb polishing.

Cation-exchange (CEX) chromatography is commonly used to remove aggregates, charge variants, and HCPs from mAbs in a bind-and-elute mode. Loading at a pH of 6.0 allows high pI HCPs to have a negative charge and remain unbound in the flow-through fraction.[132] Usually, the pH, salt concentration, and gradient length of the CEX elution step are optimized for aggregate removal, but this also influences HCP removal.

At feed HCP levels of 10^4 to $10,^5$ CEX clears about 2–3 LRV with HCPs mainly in the flow-through fraction (Figure 4). When CEX was used as the first step after Protein A capture, the QbD case study working group characterized HCPs in the elution as ranging from 16 to 91 ppm (~2.2 LRV), depending on the critical parameters of loading (10–30 mg mAb/mL) and elution conductivity (3–7 mS/cm).[17]

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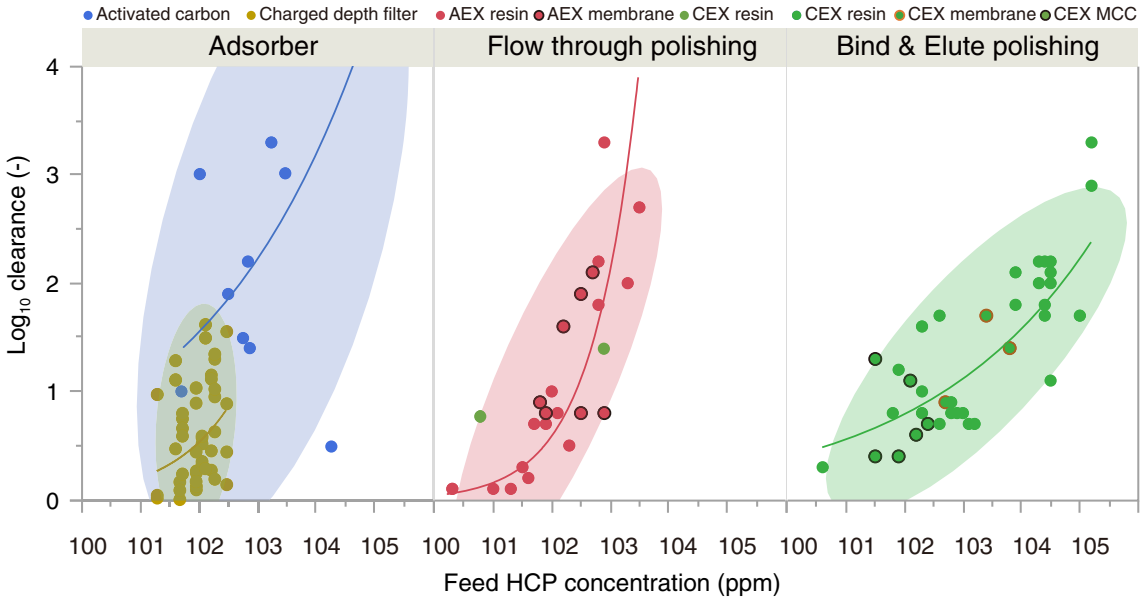

FIGURE 4 Comparison of HCP clearance technologies in downstream polishing step as a function of feed HCP concentrations. Data were read from references: David et al. (2020),[101] Ichihara et al (2018),[115] Ishihara et al. (2019),[75] Slocum et al. (2021),[116] Kateja et al. (2018),[74] Liu et al. (2011),[117] Mothes et al. (2016),[95] Riordan et al. (2009),[118] Steinebach et al. (2017),[96] Trnovec et al. (2020),[119] Xu et al. (2020),[102] Yamada et al. (2017),[72] Nguyen et al. (2019),[120] MilliporeSigma AN1719EN Ver. 2.0 (2020),[121] MilliporeSigma AN4635EN Ver. 0.1 (2020),[122] Peeck et al. (2017).[123] AEX, anion exchange chromatography; CEX, cation exchange chromatography; MCC, multicolumn chromatography.

TABLE 4 HCP clearances for the integrated processes.

Bioreactor Type	Downstream process integration	Feed HCP level (ppm)	Final HCP level (ppm)	HCP log clearance (–)	Reference
Perfusion	PA(MCC)–VI–CEX(MCC)–AEX(FT)	–	<4[a]	4	Godawat et al. (2015)[124]
Perfusion	PA(MCC)–VI–mAEX(FT)–HIC(FT)–VF (TFF)–inline UF/DF	200,000	<2[a]	5[a]	Coolbaugh et al. (2021)[103]
Perfusion	PA(MCC)–VI–CEX(MCC)–AEX(FT)	500,000	2[a]	5.4[a]	Steinebach et al. (2017)[96]
Perfusion	PA(MC)–VI–Depth–inline UF–AEX(FT)–VF–inline DF	–	24	–	Arnold et al. (2019)[125]
Fed-batch	AWS–Depth–PA(MCC)–VI–AEX(FT)–CEX (MCC)–inline UF/DF	92,811	10	4	Kateja et al. (2021)[126]
Fed-batch	Depth–PA(MCC)–VI–AEX(FT)–CEX (FT)–VF–UF/DF	400,000[a]	1[a]	5.6[a]	Xu et al. (2020)[102]
–	PA(MCC)–VI–AEX(FT)–mCEX(MCC)	500,000	10	4.7	Gjoka et al. (2017)[127]
–	PA(MCC) –VI–AEX(FT)–mCEX(MCC)	200,000	1	5.3	Utturkar et al. (2019)[98]
–	PA–VI–CEX–AEX(FT)–VF–UF/DF	200,000	10	4.3	Mothes et al. (2016)[95]
–	AC–AEX(FT)–CEX(FT)	567–593	8.7	1.8	Ichihara et al. (2019)[128]
–	Depth–CEX(MCC)–mAEX(MCC)–AEX(FT)	95,772	<10	4.0	Kateja et al. (2018)[74]
–	DF–Depth–Depth–AC–AEX	65,194	<4	4.2	Yamada et al. (2017)[72]
Fed-batch	[Depth–PA-VI-CEX-AEX(FT)-VF-UF/DF] not integrated	300,000–800,000	2–29	4.4–5.6	CMC Biotech Working Group (2009)[18]

Abbreviations: AC, activated carbon; AEX, anion exchange chromatography; CEX, cation exchange chromatography; Depth, depth filtration; DF, diafiltration; FT, flowthrough; mAEX, mixed-mode anion exchange chromatography; MCC, Multi-column chromatography; mCEX, mixed-mode cation exchange chromatography; PA, Protein A affinity chromatography; UF, ultrafiltration; VF, virus filtration; VI, virus inactivation.
[a]Data from the figure visually in reference.

This is comparable to HCP clearance from Protein A without a wash step. CEX clearance at feed concentrations below 10^2 is higher than the value reported for AEX. Resin and membrane chromatography show no significant difference in HCP LRV performance.

Hydrophobic interaction chromatography (HIC) significantly reduces HCP levels, as does mixed-mode chromatography,[133,134] which employs both hydrophobic and ion-exchange interactions. In cases of bind-and-elute polishing chromatography, implementing an

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intermediate wash step with additives (e.g., arginine, urea, ethylene glycol, hexylene glycol, sodium caprylate, etc.) has been shown to improve HCP clearance, similar to Protein A capture chromatography.[74,135,136] The conditions of the mobile phase for HIC can be set for non-binding of the target protein, allowing for flow-through separation while removing bound HCPs. Mixed-mode or multimode chromatography (MMC) employs novel ligands (e.g., sulfhydryl, primary amine, hydroxyl) or combinations of charged (positive or negative) and hydrophobic (e.g., alkane, phenyl) groups in the same ligand for multipoint interactions (e.g., hydroxyapatite). These ligands can improve binding selectivity (purity vs yield trade-offs) and binding solution salt tolerance. Some have even used MMC to replace Protein A. However, multipoint interactions require extensive screening using DoE with high-throughput screening (HTS) methodology to find a "sweet spot" of loading, binding, elution buffer pH, and conductivity for operation. The higher cost and lengthier process development tend to make MMC a secondary option, employed for problematic therapeutics where conventional resins are unable to meet performance targets.[137]

Strong binding impurities are preferentially retained, displacing weak binding mAbs and impurities and significantly improving resin utilization. Enhanced displacement among mAb products and low-prevalence HCPs on CEX resins allows for the selective separation of HCPs.[138] Frontal chromatography takes advantage of this displacement of impurities to allow flow-through separation in CEX, thereby contributing to process integration and continuous processing of downstream purification. This mainly targets aggregate removal but also contributes to the removal of HCPs.[102,115,139]

Packed columns of activated carbon (AC) utilize a porous structure with a high surface area for adsorptive binding of HCPs through non-covalent interactions.[140] Low molecular weight proteinaceous impurities are effectively removed using AC.[141] However, it is difficult to make definitive conclusions on the adsorption mechanism of activated carbon, since this will also depend on the charge, shape, and hydrophobicity of the protein. AC has maximum protein binding capacity when the pH is at the protein's pI, where protein charge and electrostatic forces are minimized, and hydrophobic interactions dominate.[141] A reduction of 3 LRV was observed for a recombinant enzyme with sensitivity to loading, salinity, and pH.[116] AC treatment performance has also been reported to improve with low conductivity.[75] Given the diverse properties of HCPs, a DoE approach based on pH and conductivity can be applied.[115]

HCP LRV clearance by AC is comparable to AEX (Figure 4). AC has been evaluated post-clarification without Protein A and post-Protein A prior to CEX or AEX. For a purification train with acid precipitation, diafiltration to reduce conductivity and AC, robust HCP removal was superior to that of Protein A.[75] HCP removal by flow-through AC is orthogonal to AEX, that is, removes different HCP components.[142] This AC step intensifies the subsequent anion exchange column to reduce the impurity burden in the post-Protein A polishing step.[72,115]

6 | HCP GENERATION AND REMOVAL IN INTEGRATED AND CONTINUOUS PROCESSING

Continuous bioreactor operation using perfusion is under active investigation and is increasingly applied as a method to increase bioreactor productivity, reduce costs, and improve product quality. A study compared HCP levels in HCCF from mAb-producing CHO cells for 12 K fed-batch (FB), 2 L steady-state perfusion (SS), and 2 L non-steady state (NSS) perfusion bioreactors.[61] The FB lasted 11 days with a maximum viable density at day 7 of 15.6×10^6 cells/mL, 25% viability at Day 11, and a titer of 1.5 g/L. Continuous perfusion cultures use MF-TFF for harvesting. Alternating Tangential Flow (ATF), where samples flow over a TFF membrane surface in alternating directions using a diaphragm, is used to control membrane fouling, maintain mAb transmission, and reduce shear damage. SS used 1 vessel volume per day (vvd), increasing to 2 vvd in days 3–14, maintained 40×10^6 viable cells/mL (using a bleed stream) with 95% viability throughout and a titer of 7.5 g/L. The NSS used 1 vvd increasing to 0.5 vvd for 3 feed concentrates (no bleed) and reached a peak of 221.7×10^6 viable cells/mL at day 7 before dropping to 37% by Day 11 with a titer of 21 g/L. HCCF 2-D gels showed comparable spot patterns and mAb spot intensities with overall HCP levels reduced for SS. Levels in HCCF found by ELISA were 4.5×10^5 ppm for FB, 5.8–7.4×10^4 ppm for SS, and 2.1–6.3×10^5 ppm for NSS by LC–MS and attributed to reduced lysis in the SS. Perfusion also lowers the number of HCP components in the HCCF, from 1310 to 1903 in the FB or NSS to 165–347 in the SS, shown by high-resolution LC–MS. In the fed-batch, most impurities accumulate during the culture and are removed downstream after a single harvest. Continuous perfusion systems exchange cell culture media and prevent the accumulation of impurities.[143]

The HCP levels in Protein A eluate were 263 ppm for FB, 63–86 ppm for SS, and 115–123 ppm for NSS. The low level for SS suggests that the work to develop and validate additional polishing steps may be reduced. The number of different HCP components was further reduced to 319 in FB, 16–27 in SS, and 14–37 in NSS. Lipase levels were also reduced. The lower levels in SS or NSS were attributed to reduced binding to the mAb due to a smaller number of charged mAb variants and a reduction in acidic HCPs.[20,61]

A comparison of continuous perfusion and fed-batch processes (Table 4) shows comparable final HCP levels and LRV. Continuous downstream steps employ depth filter cycling, multicolumn cycling (MC) for bind-and-elute operations, and flow-through adsorption for HCP removal.[95,144] The high cell mass of intensified fed-batch processing with increased HCPs and high titers could potentially affect the continuous downstream clearance of impurities. Since the columns are usually loaded according to the mass of the product, achieving higher specific productivity requires both a maximization of product output and a minimization of impurities.[145] In a reported case where an upstream fed-batch culture with a high seed train was combined with continuous downstream processing, the HCP level in the

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intensified process was reliably controlled to be below that for a traditional process; a significant improvement in the titer was also achieved.[102] The HCPs observed for integrated continuous processing, comprised of high-density perfusion cell culture and a directly coupled continuous capture step, were comparable to those with a batch-column operation over the entire period of continuous harvest capture over 30 days.[146,147] The continuous 50 L perfusion over a 30-day process with continuous downstream processing produced comparable host cell proteins to the 500 L fed-batch conventional 14-day process.[125] In the continuous integrated process, there is no retention of HCP quantities over the cell retention device of the perfusion reactor, resulting in the same level between the bioreactor and harvest. Subsequently, the main reduction in HCPs takes place during Protein A chromatography.[96] The remaining HCPs are then removed by polishing chromatography.

Protein A chromatography is known to be a core step in the mAb processing platform, but it still has the bottleneck of cost and robustness as well as caustic stability in continuous processing.[148] Non-Protein-A continuous downstream processing has been explored as an alternative, involving combinations of precipitation, depth filtration, and polishing chromatography. Acceptable HCP limits have been reported with consistent product quality attributes.[72,74]

The flow-through mode is also an easy link to unit operation for intensified polishing in continuous downstream processing. The continuous operation of integrated connected processes requires a good understanding of the processes to ensure that they can be properly controlled from a design window (For example, integration of AC-AEX-CEX).[139] Indeed, during continuous downstream processes, HCP breakthroughs are detected by flow-through chromatography.[101,128] The intrinsic consistency of steady-state operations provides high and constant product quality. The results of HCP clearance are almost identical when comparing batch and continuous processes.[101]

The intensified use of single-pass tangential flow filtration (SPTFF) as an inline concentration step has been adopted in some integrated processes. Inline dilution using SPTFF can adjust conditions such as pH and conductivity to couple unit operations. The implementation of SPTFF has demonstrated "facility debottlenecking" advantages by reducing harvest volume. However, a need to remove HCP using pre-treatment, such as adsorptive filters, has been reported for the SPTFF to be robust.[97] In the downstream application, SPTFF has been suggested to improve HCP column binding in trace concentration polishing. For example, inline feed concentration (mAb and HCP content) prior to AEX results in high binding of HCPs to AEX resin by running at lower loading. This kind of integrated process has been reported to reduce column size, buffer volume, and tank volume requirements.[130]

7 | HCP CLEARANCE: TECHNOLOGIES OF THE FUTURE

Product quality and the regulatory drivers to monitor and control HCPs for therapeutic safety, efficacy, and purity remain in the future (Section 2). HCPs that are difficult to remove in downstream, especially "high-risk" HCPs, must continue to be addressed. We expect that future implementation will be a natural extension of historical trends, with incremental technology changes and new technologies playing a key role. One can expect performance improvements for assays, reductions in HCP expression, improvements in HCP clearance, and changes due to new therapeutic modalities and expression systems. This would lead to easier and more widespread implementation, easier validation, easier and faster use, and lower costs.

As described in Section 3, improvements in detection should allay concerns that HCP assays may not pick up small concentrations of highly immunogenic molecules that make it through the purification process and pass into the final product. GC–MS methods move past immunogenic detection and identify the molecular components of HCP. As mass spectroscopy and Raman technologies improve by further shrinking in size to permit their implementation on a manufacturing floor, while also providing more accurate detection, they could be used for process monitoring of HCPs and possibly product release.[149] These are currently being used in pilot operations to characterize and optimize processes. Use for process monitoring and control in PAT can enable better yield/purity/cost trade-offs in manufacturing processes, such as bioreactor operations, the pooling of adsorber eluate fractions, or the setting of diavolumes. Implementing more measurements requires associated action limits and corrective actions. This will limit GMP use to valuable critical functions. Improved assays enable process improvements to reduce HCP levels and variability in the purified therapeutic product. The implementation of quality assays in manufacturing may require some organizational changes to reporting and calibration.

Novel mAb modalities, such as antibody-drug conjugates (ADC), show significantly increased potency such that therapeutic doses can be much smaller. This reduces the load of HCPs and any consequent immunogenic responses. In the case of mAb vaccine manufacturing, an immune response to the vaccine is the desired outcome. Adjuvants are used to boost this immune response. In such cases, HCP reductions may lead to a lower-performing therapeutic.

As described in Section 5 and Table 2, changes in the bioreactor, such as perfusion operation, higher cell densities, and growth media composition, can also impact HCP levels and composition. As a general guide from this review, it appears that higher cell densities lead to both higher therapeutic mAb and HCP expression in CCS, roughly in the same ratio of about 106 ppm HCPs (Figure 2 and Table 4). The continuing replacement of animal-derived growth media and synthetic protein components with smaller-sized chemically defined components that are easy to separate will reduce protein contaminants, improve harvest consistency and safety, and lower costs. Cell-free synthesis employing amino acids and enzymes is emerging as a method for producing proteins up to mAbs in size with different populations of impurities requiring characterization.

Improvements in the accuracy and ease of use of genome editing technologies such as CRISPR can facilitate deletion, or "knocking-out," of the expression of problematic HCP components. This may involve a trade-off with HCP components critical to cell growth, cell

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stability, product expression, and post-translational modification. mAb candidates can also be engineered and screened for stability and limited HCP binding.

For mAbs, HCP clearance occurs primarily in the Protein A capture step with contributing removal from the purification and polishing chromatographic steps, TFF, and depth filters (Section 5). Even though special problematic cases can arise, this template provides robust HCP clearance for most mAbs. Clearance from these steps will improve with the implementation of both wash steps, which reduce HCPs bound to mAbs, and novel adsorbers, such as mixed modes, which clear more problematic HCPs using multiple interactions (e.g., in case of low solubility, low pI, bi-specifics, fusion products, ADCs). The use of dual salt/pH gradients may improve performance in some cases.

A widely applicable flow-through multi-mode adsorber that binds HCPs would be a useful product, but its development is challenged by the diversity of HCPs and therapeutic surface binding properties. One possibility is to screen a ligand library (e.g., hexamer amino acid ligands[35]) in order to identify and immobilize an ensemble of ligands that bind to "high-risk" HCPs. This would provide a robust clearance step over a range of pH and conductivity values for use in processing templates requiring less validation and process development than current methods.

Since there is no flow distribution assessment of installed depth filters, their use for chromatographic removal of HCP in large assemblies may present a validation challenge. Some depth filters are more fragile and sensitive to cracking during shipping. Poor sealing on an installed lenticular filter showed early turbidity breakthrough.[68] A reduction in turbidity may be used as a measure of manufacturing system integrity. The use of an integrity test would facilitate validation of HCP removal claims of 1–2 LRV in clarification. Flow-through adsorbers (CEX resin, intensified AEX membrane) with SPTFF provide additional clearance in an easier-to-use format.[130] Continuous processing (Section 6) would benefit from more rapid, inline assays, but could also be done at risk with product pool testing offline. Running columns with partial product binding (e.g., weak partitioning) has been implemented by several biomanufacturers to reduce the number of columns without increasing HCPs in the product.[150]

TFF has not been included as an effective HCP removal technology. It has been used to concentrate harvested and clarified cell culture fluid and to remove low molecular weight media components. TFF is a size-based separation. HCPs have many components of the same size or larger than mAbs, meaning that many of them can co-purify with mAbs in this step.[130] A combined function, such as precipitation, needs to be added.[79] The use of charged UF membranes to allow more purification has shown some promise in selected cases, but they were insufficiently robust for a process template due to the variety of components in HCCF.[151]

In addition to novel manufacturing technologies, improved scale-down test devices, systems, and methodologies can provide faster and simpler process development, implementation, and characterization validation. These do not always require linear scaling, provided that they are predictive of manufacturing scale performance. Mathematical models predicting HCP clearance and mAb yield can be developed for different unit operations. These may involve both established physical laws and empirical approximations. Models can be used to reduce data requirements and predict design spaces more accurately. This can save time, feedstock, and assay requirements. These models are sometimes referred to as digital twins for process simulation or as soft sensors to infer unmeasured values. Process simulation on a computer for optimization is easier than an experimental study. Care must be taken to validate models and not to extrapolate empirical models. In many cases, modeling can also improve understanding of current technologies to enable significant improvements.

8 | CONCLUSION

This comprehensive review describes HCPs throughout the mAb manufacturing process and characterizes current unit operations and their combinations providing a robust HCP control and clearance approach (Table 5). Due to the trade-offs between product yield and HCP removal, a holistic approach through multiple unit operations is still needed. Clearance relies mainly on multiple chromatography steps (i.e., Protein A, CEX, AEX) that have been optimized with flow-through or load, wash, and elution conditions using multi-well scale-down test systems. The process is to deliver the 4–5 LRV, moving from a typical

TABLE 5 Technology summary.

Step	HCP LRV	Best practices	Notes
Bioreactor	–	Chemically defined media, viability vs titer	Gene editing[a], PAT[a]
Clarification depth flocculation	0–3	Adsorptive no claims High titer feeds	Installation test[a] Limited use—low pH, pDADMAC
Protein A	2–5	Intermediate wash step	Wide use
Novel carbon precipitation			Limited use Limited use
Chromatography purification IEX Other	0–2.5	Flowthrough preferred Mixed mode media	Widely used, Bind-elute novel gradients with PAT[a] Selective use

[a]Future technology.

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bioreactor level of 10^6 ppm down to the typical level of 1–100 ppm in the final product, subject to variabilities in performance. HCP clearance in the Protein A capture step amounts to approx. 2–3 logs and at least two further subsequent removal processes are required. The removal of HCP in flow-through polishing still requires a strategic combination of processes, because the performance of HCP log reduction decreases with decreasing feed HCP concentrations.

The main challenges with the fixed-process template are robustness across different mAbs and the removal of "high-risk" HCPs. In the event of limited conditions for HCP removal due to product stability, HCP-mAb co-purifying due to binding, or other factors preventing HCP targets from being met, it is useful to have more technologies and techniques available in the toolbox to handle the various cases that arise.

Although clarification can provide additional clearance, most biomanufacturers do not claim clarification as a robust HCP removal step. A depth filter qualification test is suggested here to facilitate claims. The inclusion of a low pH flocculation step would require minor process modifications and could be sufficiently consistent with controls on the bioreactor to enable its validation. The use of other technologies may require more extensive process development to characterize their operation. Implementation would likely be limited.

HCP levels have been significantly reduced with improvements in bioreactor operations. It is likely that future improvements utilizing perfusion, new growth media, and gene engineering will continue to provide improvements in the long run. Even in advanced bioprocesses such as continuous manufacturing, the same HCP clearance has been obtained as that achieved historically.

While biomanufacturers have identified process pain points and adapted technologies from other industries (e.g., TFF, depth filtration), vendors have developed and supplied new products to the industry. These advances can be continuously improved through communication of process needs and the adoption of new technologies.

AUTHOR CONTRIBUTIONS

Takao Ito: Conceptualization; methodology; writing – original draft; writing – review and editing; formal analysis; visualization; data curation; validation; investigation; resources. **Herb Lutz:** Writing – original draft; writing – review and editing; resources. **Lihan Tan:** Writing – original draft. **Bin Wang:** Writing – original draft. **Janice Tan:** Writing – original draft. **Masum Patel:** Writing – original draft. **Lance Chen:** Writing – original draft. **Yuki Tsunakawa:** Writing – original draft. **Byunghyun Park:** Writing – original draft. **Subhasis Banerjee:** Writing – original draft; conceptualization; methodology; project administration; writing – review and editing; supervision; resources.

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CONFLICT OF INTEREST STATEMENT

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

PEER REVIEW

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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