Selection of Clarification Methods for Improved Downstream Performance and Economics

By Sarah Le Merdy

Introduction

The production of biopharmaceutical drugs typically involves a biological expression within a bacterial, yeast, or mammalian cell expansion system. Getting to the final product requires multiple purification steps, from primary clarification to the final formulation and sterile filtration. The aim of the initial purification steps is not to purify the stream perfectly but rather, to prepare the stream for finer and more specific purification steps further downstream. Apart from efficiently removing contaminants, the clarification stages also need to maintain high product recovery whilst being consistent and robust.[1] The finer downstream purification stages represent the largest portion of production costs; and for monoclonal antibodies (MAb), they can represent up to 80% of the overall cost. This cost is, however, heavily impacted by the quality of the feed stream that has been produced.

Early clarification and purification steps, although seemingly crude, are critical in removing contaminants and maintaining a cost-effective process further downstream. Selecting them scientifically, based on chosen performance markers, could thus improve the performances of downstream operations. After reviewing the typical feed stream compositions, this article reviews the existing literature and shows the advantages of thoroughly investigating and then selecting a clarification method that provides the best downstream performance and economics.

Typical Characteristics and Expected Purity of a Feed Stream Product

Feed Composition and Characterization

Depending on the nature of the product being produced using live cells (e.g., plant, microbial, or mammalian) to extract biopharmaceutical active ingredients, the composition of a feed stream to be clarified and purified can vary significantly. An example of the feed characteristics for a MAb process is shown in Table 1.

Based on the cell viability and the primary clarification (centrifugation, flocculation) or lysis (chemical, mechanical) methods selected, the impurity profile may differ considerably. For example, low cell viability can mean high levels of contaminants released in the feed stream due to cell lysis.[3]

Among the typical main contaminants, DNA, host cell proteins (HCPs), lipids, and bigger particles such as cell debris can be identified. The proportion of solids in the feed are usually a good indicator of the challenges ahead in purifying it (~6–8% in Chinese hamster ovary [CHO], 10–13% and greater, up to 40%, in yeast).

HCPs compose a very heterogeneous family of molecules, in terms of molecular weights and properties (pI, immunogenicity, etc.). They are released during both the cell culture itself and the lysis process. Although HCPs are rarely fully characterized, a better knowledge of their properties could help in their removal during the downstream steps.

As for HCPs, nucleic acid material content depends on the type of biological product and its manufacturing process. Different strategies can be put in place to reduce the average size (measured in base pairs [bp]) and con-

<table>
<thead>
<tr>
<th>TABLE 1. Typical process feed characteristics for MAbs.[2]</th>
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<tbody>
<tr>
<td><strong>At Various Stages</strong></td>
</tr>
<tr>
<td>Clarified Harvest</td>
</tr>
<tr>
<td>Protein A (capture)</td>
</tr>
<tr>
<td>CEX (purification)</td>
</tr>
<tr>
<td>AEX (polishing)</td>
</tr>
</tbody>
</table>

NOTES: Chromatography processes – protein A removal, cation exchange (CEX), and anion exchange (AEX)
centration of DNA in the final product. Endonucleases (such as Benzonase®) can be used for their digestion, and techniques like chromatography or tangential flow filtration (TFF) have been proven to efficiently reduce DNA contaminant levels.\[^4\,^5\]

Although the average particle size of a CHO preparation is around 10 µm,\[^6\] the larger particles are easier to remove by either centrifugation or depth filtration. The smaller particles, below 5 µm, are more likely to impact capacity or penetrate the primary clarification process and present challenges to membrane filter protection or chromatography performance downstream.

Before primary clarification begins, it can be found that specific pretreatment of less common feed streams, such as those with high cell densities or high levels of specific contaminants, may well generate feedstocks with properties compatible with effective and efficient downstream purification steps.

**Feed Pretreatment**

Feed pretreatment is a well-known technique widely applied to wastewater treatment to build up sludge for the biomass removal, or in the food industry for cheese production.\[^7\] In biopharmaceutical production, specifically with mammalian cell-based feed, pretreatment may lead to a shift in average particulate size from below 10 µm to between 20 and 60 µm after pretreatment (depending on the strategy used). Remaining particulates can be removed by depth filtration and improve the efficiency of a centrifuge step. A secondary effect can be the selective precipitation or flocculation of contaminant species.\[^8\]

Pretreatment agents are diverse. For example, acids (acetlic or citric), salts ([NH₄]₂SO₄, K₂SO₄, KH₂PO₄), cationic polymers (chitosan, pdADMAC, PEI) or other artificial polymers (PEG) are frequently used for such purposes.\[^9\]–[^11\]

In order to assess the efficiency of the pretreatment agent, several parameters can be monitored, including supernatant turbidity and particle size distribution, for which a combination of absorbance at 600 nm and dynamic light scattering can be used. Product yield and potency is, of course, a key indicator of the impact pretreatment will have on product quality, and this must be monitored by in-house assays developed specifically for the feedstock.

Acidic treatment is one of the simplest methods to implement and is widely accepted by the regulatory authorities: pH is lowered down to ~5.0 (to be optimized during trials) by adding acid, thus modifying the charges of the solutes and leading to the aggregation or precipitation of medium-sized particles (20–30 µm).\[^12\] From a regulatory point of view, it is not necessary to demonstrate the absence of any added agent in the final product as only the pH has been modified.\[^13\]–[^14\] It is generally readjusted after filtration.

Using salts for protein precipitation is a broadly used method in biochemistry. Salts interact at the surface of contaminants, decreasing their solubility, which leads to their precipitation.

However, these two methods can denature the product of interest and decrease its stability, and/or have a negative impact on product recovery during the filtration step. Losing product via precipitation is also a possibility, and some level of yield loss has to be expected.

Flocculation agents such as cationic polymers act as a binder to aggregate contaminants in the solution, resulting in the formation of a loosely bound floc. Polymers have a positive or negative charge and bind cell debris, host cell proteins, nucleic acids, and other contaminants. Flocs are generally larger than particulates obtained via acidification, ranging from 30–60 µm. The success of a flocculation step depends mainly on polymer dosage. Its removal has to be validated, per regulatory standards, as polymer remnants are considered to be contaminants.\[^15\]

**Final Product Purity Levels**

Regulatory agencies provide recommendations and requirements regarding acceptable residual amounts of contaminants in the final product. Host cell DNA in the final product, for patient safety and tolerance reasons, must be reduced to appropriate levels. In 1998, the World Health Organization specified the maximum residual DNA content in a vaccine to be below 10 ng/dose. More recently, the European Medicines Agency (EMA) proposed more stringent conditions based on the type of cell line (tumorigenic origin) used in the vaccine manufacturing, whereas the U.S. Food and Drug Administration (FDA) follows a case-by-case evaluation approach and recommends that manufacturers reduce both the size (~200 bp) and amount of DNA per dose. To date, a final DNA content of <10 ng/dose is commonly accepted for most biologics.\[^16\]–[^18\] Similarly, a recombinant MAb product must reach clearance of impurities down to <100 ppm of HCP, ≤10 ng/dose of DNA, and ≤5% of immunogenic aggregates.\[^19\] A summary is shown in Table 2.

**TABLE 2. Acceptable remaining impurities in vaccine and MAb products.**

<table>
<thead>
<tr>
<th>Requirements</th>
<th>Specification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reduction of DNA amount</td>
<td>≤10 ng/dose</td>
</tr>
<tr>
<td>Reduction of DNA strand size</td>
<td>~200 bp</td>
</tr>
<tr>
<td>Reduction of HCP</td>
<td>&lt;100 ppm</td>
</tr>
<tr>
<td>Immunogenic aggregates</td>
<td>≤5% (for MAb)</td>
</tr>
</tbody>
</table>

**Feed Quality Evaluation Criteria**

Several parameters can be used in order to assess the clarity or quality of a product, either during process development work or after each manufacturing process step. Some of them will be described in this article. Turbidity is an easy parameter to monitor and provides an immediate assessment of the feed quality. It allows for the detection of depth filter breakthrough, for example.

There are two primary methods of ascertaining the
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Factors Impacting Chromatography Performance and Processes

Common parameters generally considered in evaluating chromatographic performance are dynamic binding capacity, recovery, product purity, and resolution. What affects them can be different depending on the type of chromatography applied. For example, packed bed chromatography is especially sensitive to changes in feed stream characteristics, particularly to fouling species like lipids, DNA, and solids. We recommend early removal of HCPs and DNA, prior to selected chromatography processes. Among others, depth filtration can be an efficient method for downstream protection.

Protein A Chromatography

Protein A chromatography is one of the most effective technologies to capture and efficiently purify antibodies. However, the cost of the media used in this step is significant and users attempt as many reuses/regeneration cycles as possible whilst still retaining acceptable binding capacities and contaminant clearance profiles, often up to several hundred cycles.[2] Predictably, product yields decrease while contaminants—such as DNA and HCP—rise. Low pH conditions are mandatory for elution of product and may lead to the formation of proteins aggregates or precipitates (antibodies or HCPs). This can lead to fouling and may reduce the resin lifespan.

Precipitates are composed of the product and HCPs, highlighting an interest in their early removal or prevention of formation in the first place.[25] In parallel, some studies show that HCPs in the feed can interact with the product of interest on the column and lead to their increased retention.[3] Therefore, an efficient reduction of contaminants prior to protein A is key to maintaining the longevity and efficiency of the resin.[3]

AEX Chromatography

It has been shown that after the protein A step, fouling agents impacting the AEX polishing step are mainly composed of HCPs. Excessive contaminant binding can seriously impair cleaning effectiveness and reduce the lifetime of the resin, which is why such contaminants should be removed prior to chromatography.[23]

CEX Chromatography

Feed quality has been shown to dramatically impact CEX performance as the complexity of the product loaded on the column increases with the concentration of contaminants. Competitive binding is indeed more pronounced in less clarified feeds with cell debris components acting as adsorbents. This also reduces the window of operation for good performance.[6]

Hydrophobic Interaction Chromatography (HIC)

Lipids are a specific challenge for HIC when used in yeast applications, for example. They tend to accumulate run after run and coat the outer surface of the beads.[26, 27] They can impact the HIC capacity by competing or even shielding the binding surface and changing the binding properties of the matrix. Harsh clean-in-place conditions do not represent a robust method to recover column capacity. Therefore, another method for lipid removal prior to column loading must be considered.

Depth Filter Clarification Effects on Feedstream Characteristics

Structure and Composition of Depth Filters

Included in the Millistak® family of primary and secondary clarification filters are traditionally depth filters...
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composed of cellulose fiber (with a high permeability matrix and good mechanical properties), an inorganic filter aid (such as diatomaceous earth) presenting an inherent binding capability for biological material, and a positively-charged polymeric binding agent (with adsorptive charge-based properties) (Figure 1).

Other materials are used for prefiltration like glass, cellulose ester, or activated carbon. Their structures are made of a single layer, or specific combinations of media layers, for different feed qualities and origins. Furthermore, specific media aimed at a higher filtrate purity are constantly being developed. For example, Merck Millipore recently launched a new media (Clarisolve®) for handling pretreated feed that is designed to more fully optimize the overall media depth, and therefore, enhance retention and capacity (Figure 2).[28]

Mechanisms of Retention

Depth filters ensure a normal flow separation of particles and solutes via two main mechanisms: sieving and adsorption. Their high capacity is due to the fact that contaminants are trapped and retained within the whole depth of the media rather than being held only on the surface of the filter.

Sieving is based on size exclusion, where rigid particles are retained at the surface and within the depth of the filter. Adsorption works independently from size. It is based on the interactions between filtration media and particles through electrostatic attraction, hydrophobic interaction, or Van de Waals forces. Adsorption is often effective for the removal of smaller particles (below 1 µm) but is limited by available adsorption sites within the media.

FIGURE 1. Main components of depth filters: (A) cellulose fibers; (B) diatomaceous earth; and (C) polypropylene fibers.

FIGURE 2. Media grades of the Clarisolve®/Millistak+® family.

Legend:
- Clarisolve grades (for pretreated feeds)
- Millistak+ grades with diatomaceous earth only
- Millistak+ grades with diatomaceous earth and cellulose
- Millistak+ grades with cellulose
- Millistak+ grade with activated carbon
Particles
The largest particles present in feed stocks can be retained via sieving at the surface of depth filters. The smaller ones penetrate the depth of the filter and are retained by entrapment. When any pretreatment is applied to the feed, the average size of particles shifts significantly (up to 60 µm), leading to an increase in sieving/entrapment and therefore may require the use of specific media.

DNA
DNA removal by depth filtration occurs mostly via the mechanism of adsorption. DNA adsorption is usually due to both hydrophobic interactions and electrostatic effects. Electrostatic interactions take place along the phosphate backbone of the DNA, whereas hydrophobic interactions happen within the grooves of the DNA helix. Therefore, adsorption depends on feedstock solution composition, with pH and conductivity (lower conductivity suggests higher retention) playing a main role.

HCP
Similarly to DNA, the removal of HCPs by depth filtration occurs by adsorption. Absorbance can be a good indicator of their removal as studies show that absorbance at 410 nm is mainly due to HCP (increased spiking with DNA does not affect its value). Also, in a CHO process, most HCPs are negatively charged due to their pI < 7.0. Therefore, slightly basic pH and lower conductivity is preferable for efficient HCP removal by depth filtration. The efficiency of depth filters has been shown at different stages (prior to or after protein A) and highlights the necessity for a thorough selection of the media used for optimum clearance. As absorbance is a good tool to monitor the presence/absence of HCPs, it can be utilized effectively at scale and is one of the major parameters typically monitored during filter sizing trials.

Discussion
Column Protection with Clarification Depth Filters
Different chromatography modes have been shown to be sensitive to the nature and/or content of contaminants loaded onto them. Logic says that removing them earlier, and in a more effective way, would improve the protection and performance of the chromatography steps. However, available literature that clearly illustrates the cause/effect link is scarce.

Among existing data, the choice of the secondary clarification by depth filtration indeed impacts the HCP profile post-protein A column, showing that the retention properties of the depth filters could be used to reduce and influence this profile. HCP profiles could also be used early in process development to select the most suitable clarification step for a specific feed. As well, the positively charged depth filtration of feed streams was shown to result in less turbid protein A eluates, a result of its contaminant removal qualities. In another study, performed on AEX chromatography, data show that applying clarification by depth filtration on the feed stream beforehand improves the AEX capacity, robustness, and media longevity. Also, a multi-stage depth filtration is more efficient in removing contaminants and the use of pretreatment shows promise for contaminant removal and downstream protection.

These published examples prove that there is a real interest in investigating further how to best apply clarification by depth filtration for early purification of feeds. It should still be kept in mind that to ensure consistent and robust clarification, variability of the feed stream and the clarifying media should be taken into account and built into the safety factors for designing and sizing this protective step. For comparable performances, the selection of a clarification train may depend on scale and/or preferred technologies. For example, at scales from 2,000–5,000 L of bioreactor capacity, using depth filtration (single or multi-stages) versus centrifugation for primary clarification is cost-equivalent. For smaller scales (<1,000 L), it offers real financial advantages.

However, in all of the clarification scenarios involving depth filtration (clarification in one or two steps following pretreatment), further work should be performed to assess the overall impact on the downstream process. A number of questions can be raised:
- Does it affect the design of the downstream process itself or only its lifetime/performance?
- Does this increased early removal of contaminants impact only the first stage of the downstream cascade or the complete downstream train (to the point of modifying it by reducing its stages)?
- Could the performance of the clarification steps be pushed so far as to lead to a redesign (and ideally simplification) of the downstream cascade?

Until results from further clarification process efforts become available, and given the variability of feeds, case-by-case testing, supplier experience, and in-house process development remain the best ways to assess the actual impact of depth filtration for contaminant removal and to design an economical downstream process.

Conclusion
In this paper, the referenced literature illustrating depth filtration properties for the removal of contaminants such as HCP and DNA has been reviewed. Even though the impact on the overall downstream purification process, and notably the chromatography steps involved, is rarely made clear, published data show that resin lifetime, robustness, and/or capacity can be improved by using cleaner clarified feeds on different chromatography types. This opens distinct possibilities for further investigations on the advantages of improved clarification for downstream economics or redesign.
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References


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