Technical Note

Development Strategy for a Cation Exchange (CEX) Chromatography Step in a Monoclonal Antibody (mAb) Process

Objective

The aim of this technical note is to provide a description of the steps followed and considerations taken during the development of a CEX chromatography step in a mAb process.

CEX in a mAb Process

Capture with a Protein A resin is typically the first chromatographic step in a mAb process. While this purification step generally results in a product with >95% purity, the remaining impurities, such as host cell protein (HCP), DNA, product related species (e.g., clips, aggregates), and leached protein A need to be cleared in the next chromatographic steps. The CEX step offers a powerful mechanism to remove these product and process related impurities.

In a mAb process, a CEX step often follows the capture step to further reduce the previously mentioned impurities. However, in some cases, this step is positioned downstream of a second chromatography step. Regardless of the position, the CEX step is normally operated in a bind and elute mode since most humanized mAbs have pIs > 7, allowing them to bind CEX resins at pHs between 4 and 6. Under these conditions, most impurities bind more tightly than mAb monomer to CEX resins, allowing for separation conditions to be established.

Resins Tool Box

The first step in the development process is to establish the resins that will be evaluated for each molecule or platform. It is important to keep in mind that the optimal resin can vary for different mAbs. Each mAb, even those using a common molecular platform, will have different charge properties that will influence its chromatographic behavior in relation to the impurities present. For example, leached Protein A, which is primarily present as a complex with the IgG molecule[1], will elute at different positions when run under identical conditions due to the IgG differences.

In the case of Merck Millipore chromatography media, the recommended resins for screening are the strong cation exchangers: Fractogel® EMD SO_3^- (M), Fractogel® EMD SE Hicap (M), Eshmuno® CPX and Eshmuno® S resins. The selectivity of weak cation exchangers is usually different than strong ion exchangers at any given pH since they have a titratable functional group of higher pKa. Therefore, weak cation exchangers may provide better aggregate removal but generally have a lower binding capacity. To this end, Fractogel® EMD COO^- (M) resin is often evaluated as well. Please note: usually weak ion exchangers are only tested when challenges in resolution of impurities arise. If the number of resins to be tested is limited and resolution of aggregates is one of the main purification goals, Fractogel® EMD SO_3^- resin and Eshmuno® CPX resin are the initial resins recommended for evaluation due to demonstrated success across a wide range of mAb processes.

Table 1 shows a flow chart for the overall CEX development strategy including the details for the elution optimization studies described next.

Considerations for scale up

At this early stage in the development, end users take into account factors such as: previous experience with the vendor (security of supply), the resin (ease of packing), and commercial availability of the product (when resins in development are tested).

Column bed height, flow rate, and system pressure should be considered during the resin evaluation process. Although these factors may not be as critical at the initial purification scale, they may become more important at commercial manufacturing scale. Selection of a resin that can be used throughout manufacturing—from clinical to commercial scale—simplifies process development and leads to a more robust final process.

Initial Screening—Window of operation for binding (static)

The first evaluations of the resins in the tool box involve binding capacity determinations under a range of pH and conductivity conditions. For mAb processes, the pH range is generally between pH 4 and 5.5, although in some cases, pH values as high as 6 have been employed. The ionic strengths tested are usually between 3-5 mS/cm at low buffer concentration (e.g., 50 mM acetate). Dilution and/or pH adjustment of the feed material are usually needed depending on the specific process conditions for the Protein A elution and low pH viral inactivation steps that generally precede the CEX step.

At this stage, the binding capacity is generally determined under static conditions to maximize the number of conditions tested. The use of High Throughput Screening (HTS) tools, such as 96-well plates or micro-columns, can expedite the evaluation. Additionally, the small volumes required for these experiments allow the exploration of a wider experimental space. In cases where these HTS tools are not available, similar batch binding experiments can be performed manually using small resin volumes (e.g., in centrifuge tubes).

Although batch experiments can also be conducted to evaluate elution conditions, these evaluations are generally done in dynamic mode in a column format since linear gradients cannot be performed in batch mode and other parameters (e.g., protein loading) can also impact the resolution of impurities as described later in this document. The results from these initial screenings should narrow the number of resins and operating conditions to be tested in the next step of the development.

Considerations for scale up

Factors to consider before and during this step include buffer characteristics (e.g., pKa, type) and buffers used in the previous and subsequent steps. Buffers generally used for equilibration, binding and elution in a CEX step in a mAb process are acetate, citrate or phosphate. Other buffer related factors such as cost, ease of use and disposal (e.g., phosphate), particularly at large scale, should be evaluated as well.

Dynamic Binding Capacity

The static binding capacity studies narrow the binding conditions (pH and conductivity) and establish the foundation for the optimization of capacity and impurity clearance in a dynamic mode. However, it may be preferred to omit the static binding capacity studies and start the initial resin screening in a packed column format if time and/or mAb feed stock are limited, or if there are budgetary constraints.

The binding capacity of the selected resins is then evaluated in a dynamic mode in the narrowed range of pH and conductivity to establish the optimal binding conditions. A breakthrough curve of the mAb is generated and the dynamic binding capacity (DBC) is determined at a percent breakthrough. A 5% breakthrough is more relevant to use in DBC measurements since the shape of the breakthrough curve is not as sharp as for some affinity resins and can also be influenced by impurity load. Generally, residence times on the order of 4.5 to 6 minutes for Fractogel® EMD (M) resins and 3 to 5 minutes for Eshmuno® CPX and Eshmuno® S resins are good starting points for DBC evaluations. The preferred column format for these studies is at a bed height that would be utilized upon scale up (typically 15-25 cm). If feedstock is limited, a shorter bed height can be used for screening conditions and the DBC can be later confirmed at the desired bed height with the selected resin. In addition, the column diameter generally utilized is 1-2.5 cm whenever possible to minimize potential wall effects. Each resin should be packed according to the supplier's recommendations to obtain the best assessment of a resin's capabilities.

Considerations for scale up

At this point, an important consideration relates to the system and hardware capabilities at large scale, particularly pump capacity. Semi-rigid or rigid media, like Eshmuno® S media, have relatively high permeabilities (e.g., pressure drops below 2 bar at linear velocities higher than 1000 cm/h for a 20 cm bed height). However, at large scale, chromatography systems can generally deliver flow rates corresponding to linear velocities < 400 cm/h for 1 m diameter columns or larger. Moreover, semi-rigid resins such as Fractogel® EMD resins exhibit a linear pressure-flow relationship at low linear velocities (e.g., < 250 cm/h for a 20 cm bed height), but this relationship is non-linear when exceeding the critical operating pressure (approximately 280-300 cm/h for a 20 cm bed height at 25% compression with a fluid with a viscosity comparable to water). Therefore, the residence times chosen for DBC evaluations should meet both resin and large scale limitations. Finally, the system pressure as contributed by the piping, column hardware (screens, flow distributor, etc.), and head pressure due to tank height in large scale facilities must also be considered in addition to the resin bed pressure drop when designing large scale processes.

Optimizing Impurity Clearance

Before beginning impurity clearance studies, it is critical to establish the impurity removal goals of the CEX step. This will depend somewhat on whether the CEX step is the second or third chromatography step. In the second position, the impurity levels in the CEX elution pool need to be low enough to be cleared to the final target by the third chromatography step, while in the third position, the overall impurity goal must be achieved by the CEX step. In general, the CEX step goal should be able to meet the final specification for aggregate, clipped species, and leached Protein A as well as be able to reduce the level of HCP. If charged antibody variants need to be reduced below what the cell culture process produced (a determination based on the biological impact of the variants), the CEX step can also be used.

While DBC is one key criterion for selecting the resin and binding conditions, the primary function of the CEX step is purification. The next step in the process development is to establish the appropriate binding and load conditions to achieve the target impurity clearance (e.g., aggregates, leached Protein A).

It is important to note that conditions that provide the highest DBC are not necessarily the same as those that provide the optimal resolutoin of mAb and impurities. Therefore, we suggest that one or two resins with the desired DBC be tested in a resolution study. This type of study will initially involve more work, but will provide an extensive amount of data that will allow the optimal conditions for impurity clearance to be determined.

Figure 1 illustrates the separation of a mAb from an impurity (e.g., aggregates) during a linear elution gradient. The resolution of mAb and impurities is usually incomplete (i.e., not down to baseline), thus, the volume of the elution pool collection has to be established such that both yield and purity are maximized. In Figure 1, a collection up to point A would achieve a high purity but the yield would be low. In contrast, collection of the elution up to point C would result in a high yield but minimal resolution of the impurity. An elution pool collection up to point B would maximize both yield and purity.

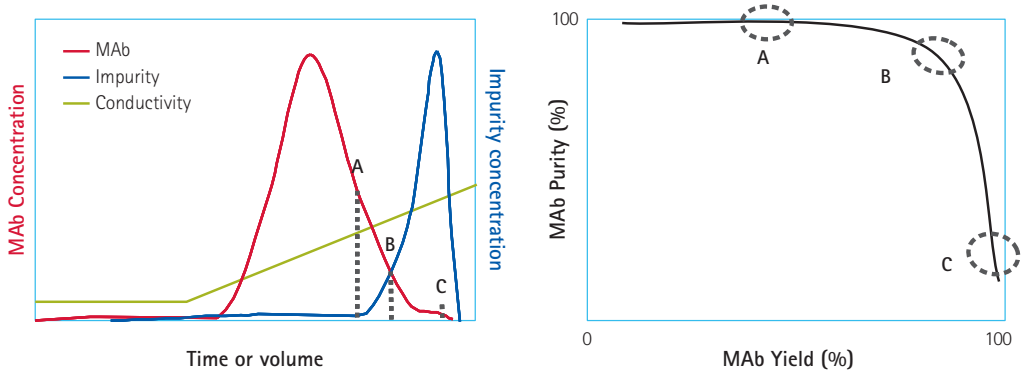

Figure 1. Left. Qualitative example of mAb elution (red) and resolution of impurities (blue) using a linear gradient of increasing conductivity (green). The scales in the two y-axis do not have the same magnitude since impurities are in trace amounts.

Right. Relationship between resolution of impurities and yield for different elution collection points as described in the text.

Figure 2 shows an example of the gradient elution and fractionation described above and the corresponding HCP and aggregate levels for each of the fractions. Since some of the HCP elutes in the early part of the mAb elution, a similar optimization of peak collection considering both ends of the elution peak can be done to maximize purity and yield.

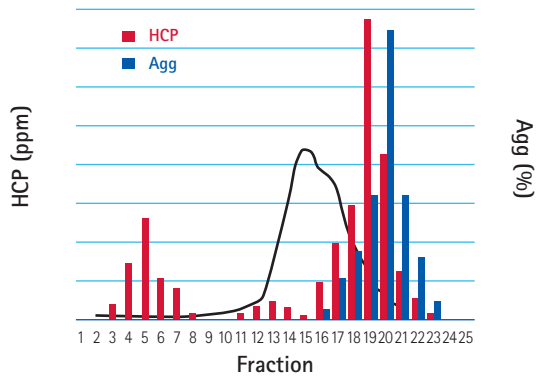

Figure 2. Example of gradient elution fractionation. HCP and aggregate (Agg) levels for each fraction are shown relative to the mAb elution peak (black line).

Study Design

Pack a column with the desired bed height as can best be estimated from the final manufacturing conditions. If time permits, the linear gradient study described should be tested at a variety of loading pHs ideally covering at least 1 pH unit (e.g., 4.0-6.0) in 0.5 pH unit increments. A linear conductivity gradient is generally used for elution and fractions across the gradient are collected for impurity analysis (e.g., HCP, aggregates, leached protein A). Load representative feedstock at the desired pH and wash the column with the loading buffer until the absorbance reaches baseline (collect this wash fraction). Begin the elution by performing a gradient from 0 to 100% B buffer over 20 column volumes with the A buffer being the load buffer and the B buffer being the load buffer plus 0.5 M NaCl. Over the course of the gradient, collect 1 column volume fractions for analysis. It is then generally sufficient to perform a final elution with 1 M NaCl to remove any remaining protein followed by 0.5 N NaOH and storage in 0.1 N NaOH. Analyze the fractions for HCP, leached Protein A, host cell DNA, antibody charged variants, antibody aggregate and total IgG. Based on the separation of the various impurities and the antibody yield, further studies can be used to optimize the conditions. The goals will be to obtain the desired purity targets for the CEX step, optimize the antibody yield, develop manufacturing pooling conditions, and obtain manufacturing plant fit. At this point, ideally the optimal resin and perhaps two different elution pHs can be selected for further development. A key decision is if the manufacturing scale process will use a gradient. A step elution will be used if there is no gradient capability in manufacturing or buffer tank volumes are limiting. The study for both a step elution and a gradient elution will be briefly discussed.

Resin Toolbox and Step Targets

- Shortlist of resins (based on experience, security of supply, etc.)
- Define success criteria (purity, recovery, facility fit, cost of goods)

Initial Screening

- Static (optional) – 96-well plates or centrifuge tubes
- Dynamic – Packed bed column (target manufacturing scale bed height)
- Determine capacity in window pH and conductivities (e.g., pH 4–5.5, conductivity: 3–5 mS/cm)
- Narrow resin options

Optimization of Impurity Clearance

- Perform study with selected resins
- Load to 80% of DBC
- Perform 20 CV gradient (0 to 0.5M NaCl)
- Fractionate elution and assay
- Optimize conditions to meet purity targets and yield
- Select optimal resin

Elution Optimization

- Define elution type to be used at manufacturing (e.g., gradient or step)
- Determine conditions for step elution or optimize slope and length of elution gradient (e.g., 5 CV)
- Determine pooling criteria that will maximize yield and purity as well as ease of scale up

Process Robustness and Integration

- Assess the performance of CEX step in process window of operation including potential failure areas.
- Confirm performance of overall process with cell culture material from pilot scale (or larger)

Table 1. Overview of CEX step development strategy.

Gradient Elution

The 20 column volumes gradient used in the screening study is likely too long and uses too much buffer for use in manufacturing. Make the A buffer at a conductivity slightly below (e.g., 2-3 mS/cm) that at which the antibody begins to elute and the B buffer at a conductivity higher (e.g., 2-3 mS/cm) than that at which the antibody stops eluting. Perform a 5 column volume gradient collecting fractions and determine if the level of separation and yield are acceptable. If necessary continue to vary the pH and conductivities of the two buffers for further optimization.

Step Elution

Make the elution buffer at a conductivity that is approximately in the midpoint of the antibody peak. If there are impurities that are bound to the column after washing to baseline with the load buffer, and then make an additional wash buffer (wash buffer 2) with a conductivity below that of the elution buffer. Load the column, wash to baseline with the load buffer (wash 1), wash to baseline with wash buffer 2 followed by the elution buffer. Collect the elution peak from baseline to baseline and determine purity and yield. Adjust the conductivities of wash buffer 2 and the elution buffer to obtain the desired purity and yield. Other model-based methods[2] available in the literature can be applied to transition from gradient to step elution.

Pooling Criteria

The parameters used to start and stop collecting the elution pool containing product are critical to achieving high yield, high purity and a successful scale-up to manufacturing. Due to the operational complexity and quality control work involved at manufacturing scale, collecting fractions for testing and pooling is rarely done. Instead easily measured output parameters such as absorbance and/or volume are usually used. A thorough understanding of the impurity profile across the elution peak is necessary to choose the appropriate starting and ending conditions.

Additional approaches

If further purity optimization is needed, changing the salt or buffer type can affect impurity separation from the product. Changing the pH of either the wash buffer or elution buffer can also result in better impurity clearance. If an impurity is present on the leading edge of the elution peak, increasing the absorbance when pooling is started will lower this impurity in the elution pool, although with slightly reduced yield. Conversely, increasing the absorbance of the pool end conditions will lower impurities on the trailing edge of the elution peak.

Process Optimization

Once the optimal conductivity for the separation has been established, the length and end point of the conductivity linear gradient can be reduced (e.g., 5-10 CV up to 250-300 mM NaCl in equilibration buffer) to reduce the total pool volume since shallow gradients result in larger pool volumes. This elution pool collection has to meet tank volume limitations that can exist at pilot or large scale. In addition, the resolution of impurities under these conditions has to be confirmed since the slope of the gradient also affects the resolution of mAb and impurities.

It should be noted that the loading and bed height can also affect the separation of impurities. In the evaluations described above, a loading of approximately 80% of the DBC at 5% breakthrough is generally used. A lower loading may result in an uneconomical process and, while a higher loading may provide the desired resolution, a safety factor is generally used (e.g., 80% of the 5% breakthrough). Longer bed heights can also improve the resolution and impurity clearance, but generally the condition optimizations are performed at the bed height that will be used at large scale, i.e., 15-20 cm. In some cases, up to 30-40 cm bed heights may be used. The desired bed height is based on the optimal height for desired resolution and the overall column volume (bed height x cross-sectional area) needed based on expected protein load and resin capacity (g/L).

Although pH gradients can also be employed, these are not commonly used due to the difficulty in controlling the pH change. In addition, establishing a robust pH gradient during scale up can also be more challenging compared to a conductivity gradient. However, in some cases a pH gradient can improve the resolution of some impurities, such as aggregates, and could facilitate the need for conditioning before the next step (e.g., dilution and pH adjustment prior to an anion exchanger). Another approach is to change the pH between the load conditions and wash and/or elution conditions as a step in conjunction with conductivity changes. The control of pH step changes is usually more robust than a pH gradient. A step elution with a fixed NaCl concentration can also be employed, but the impurity clearance needs to be robust within the variability that can be encountered in a process.

In the evaluations described in this section, the wash step is usually conducted with equilibration buffer (e.g., 5-10 CV). In cases where a step elution is utilized, an

intermediate wash with a conductivity between that of the equilibration and elution buffers can be utilized to remove loosely bound species. In addition, 0.5 M-1 M NaCl is usually used for regeneration of the column and 0.1-0.5 N NaOH for cleaning. Alternatively, 0.5 N NaOH can be used simultaneously for regeneration and cleaning. This approach can be efficient in processes where there is not a significant amount of protein remaining in the column after the elution step. In addition, one buffer can be removed from the process along with its potentially corrosive effect on equipment.

The effectiveness of the cleaning regime needs to be evaluated as part of the step optimization. A blank cycle, (all the step buffers except the load) is generally performed after a few cycles (e.g., 5, 10) and the elution is collected and analyzed for carryover of product or impurities. If impurities are found in this mock elution, it is necessary to identify them and also assess the effect of the carryover with regards to performance (e.g., yield, purity) in the subsequent cycles. For CEX chromatography in mAb processes for non-capture steps, standard cleaning with 0.5 N NaOH is generally sufficient since the impurity loads are much lower compared to capture steps. Carryover of product or impurities generally necessitates an improved cleaning regime, for example a higher concentration of NaOH may be needed. In addition, if the carried-over impurities and their nature have been identified, more specific cleaning strategies can be evaluated, e.g., detergents for hydrophobic impurties. An ineffective cleaning regime can have an impact on chromatographic performance and/or ease of packing used resin.

The evaluation of resin lifetime is another important part of the development process. In the early stages, this goes hand in hand with the cleaning optimization described above since at least a few cycles are needed to ensure an acceptable cleaning regime. A truncated/short lifetime study can be conducted for products in early stage that would at least cover the expected number of cycles for a batch of clinical material. In later stages, once the process conditions have been finalized, the useful lifetime of the resin needs to be confirmed for the number of cycles that will be validated, generally > 100 cycles.

Process robustness

In addition, once the working parameters for pH, conductivity and product load have been established, the robustness of the process needs to be evaluated as well. Generally, an acceptable window of operation would be within ± 0.2 pH units, ± 1 mS/cm and loading between the minimum and maximum load expected at this step based on variability in cell culture expression. The pH and conductivity ranges tested should align with the manufacturing capability for these parameters. Impurity clearance and yield can be significantly impacted over a wide range of protein loading. Design of Experiment (DOE) studies are valuable at this point to examine the ranges in which parameters (e.g., pH, conductivity, load) may interact with each other. The variability of the feed from the previous step and multiple lots of resin can also be evaluated at this stage. These studies will hopefully define a wide range of operating parameters or determine the "edge of failure" for a parameter with regard to impurity removal. A robust process should perform comparably with regards to yield, purity, etc. within the aforementioned window of operation.

Process Integration

Ultimately, the CEX step will need to work in conjunction with all of the other process unit operations including the cell culture process. Since during development the feedstock for the CEX studies may come from preceding process steps that are also undergoing development, it is necessary to test all of the unit operations together as all the other steps are being finalized. This will ensure the CEX step (as well as the other steps) will meet their purity and yield goals with feedstock that is most representative of the final process. Testing every unit operation with every combination of parameter ranges is not practical. However, the parameters with the narrowest ranges which (i.e., the most critical for impurity removal) can be tested using the parameter ranges from previous steps most likely to generate the highest levels of that impurity should be evaluated. While the purification process should perform the same regardless of scale, the cell culture process can be more scale sensitive. Therefore, the purification process should be tested using cell culture material produced at least at the pilot scale (e.g., ~400L) for final confirmation of process robustness.

Considerations for scale up

As mentioned above, the elution pool volume has to consider not only the resolution of impurities but also the tank size limitations that can be encountered upon scale up; an elution pool volume of approximately 5 CV is generally acceptable. When determining the fit for the pool tank, account for any subsequent conditioning operations (pH, conductivity) that may increase the final volume of the conditioned CEX pool, i.e., considering the increase in volume after dilution and/or pH adjustment required prior to the next unit operation.

References

1. Fahrner, R., et al., J of Chrom A, 1163 (2007) 105-111
2. Ishihara and Yamamoto, J of Chrom A, 1069 (2005) 99-106

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