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Application Note

Eshmuno® CMX—a novel Mixed Mode Cation Exchange Resin for the purification of Glycoproteins

Introduction

The aim of this technical note is to provide a description of the capture and subsequent purification of glycoproteins. Case studies are given for monoclonal antibodies and other glycoproteins, incl. the SARS-CoV2 Spike glycoprotein.

SARS-CoV2 Spike Glycoprotein as therapeutic option to fight COVID-19

With the outbreak of COVID-19, the most severe pandemic in the 21st century so far, a feverish search for therapeutic treatment and vaccinations has just started. A substantial number of approaches are focusing on the coronavirus S glycoprotein, a surface-exposed protein that mediates the entry of the virus into the host cells.[1] The Spike Glycoprotein might be the best suited target for the production of therapies and vaccine candidates. On April 27th, 2020 the Milken Institute COVID-19 tracker listed 28 vaccines based on inactivated viruses, virus-like particles (VLP) or non-replicating viral vectors and 28 protein subunit approaches.[2]

The SARS-CoV2 Spike Glycoprotein consists of 674 amino acids and is extensively glycosylated with 22 N-linked glycans that are important for proper folding and modulating accessibility to host proteases and neutralizing antibodies. A high percentage of those N-glycans are oligomannosylated.[3] Capturing and purification of highly glycosylated proteins is a difficult chromatographic task which is best approached by more than one chromatographic interaction principle.

References

1. Walls et al, Cell 180, 281-292, April 16, 2020
2. https://milkeninstitute.org /covid-19-tracker
3. Watanabe et al, bioRxiv, preprint Feb 21 2020 doi: https://doi.org/10.1101/2020.02.20.957472

Through combination of weak cation exchange ligands with moderately hydrophobic side chains, we have developed a novel mixed mode cation exchange resin introduced into the market as Eshmuno® CMX. The dual operation mechanism of this resin allows the purification of otherwise difficult to separate proteins, e.g., bispecific antibodies or antibody-drug conjugates (ADC) according to their Drug-Antibody Ratio (DAR).

In addition, Eshmuno® CMX shows a high binding for oligo-mannose glycosylated proteins, allowing capturing of those proteins as well as purification according to different glycopatterns. Starting with linear screening gradients, production methods can be straightforwardly developed and scaled-up.

Initial Screening – Window of operation for binding (static)

The first evaluations of the mixed mode cation exchange resin involve binding capacity determinations under a range of pH and conductivity conditions. For oligo-mannose or galactose containing antibody, the pH range is generally between pH 4.5 and 7.5. The salt concentrations (e.g., Na_2SO_4) tested are usually between 0–1 M at low buffer concentration (e.g., 50 mM acetate or 50 mM phosphate). Dilution and/or pH adjustment of the feed material might be needed.

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 glycoprotein species. The results from these initial screenings should narrow down the number of resins and operating conditions to be tested in the next step of the development. For example, as shown in **Figure 1**, the separation of galactose containing and oligo-mannose containing antibody molecule would be possible in pH 5.5 and addition of 300–600 mM Na_2SO_4, where galactose containing antibody molecule has lower binding capacity in comparison to oligo-mannose containing antibody molecule.

The initial screening is not limited to given pH or salt or its concentration range and must be selected at the best suited conditions for the target protein.

Figure 1a illustrates the window of operation for binding the oligo-mannose containing antibody molecule under a range of pH and conductivity conditions.

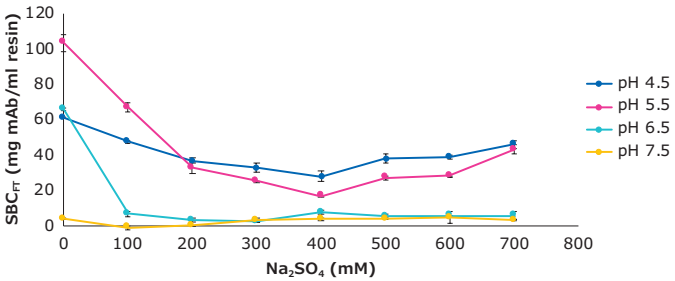

Figure 1b illustrates the window of operation for binding the galactose containing antibody molecule under a range of pH and conductivity conditions.

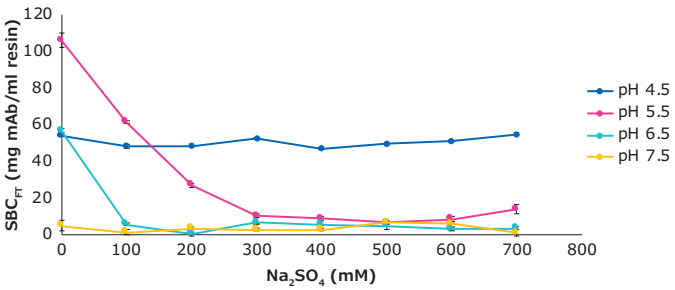

Figure 1a, 1b. Quantitative example of a) oligo-mannose containing antibody molecule and b) galactose containing antibody molecule binding under a range of pH and conductivity conditions. 50 mM acetate and 50 mM phosphate buffer system was used to achieve the pH with different Na_2SO_4 amounts. All values are average values from duplicates performed with 10 µL resin samples in the micro titer plates and using robotic Tecan system for the buffer and sample subjection and UV detection. Static incubation was done for 120 minutes in 0.2 mL sample suspension.

Considerations for scale-up

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

Considerations for scale-up: Systems and hardware

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® CMX media, have relatively high permeabilities (i.e., pressure drops below 2 bar at linear velocities higher than 400 cm/h for a 20 cm bed height). Therefore, the residence times chosen for dynamic evaluations should meet those 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.

Oligo-mannose containing antibody separation study design

Pack a column with the desired bed height as can best be estimated from the final manufacturing conditions. The linear pH gradient study described should be tested at a variety of different salt concentrations ideally covering at least 150 mM and 300 mM NaCl. A linear increasing pH gradient is generally used for elution and fractions across the gradient are collected for oligo-mannose containing antibody analysis. Load representative feedstock at the desired pH and conductivity followed by the column wash 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 60 column volumes with the A buffer being the load buffer (e.g., 0.00796 M citric acid, 0.009068 M sodium di-hydrogen phosphate, 0.021543 M glycine, 0.010668 M TRIS, 0.007666 M succinate, 0.0111 M NaOH and 0.2889 M NaCl of pH 4.75 and conductivity of ~24 mS/cm) and the B buffer (e.g., 0.00796 M citric acid, 0.009068 M sodium di-hydrogen phosphate, 0.021543 M glycine, 0.010668 M TRIS, 0.007666 M succinate, 0.046 M NaOH and 0.254 M NaCl of pH 8.5 and conductivity of ~24 mS/cm).

Table 1 illustrates an example of buffer components and the amounts thereof for the pH gradient.

Substance	Art. No.	Mol	Amount in 1 L Buffer (g)
Buffer A			
Citric acid	1.00244	0.00796	1.6727
Sodium-di-hydrogen-phosphat	1.06346	0.009068	1.2513
Glycine	1.04201	0.021543	1.6170
TRIS	1.08320	0.010668	1.2923
Succinate	8.18601	0.007666	1.2423
NaOH W=32%	1.05587	0.0111	1.3874
NaCl	1.06404	0.2889	16.8833
Buffer B			
Citric acid	1.00244	0.00796	1.6727
Sodium-di-hydrogen-phosphat	1.06346	0.009068	1.2513
Glycine	1.04201	0.021543	1.6170
TRIS	1.08320	0.010668	1.2923
Succinate	8.18601	0.007666	1.2423
NaOH W=32%	1.05587	0.046	5.7496
NaCl	1.06404	0.254	14.8438

Table 1. Major components and amounts used for the 1 L buffer A and B. All components are obtained from Merck KGaA, Darmstadt, Germany. Use sanitization agents and conditions that are suitable for the process requirements. Sanitization agents must comply with applicable local regulations.

Table 2 illustrates an example of method conditions for the pH gradient run. This example is covering a wide range of oligo-mannose containing antibody properties and needs further optimization after the first gradient run is performed to reduce the duration of pH gradient elution or to change to step elution approach.

Starting Method

Steps	Duration	Solvent
Equilibration	5 CV	Buffer A
Load	10–60 mg/mL	Feed at pH 4.5 and 24 mS/cm
Wash	10 CV	Buffer A
Gradient Elution	120 CV	0–100% Buffer B
Strip	5 CV	Buffer B
CIP	7.5 CV	1 M NaOH
Reequilibration	10 CV	Buffer A

Table 2. Sequential steps and duration for the pH gradient set-up on the liquid chromatography system including the column volumes for each step. Recommended velocity is 4 minutes residence time for 1 CV.

It is then generally sufficient to perform a final cleaning with buffer B to remove any remaining protein followed by 1 N NaOH and storage in 20% ethanol containing 150 mM NaCl solution.

Analyze the fractions for glycoprotein distribution. Based on the separation of the oligo-mannose containing antibody and yield, further studies can be used to optimize the conditions. The goals will be to obtain the desired purity targets for the mixed mode cation exchange step, optimize the oligo-mannose containing antibody yield, develop manufacturing pooling conditions, and obtain manufacturing plant fit.

An exemplary glycoprotein bind and elute separation is shown in **Figure 2**, displaying a part of the separation, namely the elution peak of the bound glycoprotein on the Eshmuno® CMX at 20 mg/mL CV loading and 250 mM NaCl. UV adsorption signal trace is displayed in blue line and pH signal trace in orange line.

Figure 2 illustrates an example of the UV and pH traces after applying the linear increasing pH gradient during an oligo-mannose containing antibody separation.

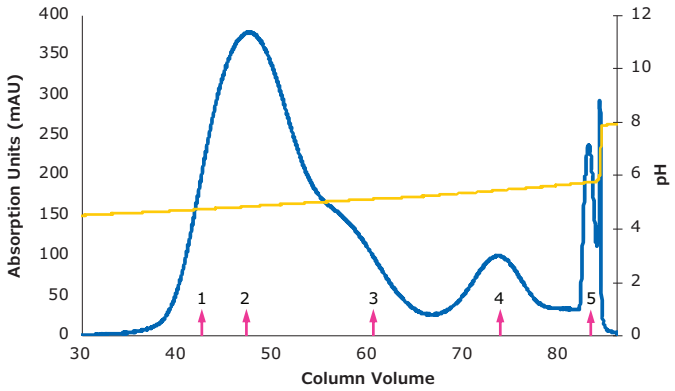

Figure 2. Example of pH (yellow) and UV (blue) traces detected for the chromatographic separation of oligo-mannose containing monoclonal antibody sample loaded to 20 mg/mL CV on the mixed mode cation exchange resin. The blue line represents protein concentration based on the UV adsorption, the yellow line the pH trace. Pink marking indicates the fractions collected during the sample elution. 150 cm/h velocity was chosen throughout all the chromatographic steps.

Additional analytics usually are required for the collected fraction analysis. Liquid chromatography methods coupled with mass spectroscopy detection methods can enable detection of various glycovariant species. In order to identify and quantify N-glycosidic oligosaccharides bound to a protein the method can be performed offline. The N-glycans can be enzymatically cleaved from the protein with PNGase F, then the protein and oligosaccharides are separated from each other and the obtained carbohydrate sample is desalted. The N-glycans can be coupled with the fluorescent marker RapiFluor-MS labeling module and subsequently analyzed by LC-MS. The analysis can be performed using the GlycoWorks RapiFluor-MS N-Glycan Kit from Waters.

Figure 3 illustrates the analytical results of fractions collected during the oligo-mannose containing monoclonal antibody sample separation on the mixed mode cation exchange resin (e.g., Eshmuno® CMX) using the LC-MS analytical method.

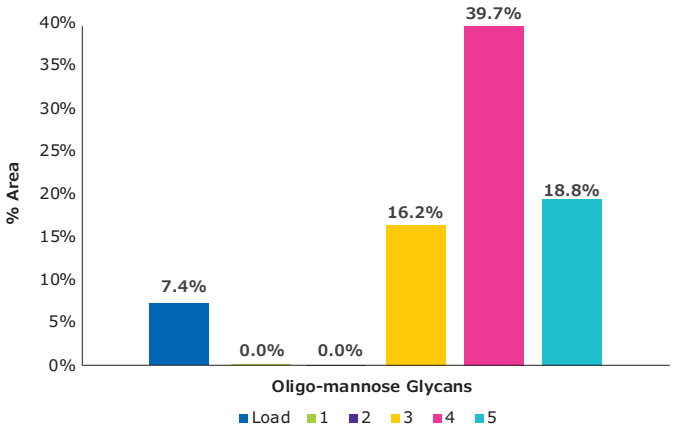

Figure 3. Analytical results of the sample fraction characterization using a LC-MS analytical method displayed in quantitative area of analyzed elution fractions (1 to 5), including loaded glycoprotein (load) at 20 mg glycoprotein/mL CV loading.

As shown in **Figure 2** and **Figure 3**, while using a linear pH gradient elution the separation/enrichment of the oligo-mannose containing glycan species is possible. In the main glycoprotein containing fractions (e.g., 1 and 2) no oligo-mannose variants were detected. Most of the oligo-mannose glycan variants eluted in a separate fraction (e.g., 4).

Alternatively, glycoprotein bind and elute separation can be obtained using buffers containing other salts, such as Na_2SO_4. It is shown in **Figure 4**, displaying a part of the separation, namely the elution peak of the bound glycoprotein on the separation matrix at 30 mg/mL CV loading and 400 mM Na_2SO_4.

Figure 4 illustrates an example of the UV and pH traces after applying the linear increasing pH gradient during an oligo-mannose containing antibody separation. UV adsorption signal trace is displayed in blue line and pH signal trace in yellow line.

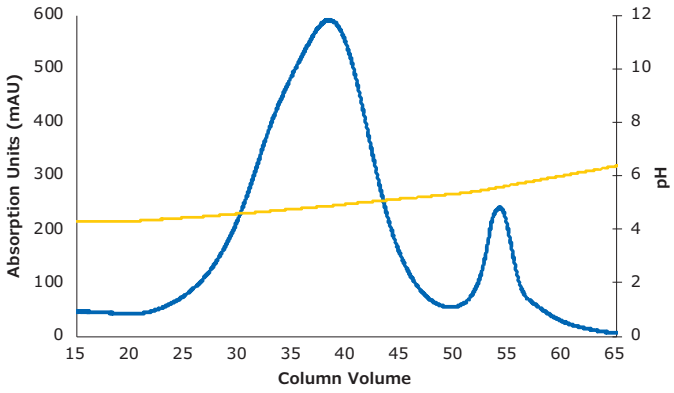

Figure 4. Example of pH (yellow) and UV (blue) traces detected for the chromatographic separation of oligo-mannose containing monoclonal antibody sample loaded to 30 mg/mL CV on the mixed mode cation exchange resin. The blue line represents protein concentration based on the UV adsorption, the yellow line the pH trace. 400 mM Na_2SO_4 was added to buffer A and buffer B instead of 250 mM NaCl. 150 cm/h velocity was chosen throughout all the chromatographic steps.

Figure 4 shows, like **Figure 2**, while using a linear pH gradient elution the separation/enrichment of the oligo-mannose containing glycan species is possible. The oligo-mannose glycovariant containing peak is completely separated from the main glycoprotein containing peak.

Alternatively, glycoprotein separation can be obtained using flow through conditions. It is shown in **Figure 5**, displaying a part of the separation, namely the flow through UV signal trace for the glycoprotein loading and the elution of the glycoprotein on the separation matrix at 10 mg/mL CV loading and 400 mM NaCl.

Figure 5 illustrates an example of the UV and pH traces for flow-through oligo-mannose containing antibody separation. UV adsorption signal trace is displayed in blue line and pH signal trace in yellow dashed line.

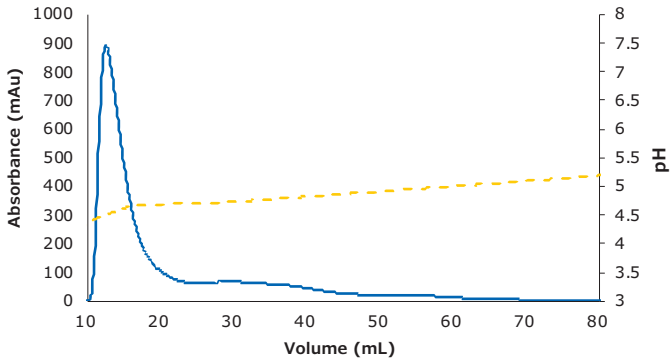

Figure 5. Example of pH (yellow) and UV (blue) traces detected for the chromatographic flow through separation of oligo-mannose containing monoclonal antibody sample loaded to 10 mg/mL CV on the mixed mode cation exchange resin. The blue line represents protein concentration based on the UV adsorption, the yellow dashed line the pH trace. 400 mM NaCl was added to buffer A and B. 150 cm/h velocity was chosen throughout all the chromatographic steps.

Figure 6 shows the analytical results of fractions collected during the oligo-mannose containing monoclonal antibody sample flow through separation on the mixed mode cation exchange resin (e.g., Eshmuno® CMX) using the LC-MS analytical method.

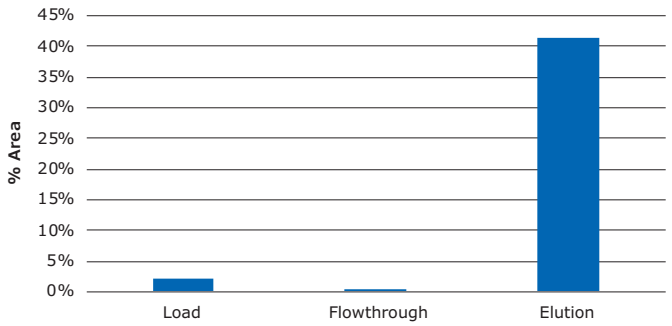

Figure 6. Analytical results of the sample fraction characterization using a LC-MS analytical method displayed in quantitative area of analyzed elution fractions (flow through and elute), including loaded glycoprotein (load) at 10 mg glycoprotein/mL CV loading.

Oligo-mannose containing protein (e.g., SARS-CoV2 Spike glycoprotein) separation study design

Pack a column with the desired bed height as can best be estimated from the final manufacturing conditions. The linear pH gradient study described should be tested at a variety of different salt concentrations ideally covering at least 150 mM and 300 mM NaCl.

A linear increasing pH gradient is generally used for elution and fractions across the gradient are collected for oligo-mannose containing protein analysis. Load representative feedstock at the desired pH and conductivity followed by the column wash 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 60 column volumes with the A buffer being the load buffer (e.g., see recommendations for oligo-mannose containing antibody separation) and the B buffer (e.g., see recommendations for oligo-mannose containing antibody separation. pH was increased to 10.5).

It is then generally sufficient to perform a final cleaning with buffer B to remove any remaining protein followed by 1 N NaOH and storage in 20% ethanol containing 150 mM NaCl solution.

Analyze the fractions for glycoprotein distribution. Based on the separation of the oligo-mannose containing protein and yield, further studies can be used to optimize the conditions. The goals will be to obtain the desired purity targets for the mixed mode cation exchange step, optimize the oligo-mannose containing protein yield, develop manufacturing pooling conditions, and obtain manufacturing plant fit.

An exemplary glycoprotein (e.g., SARS-CoV2 Spike glycoprotein) bind and elute separation is shown in **Figure 7**, displaying a part of the separation, namely the elution peak of the bound glycoprotein on the separation matrix at 1 mg/mL CV loading and 250 mM NaCl.

Figure 7 illustrates an example of the UV and pH traces for bind and elute oligo-mannose containing protein (e.g., SARS-CoV2 Spike glycoprotein) separation. UV adsorption signal trace is displayed in blue line and pH signal trace in yellow line.

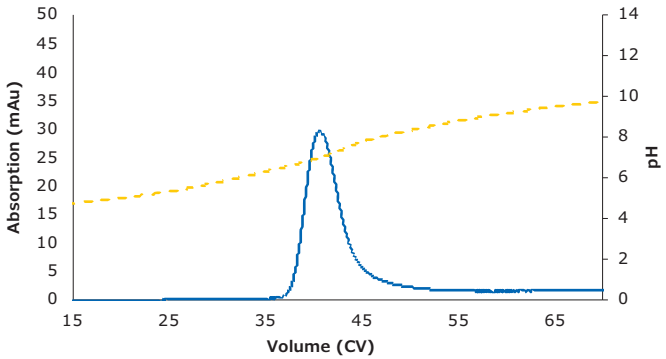

Figure 7. Example of pH (yellow) and UV (blue) traces detected for the chromatographic separation of oligo-mannose containing protein (e.g., SARS-CoV2 Spike glycoprotein) sample loaded to 1 mg/mL CV on the mixed mode cation exchange resin. The blue line represents protein concentration based on the UV adsorption, the yellow dashed line the pH trace. 150 cm/h velocity was chosen throughout all the chromatographic steps.

Figure 8 shows the analytical results of fractions collected during the oligo-mannose containing protein (e.g., SARS-CoV2 Spike glycoprotein) sample separation on the mixed mode cation exchange resin (e.g., Eshmuno® CMX) using the HIC (hydrophobic interaction chromatography) analytical method.

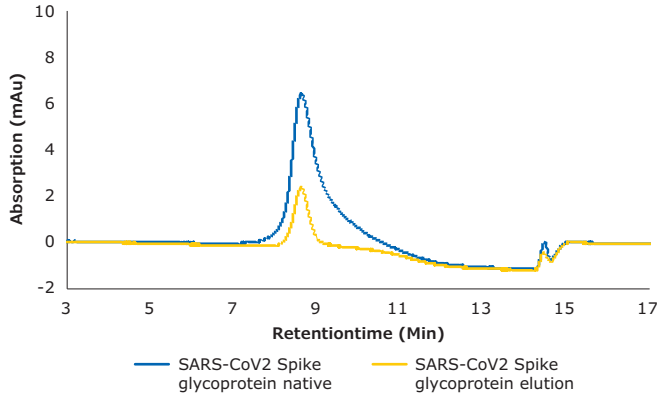

Figure 8. Analytical results of the sample fraction characterization using a HIC analytical method displayed in UV traces of analyzed elution fraction, including loaded glycoprotein (load) at 1 mg glycoprotein/mL CV loading. Blue line represents UV trace for the loaded glycoprotein and yellow line for the elute glycoprotein.

Figure 9 depicts the analytical results of fractions collected during the oligo-mannose containing protein (e.g., SARS-CoV2 Spike glycoprotein) sample separation on the mixed mode cation exchange resin (e.g., Eshmuno® CMX) using the SDS-PAGE qualitative analytical method.

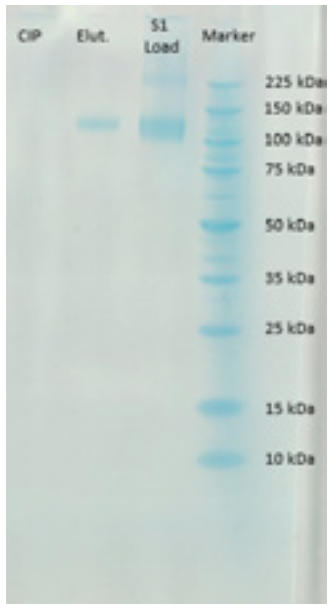

Figure 9.
Analytical results of the sample fraction characterization using a SDS-PAGE analytical method displayed in traces of analyzed elution fraction, including loaded glycoprotein (load) at 1 mg glycoprotein/mL CV loading and cleaning (CIP) with 1 M NaOH.

Additional Approaches

If further purity optimization is needed, changing the salt or its concentration as well as buffer type can affect impurity separation from the product. Changing the pH or conductivity of either the wash buffer or elution buffer can also result in better glycoprotein selectivity. If an undesired glycoprotein species is present on the leading edge of the desired glycoprotein species, increasing the absorbance when pooling is started will lower this undesired glyocprotein species in the elution pool, although with slightly reduced yield. Conversely, increasing the absorbance of the pool end conditions will lower other glycoprotein species on the trailing edge of the elution peak.

Process Optimization

Once the optimal conductivity for the separation has been established, the length and end of the increasing linear pH gradient can be reduced to reduce the total pool volume since shallow gradients result in larger pool volumes. This elution pool collection must meet tank volume limitations that can exist at pilot or large scale. In addition, the resolution of glycoprotein species under these conditions must be confirmed since the slope of the gradient also affects the resolution of glycoprotein species.

It should be noted that the loading and bed height can also affect the separation of glycoprotein species. In the evaluations described above, a loading of approximately >30 mg/mL 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. 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).

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 glycoprotein species. In addition, 1 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 assess the effect of the carryover with regards to performance (e.g., yield, purity) in the subsequent cycles. For mixed mode cation exchange chromatography in glycoprotein species processes for non-capture steps, standard cleaning with 0.5 N NaOH is generally enough 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 impurities. An ineffective cleaning regime can have an impact on the 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 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 capabilities 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 about glycoprotein separation. A robust process should perform comparably with regards to yield, purity, etc. within the before mentioned window of operation.

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