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Application Note

Regeneration and Cleaning of Eshmuno® HCX Chromatography Resin — A Screening Study

Introduction

Eshmuno® HCX resin is a salt-tolerant cation exchanger that can bind solutes in multiple modes, including electrostatic, hydrophobic and/or hydrogen bonding. While multi-modal adsorption facilitates capture at high conductivities, it also makes it necessary to optimize resin cleaning and regeneration to account for the multiple modalities of adsorption.

Chromatography resins are typically re-used multiple times to render them economically attractive for inclusion in a downstream purification process. Regenerating and cleaning the resin are critical for this purpose. During regeneration and cleaning, residual proteins and impurities are removed from the column. The terms “regeneration” and “cleaning” are sometimes employed interchangeably. Regeneration is typically needed after every loading cycle to strip the column of strongly bound impurities and/or product.

The objective of regeneration is to ensure that the chromatographic behavior in each cycle is reproducible, within tolerances. However, irreversibly bound impurities may accumulate over time, and the chromatography process performance (yield and purity) may deteriorate accordingly. In such a case, the resin would need to be cleaned to restore process performance and minimize the risk of carryover.

Sodium hydroxide (NaOH) at concentrations between 0.1–1M can be a very effective regenerant. It is generally not recommended to apply high concentrations of sodium hydroxide immediately after a capture cycle due to impurity precipitation concerns. Typically, an alternate regenerant solution is employed to remove most of the impurities, followed by a final cleaning with sodium hydroxide.

The primary objective of this study was to screen multiple solutions for regenerating efficacy. This work identifies the most promising cleaning and regenerating solutions for Eshmuno® HCX resin.

Experimental Methods

The study was executed in three phases:

Phase I

In this first phase, lysozyme was employed as a model foulant. Lysozyme has a high isoelectric point (pI ~ 11) and a strong hydrophobic character. Thus, it is expected to bind strongly to the resin in a multi-modal manner

A report[1] indicated that it was not eluted in a salt gradient on another multi-modal cation exchanger at a pH of 5, confirming that it can bind in a multi-modal manner.

For these tests, a 1.7 mL column (Omnifit 0.66 cm i.d. x 5 cm) was packed and qualified using an ÄKTApurifier®. The basic test procedure (residence time = 3 minutes) in this phase was as follows:

1. Equilibrate the column with 30 CV 50 mM acetate buffer, pH 5.
2. Load 50 mg lysozyme on the column (lysozyme feed: 2.5 mg/mL in equilibration buffer).
3. Wash the column with 3 CV equilibration buffer.
4. Flush the column with 10 CV regenerant solution.
5. Flush the column with 5 CV 6M Gu-HCl (control) (see **Table 1**).
6. Flush the column with 5 CV 0.5M NaOH.

Metric for evaluating cleaning effectiveness: The area under the UV (A280) peak during the cleaning step (Step 4) was used as a measure of the mass of lysozyme removed from the column and, thus, the cleaning effectiveness.

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Regenerants:
A recent publication[2] suggests that a mixture of two different regenerants was much more effective than either of the agents alone on a mixed-mode resin. This is not surprising, given the multi-modal nature of the adsorption process in these resins. Chemicals such as guanidine hydrochloride and arginine affect both electrostatic and hydrophobic interactions. Thus, these chemicals were not employed in combination with others.

	Regenerant	Levels
Control	6M Guanidine HCl, pH ~ 4	—
1	Arginine	pH 7, 8, 0.5M,1M
2	1.5M NaCl + 20% propylene glycol	—
3	NaCl + 20% isopropanol	1M, 1.5M, 2M NaCl
4	NaCl + Urea, pH ~ 4	4M, 6M, 7M urea; 1M, 1.5M NaCl

Table 1.
Summary of regenerants tested in Phase 1.

Phase II

In this second phase, the most effective regenerants from Phase I were compared using a complex feed stream of clarified Escherichia coli (*E. coli*) lysate.

For these tests, a 0.7 mL column (Omnifit 0.66 cm i.d. x 2 cm) was packed and qualified using an ÄKTApurifier®. The basic test procedure (residence time = 3 minutes) in this phase was as follows:

1. Equilibrate the column with 5 CV 50 mM acetate buffer, pH 5.
2. Load 20 CV of clarified *E. coli* lysate.
3. Wash the column with 3 CV equilibration buffer.
4. Flush the column with 10 CV regenerant.
5. Flush the column with 5 CV 6M Gu-HCl.
6. Flush the column with 5 CV 0.5M NaOH.
7. Re-equilibrate with 10 CV 200 mM acetate buffer, pH 5.

Repeat steps 1–7 for next regenerant.

Frozen *E. coli* lysate was obtained from the University of Massachusetts, Lowell. This was subsequently thawed and clarified using a Millistak+® Pod C0HC 540 cm^2 device. The clarified lysate was sterile-filtered using 0.22 µm Durapore® filter prior to loading onto the column.

A Bradford protein assay yielded a total protein concentration of ~ 3000 µg/mL in the lysate. The assay could not be employed for the regeneration fractions due to interference from the cleaning agents. A Host Cell Protein (HCP) Assay for this strain of *E. coli* was unavailable for these tests. Thus, as in Phase I, the A280 trace was employed as a measure of HCP content.

Scouting runs with clarified feed were executed at pH 5 and 7 to identify the appropriate loading pH. In this case, the objective was to identify the loading pH at which significant HCP binding could be expected. Based on the A280 peak obtained during regeneration with 6M Gu-HCl, the loading pH was identified as pH 5. The thawed lysate was at pH 7. The pH was adjusted to 5 prior to clarification using 1M acetic acid.

Metric for evaluating cleaning effectiveness:
The area under the UV (A280) peak during the cleaning step was employed as a measure of the HCPs removed from the column.

Phase III

At the end of Phase II, the list of regenerants was further reduced. In Phase III, the effectiveness of the remaining regenerants was compared using the following process metrics:

Restoration of Dynamic Binding Capacity (DBC) following regeneration: A 0.34 mL column (Omnifit 0.66 cm i.d. x 1 cm) was cycled 10 times with a clarified *E. coli* lysate. The basic procedure for each cycle was as follows:

1. Equilibrate the column with 10 CV 100 mM acetate buffer, pH 5.
2. Load 44 CV of clarified *E. coli* lysate.
3. Wash the column with 10 CV equilibration buffer.
4. Flush the column with 10 CV regenerant.

After 5 cycles, the column was sanitized with 1M NaOH (10 CV) and the DBC at 10% breakthrough of a model protein was determined. The variation in the DBC at 10% breakthrough was employed to evaluate the effectiveness of the regenerant solution. Horse heart cytochrome C (0.5 mg/mL) in PBS buffer (pH 7.2) was employed as the model protein. The residence time for all steps was 2 minutes. A separate column was used for each regenerant. The complex feed stream was the clarified *E. coli* lysate used in Phase II. Given the limited volume of feed stream, the DBC comparison test was limited to 6M guanidine hydrochloride and 1M arginine, pH 8 and the number of cycles was limited to 10 per regenerant.

Assessment of residual impurities after cleaning:
This experiment was performed in batch bind mode to facilitate fast throughput of samples. The basic procedure for each cycle was as follows:

A. Eshmuno® HCX resin slurry (fresh, unused media) was briefly centrifuged to pellet the media and the supernatant (transport buffer) removed. The media pellet was resuspended with ~2 volumes (i.e., 33% slurry) of equilibration buffer (50 mM Tris pH 7.5). The media slurry was again centrifuged briefly and the supernatant buffer removed. This procedure was repeated for 5 buffer changes to ensure removal of the transport buffer.

B. A batch bind was then performed by taking 1.5 mL of the equilibrated Eshmuno® HCX resin (post centrifugation) and resuspending in 25 mL of *E. coli* cell lysate pre-conditioned to pH 7.0. (Note: This was a different *E. coli* feed stream. For this feed stream, significant precipitation and, consequently, lower HCP

levels, was observed when the pH was lowered to 5. Hence, the loading pH was chosen as 7.) The batch bind was performed at room temperature for ~2 hours with frequent resuspension of the media, but without continuous stirring.

C. Using a similar procedure to that described above, the media was pelleted and washed sequentially x2 with equilibration buffer, x2 with 1M NaCl in equilibration buffer and then again x2 with equilibration buffer. After the final equilibration buffer wash, the media pellet was resuspended as a 1:1 slurry in equilibration buffer.

D. Three aliquots (200 µl slurry, containing 100 µl media) were then centrifuged and the supernatant buffer removed. Each media pellet was then individually washed (x2, using the procedure previously described) with a 10-fold volumetric excess (1 mL to 100 µl of media) using one of the cleaning agents listed below:

- 6M Gu-HCl, 50 mM Tris pH 8.0
- 2% (w/v) SDS, 50 mM Tris pH 8.0
- 1M arginine hydrochloride, 50 mM Tris pH 8.0
- 0.5M NaOH

E. The washes were collected and the pellets were then washed again (x2) with equilibration buffer and resuspended at a 1:1 volumetric ratio in fresh equilibration buffer. A sample of each resuspended media sample (100 µl) was spun down to provide a 50 µl media pellet, which was then resuspended in 200 µl of 1x SDS PAGE non-reducing sample buffer. This was incubated in a boiling water bath for 5 minutes and analysed by SDS-PAGE according to the manufacturer's directions.

Results and Discussion

Phase I – Lysozyme tests

Figure 1 illustrates the A280 curves obtained during the tests with arginine as the regenerant. The peak area in the section labeled "Flush with 6M Gu-HCl" is representative of the fraction of lysozyme that could not be eluted by the regenerant. Clearly, both the pH and the concentration of arginine are critical for its effectiveness as a regenerant. Based on the peak profiles in **Figure 1**, 1M arginine, pH 8 would be an effective regenerant.

Figure 2 illustrates the A280 curves obtained during the tests with propylene glycol and sodium chloride as a combination regenerant. The data show that this combination is ineffective at eluting lysozyme from the column and thus is ruled out as a potential regenerant.

Figure 3 illustrates the A280 curves obtained during the tests with isopropanol and sodium chloride as a combination regenerant. Clearly, the use of isopropanol or sodium chloride alone is not effective at eluting lysozyme. These chemicals are effective only when combined together. In addition, a relatively high sodium chloride concentration is needed (1.5M) for the combination to be effective. Based on the peak profiles in **Figure 3,** 20% isopropanol combined with 1.5M sodium chloride could be an effective regenerant.

Figure 4 illustrates the A280 curves obtained during the tests with urea and sodium chloride as a combination regenerant. The use of urea alone, even at 7M concentration, is not effective at eluting lysozyme. These chemicals are effective only when combined together. In addition, a relatively high sodium chloride concentration is needed (1.5M) for the combination to be effective. Based on the peak profiles in **Figure 4**, 6M urea combined with 1.5M sodium chloride would be an effective regenerant.

Figures 3 and 4 confirm that lysozyme is binding in a multi-modal manner through electrostatic and hydrophobic interactions and validate its choice as a model foulant for this resin.

In summary, the following regenerants were determined to be effective for testing with a complex feed stream:

1. 1M arginine hydrochloride, pH 8
2. 20% isopropanol + 1.5M NaCl
3. 6M urea + 1.5M NaCl

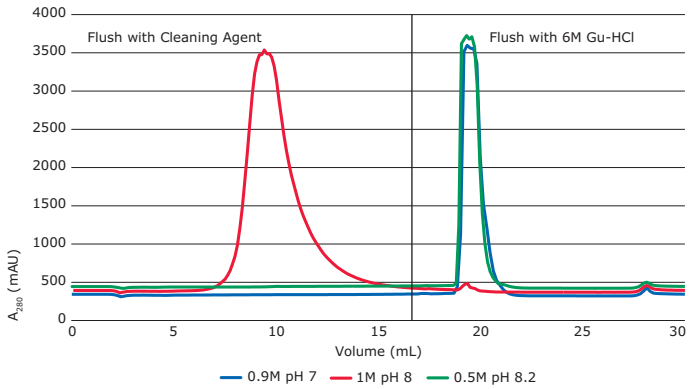


Figure 1.

A280 curves obtained with arginine.

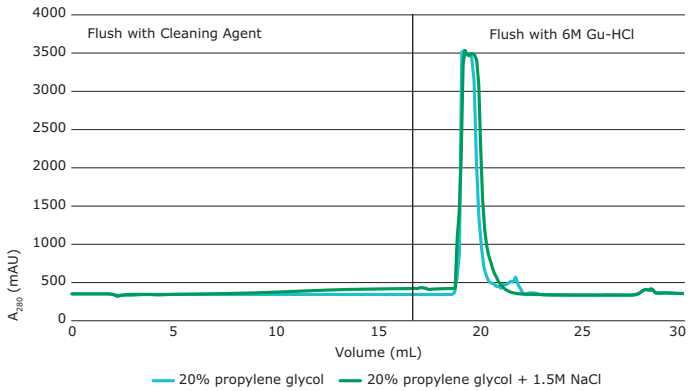


Figure 2.

A280 curves obtained with propylene glycol and sodium chloride.

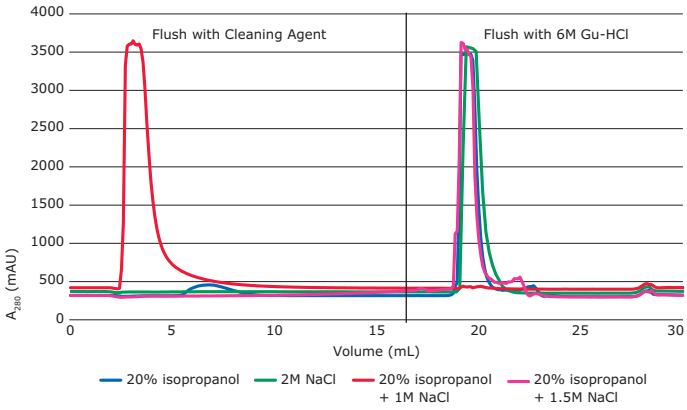


Figure 3.

A280 curves obtained with isopropanol and sodium chloride.

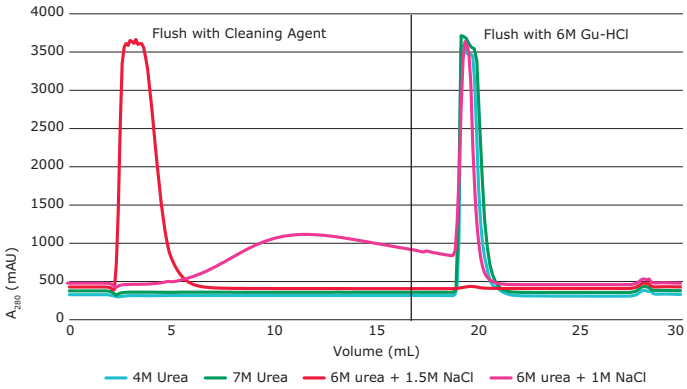


Figure 4.

A280 curves obtained with urea and sodium chloride.

Phase II – Clarified *E. coli* lysate

In addition to the regenerants identified in Phase I, 2% SDS solution was also tested as a potential regenerant in this phase. As in Phase I, the control solution was 6M guanidine hydrochloride.

Figure 5 compares the A280 traces obtained with the various regenerants. As is evident from the area under the A280 curves, the combination regenerants—20% isopropanol + 1.5M NaCl and 6M Urea + 1.5M NaCl—were the least effective of the regenerants. (Note: The SDS flush was not followed with a 6M Gu-HCl flush due to solubility issues.) On the other hand, arginine and SDS were significantly more effective as regenerants.

The effectiveness of the various regenerants is compared in **Figure 6**. In this graph, the area under the A280 curve for 6M Gu-HCl is set at 100% and serves as the control. The areas under the A280 traces for arginine and SDS suggest that they are removing ~70% of the HCPs that are removed by guanidine hydrochloride. Isopropanol and urea in combination with high salt are clearly ineffective.

The data in **Figure 6** indicate that arginine and SDS may not be as effective as guanidine hydrochloride. However, given the lumped and qualitative nature of the metric (area under A280), it is difficult to assess whether there would be a significant process impact with any of these three regenerants. In order to be truly effective under processing conditions, a regenerant should ensure that capacity is effectively restored following regeneration, and there is no carryover of impurities from cycle to cycle.

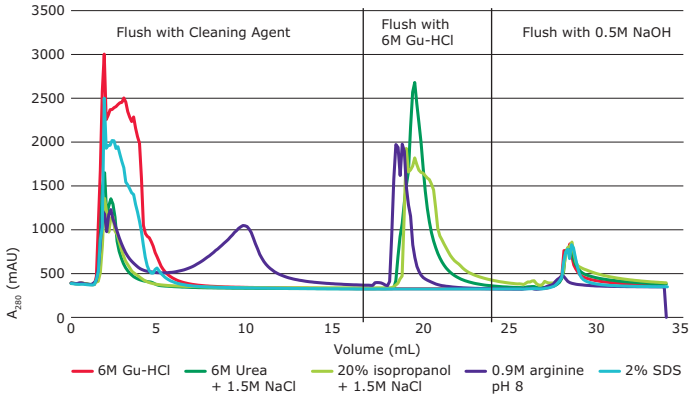


Figure 5.

A280 curves obtained with the various regenerants.

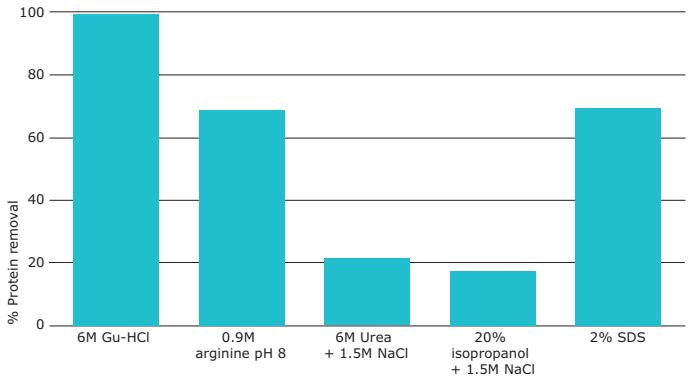


Figure 6.

Effectiveness of the various regenerants.

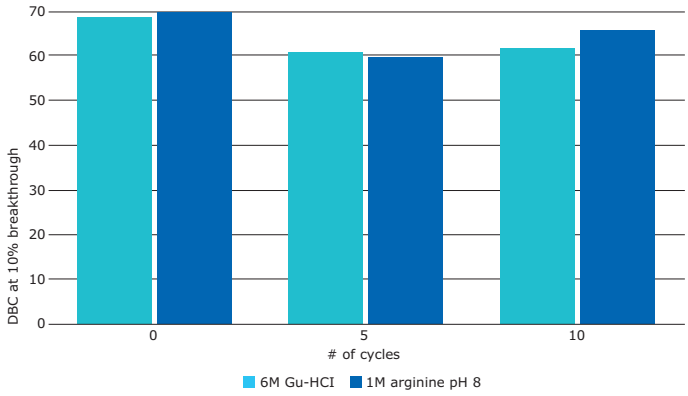


Figure 7.

Comparison of DBC at 10% breakthrough obtained with 6M guanidine hydrochloride and 1M arginine, pH 8.

Phase III

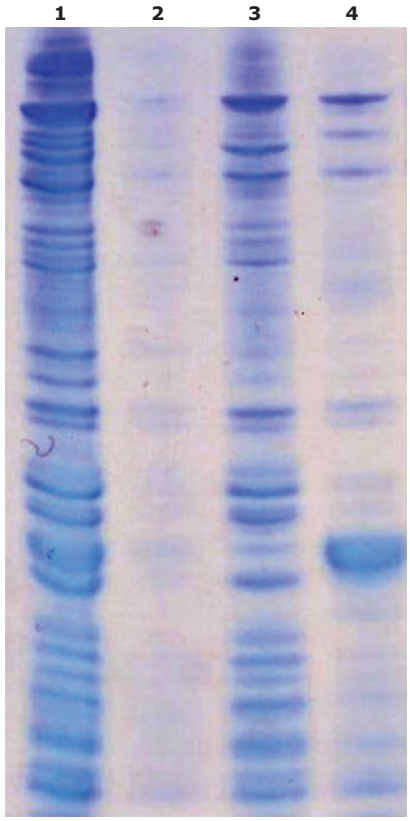
In this phase, the effectiveness of the remaining regenerants was compared using the process metrics listed above. The complex feed stream was the clarified *E. coli* lysate used in Phase II. Given the limited volume of feed stream, the comparison was limited to 6M guanidine hydrochloride and 1M arginine, pH 8. Arginine was preferred to SDS, as customers typically avoid the use of detergents to eliminate the need for removal validation. In addition, the number of loading cycles was limited to 10 per regenerant.

Effect on Dynamic Binding Capacity (DBC)
As mentioned in the experimental section, the dynamic binding capacity at 10% breakthrough of horse heart cytochrome C in phosphate buffer, pH 7.2, was employed as the capacity metric. Prior to loading the columns with the clarified *E. coli* lysate, breakthrough curves were generated with the virgin resin to establish baseline DBC data. **Figure 7** compares the DBC at 10% breakthrough after 5 and 10 cycles of *E. coli* lysate loading. Cycle 0 refers to the DBC on the virgin resin.

As is evident from **Figure 7**, the DBC data for both regenerants are very similar. Neither regenerant is effective at restoring 100% of the virgin DBC. The overall reduction in DBC is in the range of 5–10% over 10 cycles. In most capture applications, the loading is typically set to ~80% of the DBC. Thus, the observed decrease in DBC would not affect the yield of this process. Thus, **Figure 7** strongly suggests that the efficacies of 6M guanidine hydrochloride and 1M arginine, pH 8 are very similar as far as maintaining capacity is concerned.

Effect on carryover potential
The batch bind experiment described in the experimental design section was analyzed by SDS-PAGE under non-reducing conditions and visualized by Coomassie staining.

Figure 8 provides a qualitative evaluation of the risk of carryover with each of the cleaning agents. The risk of carryover is lowest with guanidine hydrochloride. The batch binding experiments clearly indicate that 6M Gu-HCl, pH 8 is the most effective cleaning agent. In fact, it is more effective than 0.5M NaOH. The current recommended cleaning agent is 0.1-1M NaOH. However, the batch binding tests strongly suggest that 6M Gu-HCl is more effective than 0.5M NaOH.



Lane	Description
1	Residual bound protein after washing with 1M Arginine, pH 8.0
2	Residual bound protein after washing with 6M Gu-HCl, pH 8.0
3	Residual bound protein after washing with 0.5M NaOH
4	Residual protein after washing with 2% (w/v) SDS, pH 8.0

Figure 8.

SDS PAGE analysis of residual media contaminants after washing with various cleaning agents.

Conclusions and Recommendations

Regeneration is typically needed after every loading cycle to strip the column of strongly bound impurities and/or product. This ensures reproducible chromatographic behavior, within tolerances. Additional cleaning cycles are required in cases where there are strongly or irreversibly bound impurities that accumulate over time, impacting product yield and purity.

The current recommendation for regenerating Eshmuno® HCX resin is to employ 6M guanidine hydrochloride. As an alternative approach, the data presented in this application note demonstrate that 1M arginine, pH 8 is a satisfactory alternative to 6M Gu HCl as a regenerant. It is more cost-effective and significantly less corrosive. For a multiple-cycle process, 1M arginine, pH 8 is recommended to be employed each cycle and the column periodically cleaned with 6M Gu HCl, pH 8. The cleaning frequency will depend strongly on the feed stream.

References

1. Holstein, M.A., Parimal, S., McCallum, S.A., Cramer, S.M., Biotechnology & Bioengineering, Vol 109, No. 1, (2012), pp 176–186.
2. Kaleas, K.A., Schmelzer, C.H., Pizarro, S.A., J. Chromatography A, Vol 1217 (2010), pp 235–242.

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