Strategies To Address Aggregation During Protein A Chromatography

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IgGs have emerged as one of the key therapeutic modalities of the biopharmaceutical industry. Due to the successful launch of several high-profile monoclonal antibodies (MAbs), increasing proportions of the clinical pipelines of several companies now belong to this class of molecules. Sales of MAbs are expected to reach >$15 billion by 2010 (**1**). In addition to MAb products, the Fc portions of antibodies are often used as tags for other proteins. A leading product in this category is Enbrel, which is a fusion of a TNFα receptor to an IgG_1 Fc (**2**).

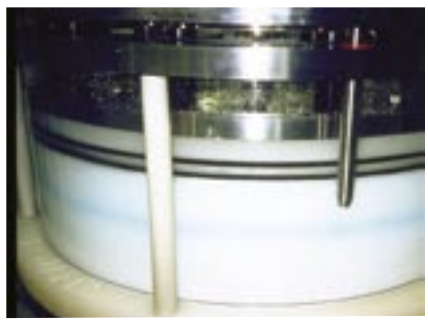
Protein A chromatography is widely used in affinity purification of both MAbs and Fc fusion proteins because it offers a high degree of selectivity in capturing those molecules from complex cell culture harvests (**3**). Protein A is usually used for the capture step in those processes and can deliver purities in excess of 99% starting from cell culture harvest supernatants. Following that step, usually only polishing type chromatographic steps are required to clear residual levels of host cell protein contaminants, leached protein A, and high molecular weight aggregates. Indeed, the use of a protein A affinity step is what makes the concept of generic purification processes possible for this class of molecules (**4–6**).

Protein A columns typically bind antibodies at neutral pH and can be eluted at low pH (typically between pH 3 and 4). It has been shown that a highly conserved histidyl residue in the center of the protein A binding region of IgG faces a complementary histidyl residue on protein A (**3, 7**). Those residues take on a positive charge at low pH, thus repelling each other and weakening the protein A-IgG hydrophobic association. That results in elution of IgG from the affinity column. It has also been shown that the Fc part of IgGs are structurally sensitive to low pH conditions in contrast with the Fab part of the molecule (**8**), which might also play a role in elution from protein A resins. Although the structural perturbation enables elution, it may also contribute to unfolding mediated aggregation of a product. In fact, proteins in general are commonly known to aggregate under low pH conditions (**9, 10**). Clearly, the low pH required to elute antibodies and Fc fusion proteins from protein A columns can pose a risk for product stability.

A few methods to reduce the risk of aggregation during protein A chromatography have been discussed in the literature. Most have centered on moderating the pH of elution for antibodies. Sodium chloride (0–1 M) has been mentioned as an elution buffer additive to increase the elution pH (**3**). Hydrophobic competitors such as ethylene glycol have been used to weaken hydrophobic interactions and thus increase elution pH from protein A columns (**11**). Urea in the concentration range of 1–2 M has also been used as a mild denaturant to facilitate elution (**3**).

A different approach has been to engineer the protein A ligand to allow for milder elution conditions (**12**). This allowed IgGs to be eluted at a pH of ~4.5 instead of ~pH 3.0, although the impact on selectivity of the ligand was not described.

Although all those approaches are useful, they do not adequately address the problem for all antibodies and Fc fusion molecules. Often, sodium chloride does not adequately modulate the elution pH, and chaotropes and hydrophobic competitors, when used at concentrations that affect elution



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pH, cause unfolding of the product and thus induce aggregation instead.

Another approach is to stabilize the product as it elutes from the protein A column by adding stabilizing excipients to the elution buffer. This strategy has been shown to be successful to stabilize antibodies by adding arginine (0.5–2 M) to the elution buffer (**13**). Using elution buffer stabilizers is one of the more practical approaches for dealing with the issue.

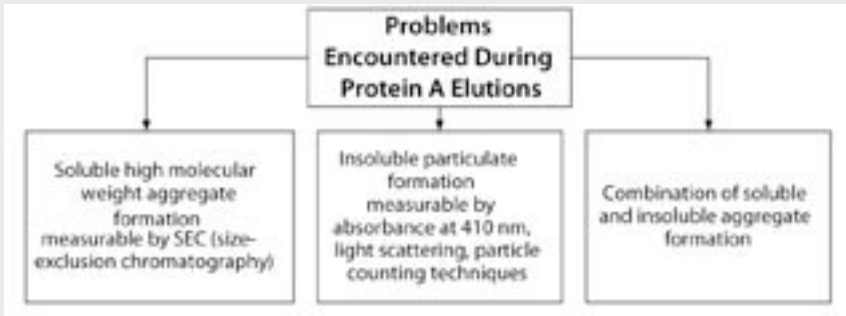
Several case studies from Amgen serve to highlight operational approaches for molecules that experienced excessive turbidity or high molecular weight formation during protein A elution.

Materials and Methods

All the protein A experiments described here were conducted on Mab Select protein A chromatographic media (Amersham Biosciences now part of GE Healthcare, www.gehealthcare.com). A variety of column sizes were used including column diameters of 1.0, 1.1, and 2.6 cm and bed heights ranging from 15 to 25 cm. Fractogel TMAE Hicap (EM Sciences, www.emdchemicals.com) was used for harvest pretreatment using a column residence time of five minutes and loading capacity of 50 mg/mL resin of product.

An ÄKTAexplorer (GE Healthcare) chromatographic system was used for the chromatographic experiments, and the column effluent was monitored using in-line UV, conductivity and pH probes that are part of the ÄKTA system. Concentrations of MAbs and Fc fusion proteins were determined using analytical protein A chromatography on a POROS 30-mm high 2.1mm-ID column (Applied Biosystems, www.appliedbiosystems.com). Off-line measurements of eluate turbidity were taken on a UV/Vis spectrophotometer (Beckman Coulter, www.beckmancoulter.com) using a 1-cm pathlength cuvette and detection at 410 nm. Analytical size-exclusion chromatography to determine the percentage of soluble high molecular weight aggregates was carried out using a 300-mm long 4.6mm-ID Tosoh G3000SWXL size-exclusion column (Tosoh Bioscience, www.tosohbiosep.com). For the aggregation kinetics experiments, the mole fraction of high molecular weight aggregate was obtained by dividing the area of the aggregate peak by the sum of areas of the aggregate and main peaks.

Figure 1: Aggregation and precipitation phenomena observed during protein A chromatography



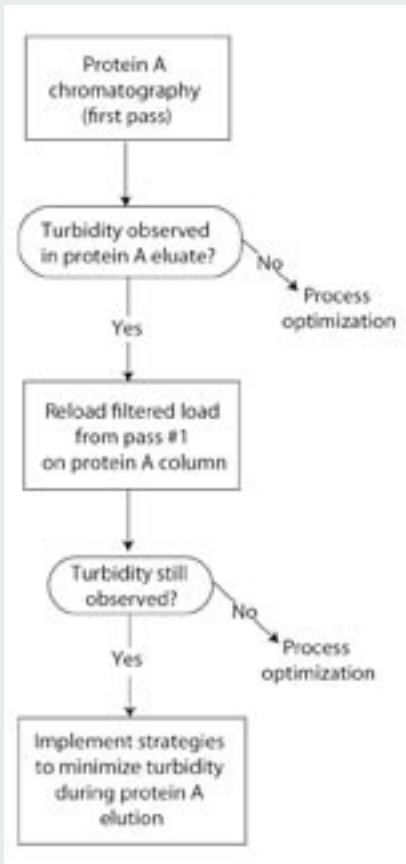
Results and Discussion

When is aggregation or turbidity a problem? Aggregation phenomena observed during protein A elution can be categorized as shown in Figure 1. The first type is the formation of soluble high molecular weight aggregates, as detected by size-exclusion chromatography. The second is the formation of insoluble particulates that diffract light and result in a turbid appearance. Depending on the specific properties of the molecule involved, either one of those two phenomena may dominate. Alternatively, both soluble and insoluble high molecular weight aggregate formation may be observed together. Additional aggregate or particulate formation may occur following protein A elution during low pH viral inactivation or during neutralization of the eluate pool. However, here we focus solely on aggregation phenomena during protein A elution.

Soluble high molecular weight aggregates pose a significant safety risk for biologics through concerns over immunogenicity. Although polishing steps subsequent to protein A chromatography usually can reduce aggregate levels, they do so at the expense of overall process yield. Thus, it is definitely undesirable to have significant increases in high molecular weight aggregate content during protein A elution.

By contrast, insoluble particulate formation during protein A elution might not always be an undesirable phenomenon to be avoided at all costs.

Figure 2: Re-chromatography over protein A as a means of distinguishing between turbidity caused by product instability or precipitation of impurities



For instance, the particulates that cause turbidity might not always consist of product molecules that have aggregated together. In several cases, host cell protein contaminants or a small subpopulation of product that is improperly folded might be the species that precipitate under the low pH conditions required for protein A elution. In such a case, the protein A step might actually be a good point in the process to remove those contaminants. The risk in this situation is largely due to the possibility of the

Table 1: Protein concentration and absorbance measurements at 410 nm for re-chromatography of a MAb over protein A

Sample	Protein Concentration (mg/mL)	Absorbance Reading at 410 nm
Protein A eluate (pass #1)	11.2	0.304
Filtered protein A eluate from pass #1	10.1	0.028
Protein A eluate (pass #2)	9.3	0.037

particulate matter clogging in-process sterile filters or to the protein A resin

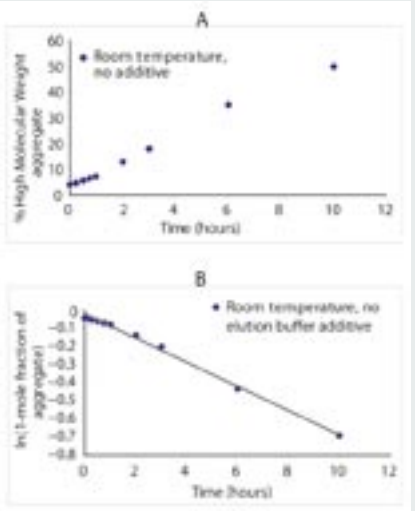
Table 2: Comparing various stabilizing additives in the protein A elution buffer, with all elution buffers at pH 3.6

Sample	Protein Concentration (mg/mL)	Absorbance Reading at 410 nm
50 mM citrate	11.1	0.329
50 mM citrate + 150 mM NaCl	10.7	0.063
150 mM citrate	10.1	0.033
50 mM citrate + 10% sucrose	13.2	0.341

Table 3: Effect of various combinations of wash and elution buffers during protein A chromatography of a MAb

Wash Buffer	Elution Buffer	pH Gradient (pH units/CV)	Elution Peak Width (CVs)	Pool pH
50 mM citrate, pH 6.0	50 mM citrate, pH 3.6	1.14	2.21	3.93
50 mM citrate, pH 6.0	10 mM citrate, pH 3.6	0.78	3.17	4.02
50 mM citrate, pH 6.0	10 mM glycine, pH 3.6	0.48	5.52	3.65

Figure 3A: Aggregation of an Fc fusion protein over time in the protein A eluate (not neutralized). Figure 3B: First-order kinetics of aggregation of the Fc fusion protein in the protein A elution buffer; slope of this plot determines the rate constant of the aggregation reaction



lifetime if the particles get entrained on the column during elution. On the other hand, if a measurable proportion of the product itself is lost through particulate formation, then that phenomenon becomes a serious problem. It would not have implications only on overall process yield, but would also pose risks for product activity and process reproducibility.

One way to distinguish between those two situations is by re-chromatographing the protein A eluate from one run on a second protein A column (Figure 2). If the turbidity/precipitation does not recur in the second pass, you can conclude that the conditions proteins are subjected to on the protein A column are not deleterious to the product itself. In such a case, the bulk of the product species itself does not form particulates; instead, an impurity or product subspecies that is removed on the first pass through protein A may be causing the problem. On the other hand, if the turbidity does recur on the second pass, it is likely that the product itself is unstable under the conditions it is exposed to during protein A chromatography. This conclusion can be better confirmed by using purified product as the protein A load. If the latter is the case, efforts need to be made to stabilize the product during protein A elution — or failing all else, you may even need to consider moving away from protein A chromatography.

Table 1 lists off-line absorbances at 410 nm and protein concentration measurements for the neutralized eluate pool from protein A purification of a MAb. The neutralized elution pool was observed to be turbid (absorbance of 0.304 at 410 nm). As can be seen from the table, filtration through a 0.2 µm filter significantly reduced the turbidity in the protein A elution pool from this experiment (from 0.304 to 0.028 AU). The neutralized and filtered eluate from this experiment was used as the column load for a second pass over the protein A column. As Table 2 shows, the off-line turbidity measurement remained low.

Thus, for this antibody, following the protein A purification step with filtration successfully removed the precipitating species from the product stream. Because the eluate turbidity did not reappear on the second pass over the protein A step, we concluded that the main product species itself was not implicated in particulate formation. That allayed some of the concerns regarding the turbidity phenomena observed with this product. Clearly, it is important to ask whether the product itself is being affected by the protein A column operation. It can be a significant step toward formulating a strategy to deal with the issue of aggregation/particulate formation during protein A column operation for a given product.

The following sections provide examples of some solutions we have used to mitigate aggregation and particulate formation during protein A elution for a variety of MAb and Fc fusion proteins at Amgen. These solutions are listed in no particular order; indeed, to a large extent the development of such solutions remains largely empirical.

Case Studies: Mitigating Aggregates and Particulates

Temperature of Column Operation: A CHO-expressed Fc fusion protein was found to be highly prone to forming soluble high molecular weight aggregates at low pHs (below 4.0). Up to 20% aggregate was observed (depending on column loading) upon elution from a protein A column with a 50-mM citrate, pH 3.7 elution buffer. Although protein aggregation is usually expected to be a higher order reaction (**14, 15**), aggregation of this protein was found to be a first-order reaction with time similar to what has been observed previously for human interferon γ (**16**). Figure 3A plots the percentage of high molecular weight aggregate against time for the protein A elution pool upon collection from the column. Figure 3B is a semi-log plot of the mole fraction of nonaggregated species against time for the data shown in Figure 3A. The rate constant of the aggregation reaction can be determined from the slope of this plot. As can be seen from the figure, a first-order reaction mechanism can fit the data well. Because the rate constant is independent of protein concentration, comparing the rate constant for aggregation in the protein A eluate can enable a better comparison of various stabilizing additives.

Several elution buffer additives were screened at various concentrations including glycine, arginine, sodium chloride, sodium citrate, and urea (data not shown). Surprisingly, urea at intermediate concentrations (0.5 to 1.5 M) was found to be the best elution buffer additive for this product. However, an even more effective solution to the aggregation problem was to operate the protein A column at cold temperatures (2–8 °C). Figure 4 shows a comparison of the aggregation rate constants in protein A eluates from three experiments using 50 mM citrate, pH 3.7 as the elution buffer at room temperature; 50 mM citrate, 1 M urea, pH 3.7 at room temperature; and 50 mM citrate, pH 3.7, with the entire column step being operated at 2–8 °C. Operating the column step at cold temperatures gave the lowest rate constant for aggregation. Even though only the elution part of the cycle needs to be run in the cold, it is preferable to avoid cycling column temperature because that can result in structural instability in the packed bed due to outgassing at lower temperatures. Using an in-line heat exchanger before the jacketed chromatographic column maintained column temperature very effectively in large-scale operation.

Clearly, operating temperature is an important variable that can have a significant impact on aggregation during protein A chromatography. That statement comes with the caveat that in some cases, lower temperature can actually enhance the formation of insoluble particulates by reducing their solubility. Interestingly, the mechanism for stabilization by cold temperature might be similar to the mechanism by which intermediate concentrations of denaturants such as urea stabilize proteins. Urea has been shown to mediate cross-linking of different parts of a protein molecule, thus decreasing its motional freedom (**17**).

Figure 4: Comparison of rate constants for Fc fusion protein aggregation under different elution conditions: 50 mM citrate, pH 3.7; 50 mM citrate, 1M urea, pH 3.7; 50 mM citrate, pH 3.7 with the entire column step operated at 4 °C. A column loading of 15 mg/mL was used in all these cases.

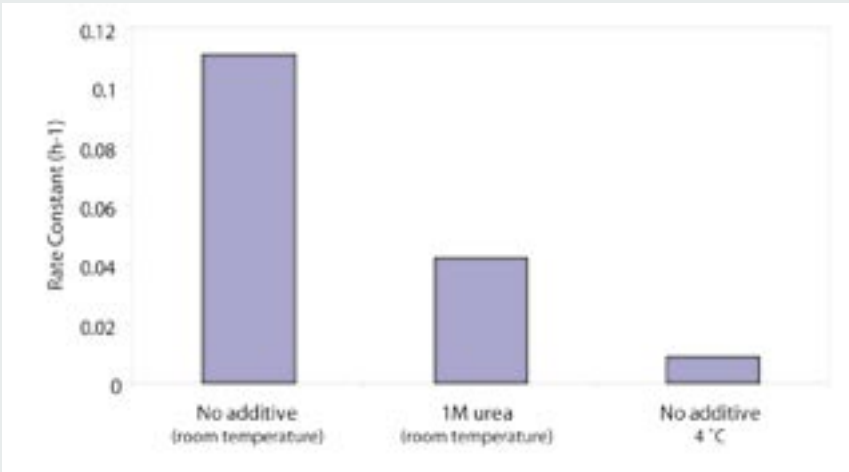
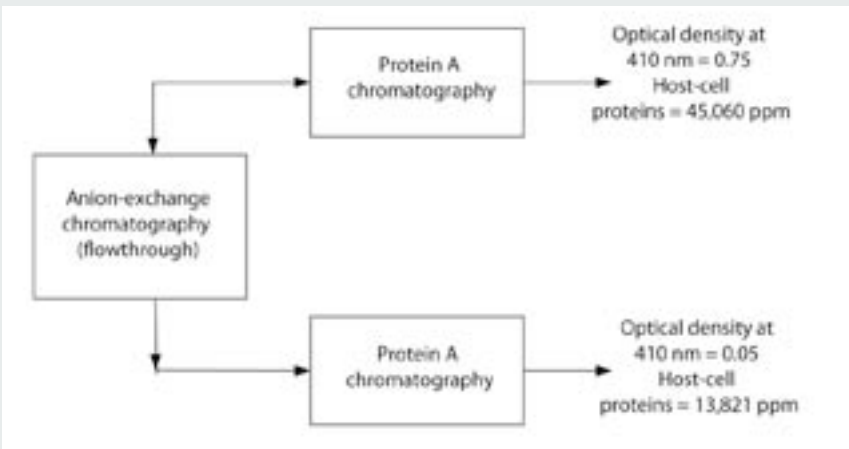


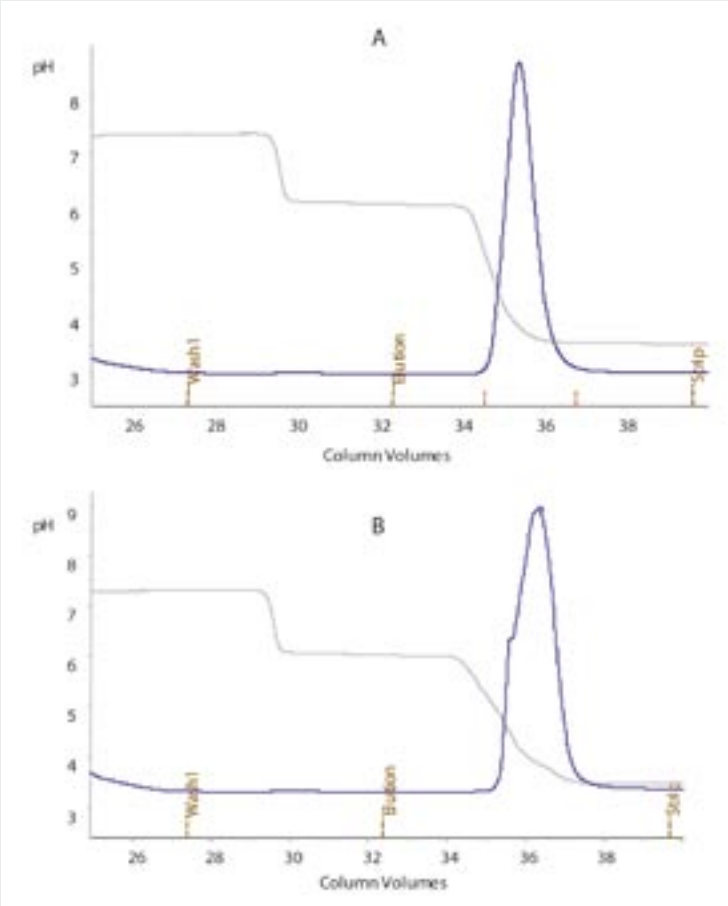
Figure 5: Pretreatment of cell culture harvest by anion-exchange flowthrough; column was a Fractogel TMAE Hicap with a residence time of 5 min. An equivalent of 50 mg/mL product was loaded over the column.



Stabilizing Elution Buffer Additives: Table 2 shows the absorbance at 410 nm and protein concentrations for the protein A elution pools of a MAb using different elution buffers, all at pH 3.6. As can be seen, increasing the salt concentration (either with sodium citrate or sodium chloride) in the elution buffer decreased column eluate turbidity. By contrast, sucrose (a widely used excipient in formulation buffers) did not succeed in controlling particle formation. Increasing salt concentration in the protein A elution buffer is a simple strategy that has had beneficial effects for several products and usually deserves a try early on when attempting to solve an aggregation problem.

Pretreatment of the Cell Culture Harvest: In some cases, turbidity observed in the protein A elution pool may be the result of host-cell protein contaminants or cell culture media additives coeluting with the product

Figure 6A and B: Protein A elution profiles for a MAb. A) Wash: 50 mM citrate, pH 6.0; elution: 50 mM citrate, pH 3.6. B) Wash: 50 mM citrate, pH 6.0; elution: 10 mM citrate, pH 3.6. Column was loaded to 15 mg/mL antibody in both cases.



though this does not pose significant risk to the product, it can pose significant (and variable) filtration challenges for the neutralized protein A elution pool and potentially decrease protein A column lifetime. This was found to be the case for another MAb during process development.

Pretreating the harvest fluid by flowing over an anion-exchange column successfully reduced turbidity in the protein A eluate (Figure 5). An additional benefit was a significant decrease in the host cell protein contaminant levels in the protein A eluate.

Other options for harvest pretreatment might also be considered, such as incubation of the harvest at low pH followed by filtration to remove the impurities that precipitate. However, in general, pretreatment of the harvest before a capture protein A unit operation is not the preferred approach. Harvest pretreatment adds an additional unit process volume is still considerably large and hence may decrease process throughput. An alternative approach is to develop a better wash procedure for the protein A column to remove contaminants before the elution step.

Manipulating the pH Transition from Wash to Elution:

One factor that is not often recognized about protein A chromatography is that even though the process is operated in a step-gradient fashion, the changeover from wash (moderate pH typically >5.0) to elution buffer (low pH typically 3.0–4.0) actually takes the form of a gradual decrease in pH rather than a sudden transition. The exact nature of that gradient depends on the two buffers used and their respective concentrations. If a strong wash buffer (e.g., citrate) is followed by a weak buffer (e.g., glycine or a lower concentration of citrate), the pH transition is gradual. Conversely, the pH transition can be made sharper by choosing a weaker buffer in the wash and using a highly concentrated, stronger buffer for elution.

The slope of pH transition can influence the width of elution peaks as well as the maximal protein concentration reached during elution. This in turn can have a significant effect on the amount of aggregate formed during elution. In addition, the slope of pH transition can also influence the overall pH of the elution pool. If the transition is sharp, a significant proportion of the product will elute before the pH has reached its lowest level, thus raising the overall pH of the elution pool.

Figure 6A shows the chromatogram obtained during protein A chromatography of an antibody by using 50 mM citrate, pH 6.0 for wash followed by a 50 mM citrate, pH 3.6 buffer for elution. Table 3 shows the resulting slope of the pH gradient, the elution peak width in column volumes (CV), and the pH of the eluate pool. For this experiment, a relatively sharp pH transition was observed (1.14 pH units/column volume). The elution peak width was 2.21 column volumes. By contrast, when the same wash buffer was followed by a weaker elution buffer (10 mM citrate, pH 3.6) the pH transition was observed to be more gradual (Figure 6B). The slope of the pH gradient in this case was only 0.78 pH units/CV, and the peak eluted in 3.17 column volumes — almost a column volume higher than in Figure 6A. Table 3 shows that this effect can be exaggerated even further if an even weaker elution buffer such as glycine is used: The pH transition is 0.48 pH units/CV, and the elution peak now stretches to over 5.52 column volumes. Accordingly, the maximal protein concentration reached during elution is lower when the pH transition is more gradual. By contrast, the pH of the elution pool follows an opposing trend. As the pH transition becomes more gradual, the pool pH also drops, although not very significantly for the set of

buffers shown in this example.

Thus, using a sharper transition of pH might be good strategy for molecules that are sensitive to low-pH conditions because it can limit exposure to low pH conditions. On the other hand, using a more gradual transition of pH might decrease the extent of aggregation for molecules that aggregate rapidly when exposed to low pH at a high protein concentration. The identity of the buffers used and the sensitivity of the product at hand need to be taken into account when deciding whether pH transition should be made steeper or shallower.

Integrating Strategies

Several strategies have been taken to avoid the problems of aggregation and particulate formation (manifested by turbidity in the eluate pool) during protein A chromatography of MAbs and Fc fusion proteins. Here, a distinction is drawn between aggregation/precipitation phenomena that involve the product and those that involve impurities. Column re-chromatography experiments are used to distinguish between those two situations.

Stabilizing additives such as salts, urea, and amino acids can be added to the elution buffer to stabilize the product as it elutes off the protein A column. Lowering the temperature of column operation can be a viable strategy for some products. Pretreating the cell culture harvest to remove certain impurities that can precipitate at low pH can address this issue for some products. Finally, manipulating the pH transition between wash and elution by varying the buffering species and their strengths can be an effective strategy to minimize exposure time to low pH conditions.

Even though the solutions identified here apply to different molecules and processes, an important goal of process development is to successfully integrate such strategies into a cohesive array of solutions for the aggregation issues during protein A chromatography. At present, the choice of which strategy works best for a given situation is still determined empirically. In the future, it will be important to establish trends to correlate these solutions with the properties of the molecules and cell culture conditions.

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