Controlling Metal Ion Contamination in Protein Purification Process Development

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Perhaps the most unique query we've received turned out to involve metal ion contamination. The symptoms described to us were lot-to-lot variable polymorphic retention on a QAE anion exchanger, remarkably poor shelf stability of the purified protein, and loss of liver weight from experimental animals treated with the product. It is unusual for metal ion contamination to cause problems of this magnitude, but rather than limiting the applicability of this example, it raises a red flag to focus attention on what is actually a widespread and usually overlooked -- even if much more subtle problem.

Proteins are highly interactive with metal ions. There are two principle types of interactions. Carboxy clusters on protein surfaces interact with metals in the same way as carboxy chelators like EDTA and EGTA. This is also the basis of protein interactions with hydroxyapatite (HA), which can be confirmed by performing the technique in 0.5M NaCl. The salt abolishes cation exchange of proteins with HA phosphoryl groups, leaving only their interaction with HA calcium. More highly carboxylated proteins have a higher tendency toward some of those carboxyls being clustered and able to chelate, hence the indirect correlation between protein acidity and strength of binding to HA. Similar selectivity is observed with immobilized metal affinity chromatography (IMAC) when iminodiacetic acid (IDA) columns are charged with ferric ions. These interactions are typically strongest at neutral to slightly acidic pH.

Metals interact with other protein surface groups as well, chiefly with the imidazolium ring of histidine. Other aromatic side groups (tryptophan, tyrosine, phenylalanine) may contribute to binding, as well as -- to a lesser extent -- arginine, methionine, and glycine. These interactions do not involve chelation in the usual sense, but an interaction referred to as metal coordination. This is the primary basis of IMAC with such metals as copper, nickel, zinc, and cobalt. The interaction is usually strongest at alkaline pH, paralleling the titration state of histidine residues. As with protein-carboxy chelation interactions, coordination interactions typically survive very high concentrations of most neutral salts.

Protein-metal interaction range from 5 to 60 times stronger than ion exchange interactions. This means that most such interactions will survive the conditions typically encountered in chromatographic purification. This has two practical ramifications. First, it means that the bound metals may alter the surface charge, hydrophobicity, aggregation state and solubility characteristics of the protein. All these factors can affect chromatographic selectivty. For example, if a divalent metal cation is bound to a polycarboxy cluster on a protein, the two positive charges on the metal will neutralize a corresponding pair of negative charges on the protein. Given that the level of metal contamination is insufficient to affect the entire product population, this will create two distinct charge populations. Native protein will bind normally to an anion exchanger, while the complexed subpopulation will bind either more weakly or not at all. Depending on the surface characteristics of the protein, there may be multiple complexation variants, with a corresponding range of ion exchange retention morphs. Up to seven metal-complexation anion exchange retention morphs have been observed for some proteins.

Metals may also mediate crosslinking, which will affect selectivity of size exclusion separations, not to mention protein solubility.

Such crosslinking may be limited to simple dimerization or it may extend to gross aggregation; even precipitation. Metal complexation may affect HIC selectivity as well, and even affect some affinity mechanisms. For example, the binding of protein A with IgG is directly dependent on interactions of histidine triplets in the binding sites on both proteins. If the histidines are complexed with metal ions, association and dissociation constants can be altered radically.

The second ramification of strong protein-metal interactions is that metals may be carried through an entire purification, where they will probably affect product stability, and may affect pharmacokinetics. Some metals are toxic outright, such as nickel, zinc, and chromium. Others may be less so, and may even be considered as nutrients, like iron, calcium, and copper. The latter group are certainly lesser evils from the perspective of validation, but they may still exert significant effects on protein conformation, stability, and product function. Metal contamination is not only a process development challenge; it is a serious validation issue..

Sources of metal ion contamination. It's probably fair to say that metals rank with endotoxin in the diversity of sources in which they can be encountered. They may be worse, since even water for injection (WFI) contains leached metal ions. Major sources include process salts. Phosphates and sulfates are often highly contaminated. USP grade salts contain astonishingly high levels of metal contamination. Stainless steel metal surfaces in process equipment are another major source. This is a particular problem with low pH buffers in the presence of halide salts. Pumps are a key point to watch out for, since the pumping action may involve internal abrasion and production of ultrafine particles that are prone to dissolution. The richest source of contaminating metals comes from their deliberate use in IMAC. Not only are the concentrations massively higher than encountered anywhere else, but metal salts are typically contaminated with other metal salts. Since different metal salts interact to different degrees with protein chelation and coordination sites, these contaminating non-primary metal ions can create diverse subpopulations of metal-complexed product.

Control. The first line of defense against metal ion contamination is to reduce it as much as possible at the source. Regulatory people tend to promote the use of USP salts in purification processes, but if you have a product with demonstrable tendencies toward metal complexation, its usually best to avoid them. Use ACS grade or better, and explain the reasons to your regulatory staff. Using USP grade salts is a cosmetic detail, and its philosophical benefits are trivial compared to the specter of the validation issues accompanying metal ion contamination. Whatever grade salts you employ, be very careful to impose metal contamination limits in your raw material specifications, and qualify vendors' abilities to meet those specifications. Many process developers are turning to sodium acetate as an ion exchange eluting ion because of its lower corrosivity. This reduces leaching, but it doesn't abolish it. If you are using stainless steel process equipment, try to limit exposure time to low pH buffers, and especially limit the combination of low pH with halide salts. Make sure the surfaces are passivated with adequate frequency. An increasing number of companies are using composite-lined process equipment.

If you are using IMAC or if your product exhibits elevated tendencies toward metal complexation, you will need to have an affirmative strategy for metal ion elimination. Otherwise metal contamination will be an uncontrolled variable in your process, and a proximal cause for potentially crippling process variation. A number of publications suggest that adding a post-column of non metal-charged IMAC gel will effectively scavenge any metal ions that leak from the primary column. If the metal ions were all free in solution this might be workable, but most of the metal ions accompanying your product are going to be protein-bound. It will be nec-

essary for the post-column to outcompete the protein for the metal. Even assuming that the association constant of the metal for the column is equal to its association constant with the protein, this becomes a far less efficient scenario. And there's no reason to make that assumption. The metal-protein interaction may be significantly stronger than the column-metal interaction.

Elimination of contaminating metal ions requires a two-pronged chemical strategy. The elements of this strategy refelect the two types of chemical interactions by which proteins interact with metals. First, include 5-50mM EDTA or some other chelator in your process buffers. This will hopefully outcompete protein carboxy clusters for soluble metal ions. Phosphate buffers (50-100mM) can significantly enhance effectiveness because of their strong interaction with calcium and iron. Second, along with EDTA, include 5-50mM imidazole, histidine, or histamine in your process buffers. This will outcompete the protein for most coordination interactions. Include these additives especially in the presence of of high concentrations of phosphate or sulfate salts such as are often used in protein precipitation and HIC. When using IMAC, pre-spike your fraction collection vessels with EDTA and imidazole. You can wash away the EDTA, phosphate, imidazole, and any complexed metals in the subsequent process step. You don't need to apply these additives in conjunction with every process step, but you should evaluate them in conjunction with any process step that involves an elevated probability of metal contamination.You can usually depend on this strategy to bring metal ion contamination within WFI levels, and it will usually ensure adequate process control to provide you with reasonable assurance of reproducibility against varying metal loads from whatever source they may arise.

Did this strategy solve the original problem that was submitted to us? Only to the extent of identifying and characterizing it. Metal contamination from stainless steel process surfaces was confirmed as the source of the retention polymorphism on QAE and as the cause of the stability problem, but the loss of liver weight turned out to be a function of the protein itself. The same surface chemistry that caused the protein to bind leached ions from purification equipment caused it to scavenge iron from the liver. The product had to be abandoned. However, even this is instructive. Don't wait for metal contamination problems to kill your product after years and millions of dollars have been invested. Make yourself aware of the potential problems as early as possible. Be alert and build in the process controls you need right fromthe beginning to ensure that the product won't fail due to an unnecessary oversight.

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