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Dissociation of Antibody–Contaminant Complexes With Hydroxyapatite

By Pete Gagnon

This article describes methods for dissociation of aggregates and antibody-contaminant complexes on hydroxyapatite. The application of decomplexing washes weakens complex stability. Because of its high affinity for apatite calcium, the DNA component of complexes is attracted more strongly to apatite calcium than it is to the antibody.

The combination of weakened complexation and strong attraction to apatite calcium apparently allows the apatite to actively extract DNA from the complexes. Once bound to the apatite, DNA cannot rejoin the antibody so that when the antibody is eluted, the DNA remains behind.

Whereas conventional application of hydroxyapatite supports effective aggregate removal, the present method dissociates non-covalent aggregates into native antibody. Non-product contaminant complexes are also dissociated and removed more effectively. The method works with both IgG and IgM. Data suggest that host cell DNA fragments are the nucleation centers for antibody aggregate formation.

Introduction

Depression of purification performance by antibody-contaminant complexes was first recognized in 2008 by Shukla and Hinckley.[1] The results of their experiments suggested that 95% of the host cell contaminants that co-elute with IgG from protein A do so because they are complexed to the antibody. The remainder bind non-specifically to the protein A media. Luhrs *et al.* focused on IgG complexation with DNA and core histone proteins.[2] They found that failing to dissociate those complexes during purification resulted in loss of assay sensitivity and false positives, with obvious ramifications for therapeutic applications. Gagnon *et al.* focused on DNA and showed that complexes create antibody populations with aberrant chromatography characteristics. Complexes depressed recovery, purification factor, and reproducibility of bioaffinity (BAC), anion exchange (AX), cation exchange (CX), hydrophobic interaction (HIC), size exclusion (SEC), and hydroxyapatite (HA) chromatography.[3]

Recent extensions of that work suggest that DNA fragments may be the primary nucleation centers for aggregate formation.[4] This is consistent with the cross-industrial recognition of higher aggregate levels in high-density cell cultures. The DNA component is apparently protected and inaccessible to most purification methods, but for methods capable of removing that DNA, non-covalent aggregates can be restored to monomer.[3, 4] This work also demonstrates the presence of complexation among contaminants, exclusive of product, creating compound "super-contaminants" that complicate purification. Decomplexation reduces them to their constituents.[3, 4]

Other forms of complexation also pose significant purification challenges. Divalent metal cations have been documented to complex with antibodies and alter their surface charge.[5] Experimental data also suggest that metal contaminants mediate formation of bridge complexes between antibodies and DNA (antibody-metal-DNA).[3] Direct metal:protein and metal-bridge complexes are both stable at high salt concentrations. Aono *et al.* demonstrated that H_2S evolved from cells during cell culture production can insert itself into antibody disulfide bonds, creating unstable trisulfide complexes and potentially contributing to the formation of mixed disulfides.[6] Such complexes are likewise unaffected by the usual range of conditions occurring in routine antibody purification.

These examples collectively reveal complexation as a pervasive problem in the field of antibody purification, and a serious barrier to achieving higher levels of product quality and reproducibility. The present article offers preliminary experimental data demonstrating the ability of HA to dissociate complexes. Practical ramifications of initial findings are discussed.

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Materials and Methods

All chromatography experiments were conducted on an ÄKTAexplorer™ 100 (GE Healthcare, Piscataway, New Jersey USA). 0.34 mL axial flow monolithic anion exchangers (CIM® QA, EDA) were obtained from BIA Separations (Klagenfurt, Austria). CHT™ ceramic hydroxyapatite types I and II (both 40 µm), UNOsphere SUPrA™ immobilized protein A, Nuvia™ S cation exchange media, and a 7.8 mm x 30 cm Bio-Sil™ SEC-400-5 column were obtained from Bio-Rad Laboratories (Hercules, California USA). A 7.8 mm x 30 cm TSK-GEL® G5000PWXL-CP analytical SEC column was obtained from Tosoh Bioscience (King of Prussia, Pennsylvania USA). Buffer components were obtained from Sigma-Aldrich (St. Louis, Missouri USA). All buffers were prepared with water for injection (WFI) and filtered to 0.22 µm prior to use.

IgM-DNA complexes were prepared as described by Gagnon *et al.*[3] In brief, IgM-containing cell culture supernatant was applied to CHT type II and eluted with a linear gradient from 10–500 mM phosphate, pH 7.0. The main IgM fraction was applied to a porous particle anion exchanger at pH 8.0 and eluted with a linear gradient to 1 M NaCl. IgM eluted first, followed by IgM-dominant complexes, DNA-dominant complexes, then DNA. The complexes were pooled for subsequent experimentation.

Protein A affinity chromatography was conducted by equilibrating the column with HEPES-balanced saline (HBS): 50 mM HEPES, 100 mM NaCl, pH 7.0. After loading with cell culture supernatant, the column was washed with HBS. The column was eluted with 100 mM arginine, 100 mM acetic acid, pH 3.8. In some experiments, a secondary wash of 1.5 M NaCl, 2 M urea, 10 mM EDTA, 50 mM HEPES, pH 7.0, was applied and followed by another wash with HBS before elution. In other experiments, the secondary wash comprised 2 M NaCl, 4 M urea, 10 mM EDTA, 200 mM histidine, pH 7.0. Activity of the eluted antibody was unaffected by exposure to these wash conditions. The column was regenerated with 2 M guanidine, pH 5.5, after each run.

IgG for CX experiments with Nuvia S was prepared by diluting filtered cell culture supernatant with two parts 100 mM MES, pH 6.0. After loading, the column was washed briefly with 20 mM MES, pH 6.0, then re-equilibrated to 20 mM Tris, pH 8.0, and eluted with a linear gradient to 200 mM NaCl. The column was regenerated with 20 mM Tris, 1 M NaCl, pH 8.0.

HA experiments with IgG

CHT type I was equilibrated with 50 mM HEPES, 5 mM $CaCl_2$, pH 7.0, at a linear flow rate of 300 cm/hr (1 mL/min). This produced an initial pH drop and gradual recovery, indicating that HA phosphates had been converted to secondary calcium residues.[7, 8] CX-captured IgG was titrated to pH 7.0 with 100 mM MES, pH 6.0, and calcium added to a final concentration of 5 mM. IgG was loaded onto the column and washed briefly with equilibration buffer. The column was then restored to native HA by re-equilibration with 10 mM sodium phosphate, pH 7.0. This produced an initial positive pH spike, then gradual equilibration to the buffer pH. The column was then eluted with a 20 column volume (CV) linear gradient to 10 mM phosphate, 1 M NaCl, pH 7.0, and cleaned with a step to 500 mM phosphate, pH 7.0.

A series of experiments was conducted in which sample was loaded as described above, then a secondary wash applied. The first employed a wash of 4 M urea, 50 mM HEPES, 5 mM $CaCl_2$, pH 7.0; the second, 2 M NaCl, 50 mM HEPES, 5 mM $CaCl_2$, pH 7.0; the third 3.2 M urea, 1.6 M NaCl, 50 mM HEPES, 5 mM $CaCl_2$, pH 7.0. The wash was followed briefly with equilibration buffer, then the column was re-equilibrated, eluted, and cleaned as described above.

HA experiments with IgM

CHT type II was equilibrated with 10 mM sodium phosphate, pH 7.0, at a linear flow rate of 300 cm/hr (1 mL/min). IgM-DNA complexes were applied and the column washed with equilibration buffer. The column was eluted with a 20 CV linear gradient to 500 mM phosphate. In a second experiment, the buffers were formulated to include 4 M urea. In a third experiment, the column was equilibrated with 10 mM phosphate, pH 7.0. IgM-DNA complexes were applied and washed with equilibration buffer, then the column was eluted with a 20 CV linear gradient to 1 M NaCl, 500 mM phosphate, pH 7.0. In a fourth experiment, complexes were applied and washed with 2 M NaCl, 10 mM phosphate, pH 7.0, washed again with 10 mM phosphate, then eluted with a 20 CV linear gradient to 1 M NaCl, 500 mM phosphate, pH 7.0. In a fifth experiment, IgM-containing cell-culture supernatant was applied and washed with 3.2 M urea, 1.6 M NaCl, 10 mM phosphate, pH 7.0, then with 4 M urea, 10 mM phosphate, pH 7.0. The column was eluted with a 20 CV linear gradient to 1 M NaCl, 200 mM phosphate, pH 7.0, then cleaned with 500 mM phosphate, pH 7.0.

Analytical AX was run by equilibrating monolithic anion exchangers with 20 mM HEPES, pH 7.0, at a volumetric flow rate of 4 mL/min. Samples were prepared by dilution with the same buffer to achieve sufficiently low conductivity to permit binding. After loading, columns were washed with equilibration buffer. QA monoliths were eluted with a ten-minute linear gradient to 1 M NaCl (20 mM HEPES, pH 7.0). EDA was eluted with a ten-minute linear gradient to 2 M NaCl.

Analytical SEC was conducted in a buffer of 50 mM MES, 200 mM arginine, 300 mM NaCl, 10 mM EDTA, pH 6.5, intended to preemptively suppress nonspecific interactions between sample components and the solid phase.[9] Sample

volume was 0.5 mL. Volumetric flow rate was 0.25 mL/min (linear flow rate of 31.4 cm/hr).

Relative DNA:IgM levels within chromatography experiments were estimated by comparing UV absorbance at 254 and 280 nm. 254 nm was used for DNA instead of the usual 260 because 254 supports nearly equivalent DNA absorbance but occurs at a protein absorbance minimum that supports better differentiation of protein from DNA. IgG and IgM gave 254/280 ratios of about 0.5. DNA gave a 254 ratio of about 2.0. This enabled easy visual estimation of relative DNA levels from chromatograms. More precise proportioning can be obtained with the equation developed by Warburg (Table 1).[10]

The extinction co-efficients for IgG and IgM at 254 were 0.45; at 280, IgG 1.4, IgM 1.18.[11] Extinction co-efficients for IgG and IgM at 254 nm were derived from comparison of the absorbance of purified protein at 254 and 280 nm. The extinction co-efficient for DNA at 254 was 20; at 280: 10. While convenient, the accuracy of this method suffers from the disproportionately high 280 absorbance of DNA.[12-14] It is also variable with respect to conductivity and pH.[15] Low level DNA contamination was measured by PicoGreen® dsDNA reagent (Invitrogen, Eugene, Oregon USA).

Other experimental details and variations are described in the following discussion.

TABLE 1. Proportioning equation for increased precision.

$$A254/280 = \frac{(e254P \times (\%P) + e254N \times (\%N))}{(e280P \times (\%P) + e280N \times (\%N))}$$

Where e = the extinction co-efficent,
P = protein, and N = nucleotide

Results and Discussion

Figure 1 illustrates the protein composition of the 1.5 M NaCl, 2 M urea, 10 mM EDTA wash from a protein A column loaded with an IgG1-containing cell culture supernatant. The IgG eluate is included for reference. Small proteins and fragments make up the largest population, followed by aggregates. Lacking the secondary wash, these contaminants

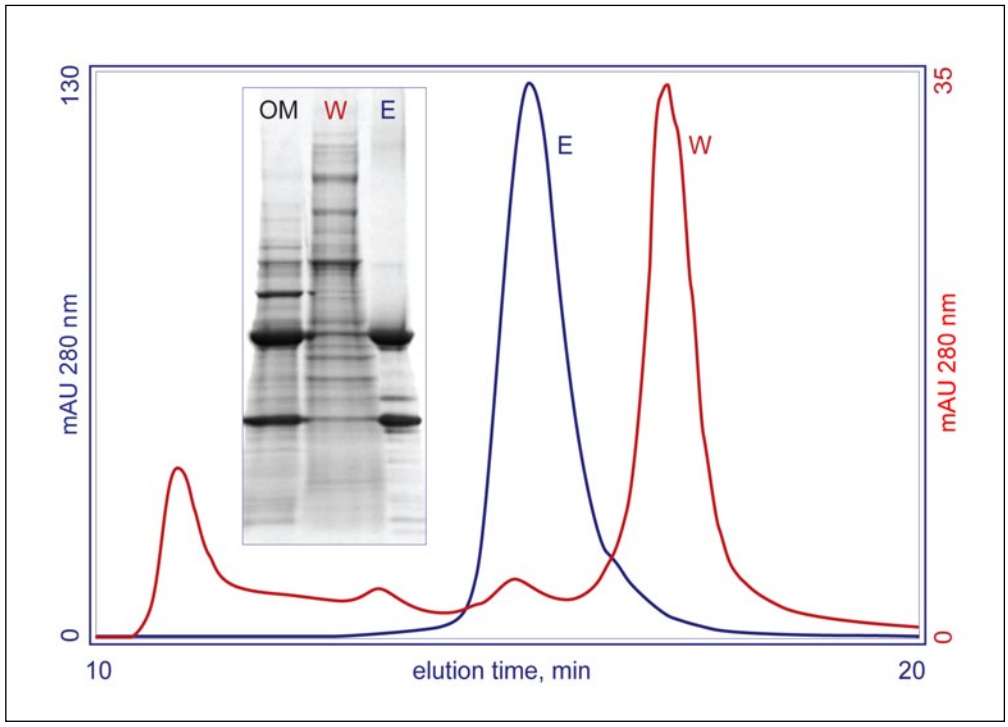

FIGURE 1. Analytical SEC and reduced SDS-PAGE of secondary wash from protein A affinity chromatography. OM: original material; W: secondary wash peak; E: elution peak.

would have remained complexed to the antibody and co-eluted with it. This is illustrated in Figure 2 which compares the SEC profiles of IgG eluates from the same column; one with a secondary wash, the other without. The secondary wash, in this case, contains 2 M NaCl, 4 M urea, and 10 mM EDTA. The sample without the wash contains dramatically higher aggregate levels, high fragment levels, and small molecule contaminants. It also contains a population of very-large size aggregates that are absent from the secondary washed IgG. Such aggregates are associated with elevated patient risk.[16] The very-large aggregate population is also noteworthy because of its elevated DNA content. According to its 254/280 ratio of 0.95, it contains about 6.1% DNA (Figure 3). This in itself implicates DNA as a promoter of aggregate formation. DNA levels are about 1.6% across the smaller aggregate and monomer populations, but still significantly higher than the monomer population from the secondary wash experiment. The profiles also differ dramatically in the late elution of very small DNA fragments in the non-secondary wash population.

Despite the clearly lower DNA content of the IgG following a secondary wash, it remained contaminated with 109 ppm. This highlights the resistance of DNA-antibody complexes to dissociation. Another IgG revealed DNA levels

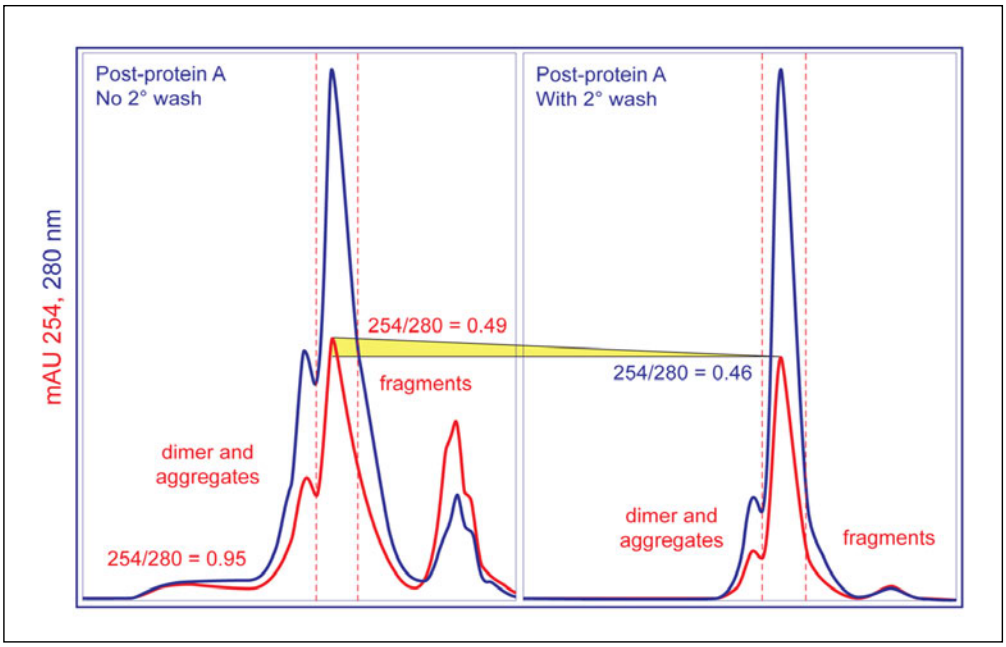

FIGURE 2. Analytical SEC of IgG eluted from protein A affinity chromatography without a secondary wash (first profile), and with a secondary wash (second profile).

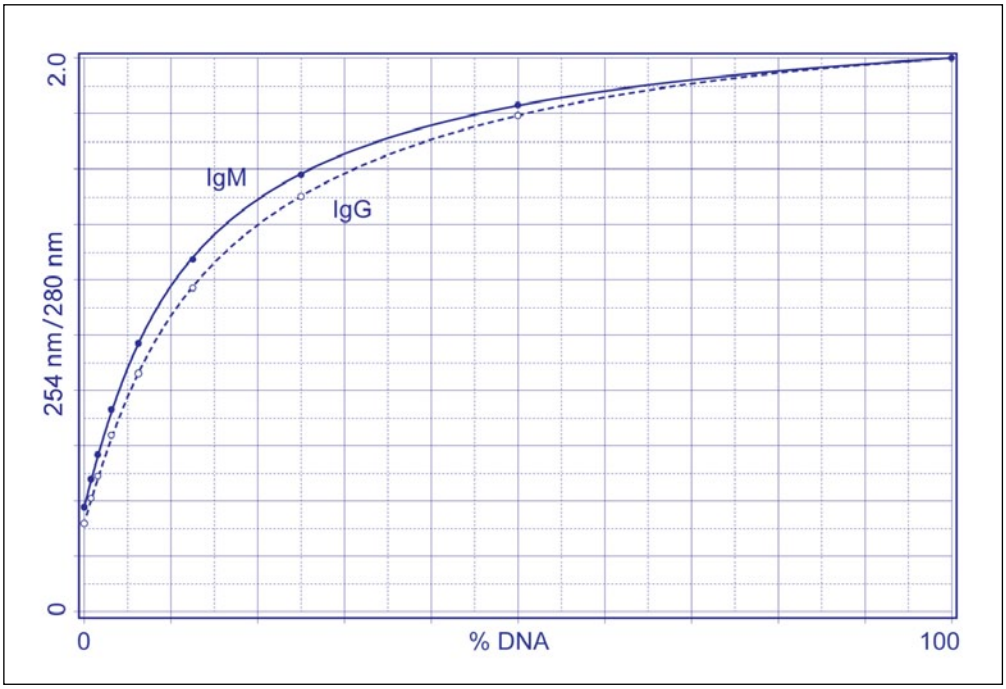

FIGURE 3. Proportioning of DNA versus protein mass according to UV absorbance ratios at 254 and 280 nm. Indicated points were calculated from the equation in Table 1.

of 39 ppm without, and 10 ppm with a secondary wash. The main difference between these antibodies was that the first exhibited a high affinity for CX. This makes intuitive sense. DNA is itself a high-density liquid phase cation exchanger, so antibodies with elevated CX affinity should be especially prone to complexation with DNA. Following CX capture, this antibody exhibited DNA contamination at 1010 ppm, nearly ten times higher than after protein A with a secondary wash. All CX capture applications probably bear this burden to some degree. Feed stream pH is often reduced, which increases antibody positive charge and enhances its affinity for DNA. Conductivity is often reduced as well. This is intended to increase antibody binding capacity, but increases potential for DNA complexation in parallel. Even as an intermediate step, CX lacks the ability to dissociate IgG-DNA complexes. DNA levels were unchanged when post-protein A samples at 10 and 39 ppm DNA were applied to and eluted from a cation exchanger.

Figure 4 illustrates sodium chloride gradient fractionation on HA at 10 mM phosphate of the CX-captured IgG with 1010 ppm DNA. Note the contaminant peak immediately before the main IgG peak. Also note the 254-dominant DNA peak at the end of the 500 mM phosphate cleaning step. Figure 5 overlays the elution gradient of Figure 4 with a run in which the IgG was washed with 1.6 M NaCl, 3.2 M urea,

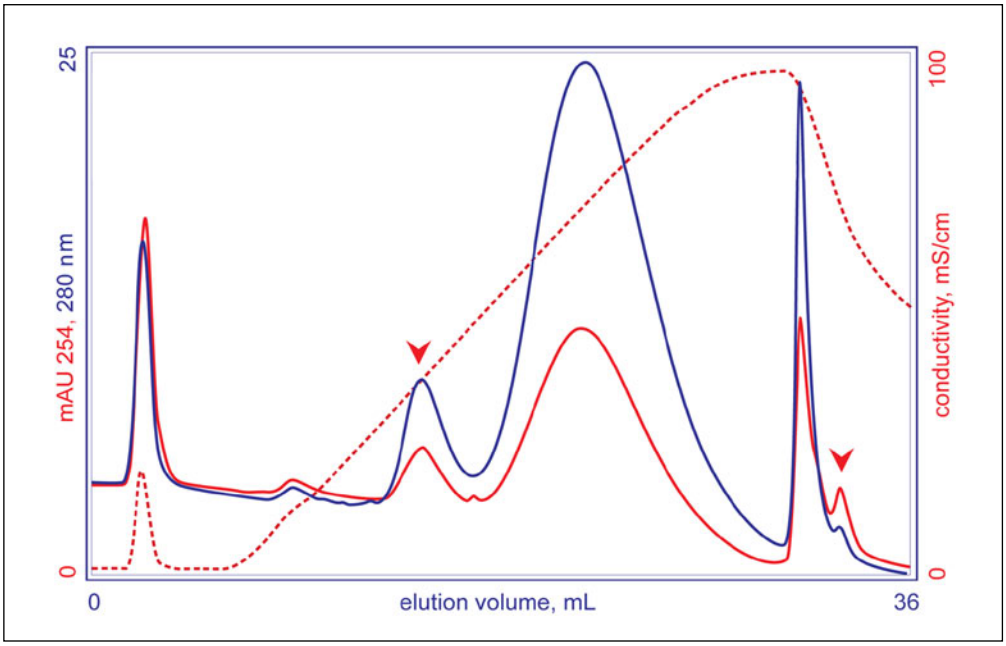

FIGURE 4. HA fractionation of CX-captured IgG in a sodium chloride gradient at 10 mM phosphate, pH 7.0. No secondary wash. Note the super-contaminant and DNA peak (red arrowheads).

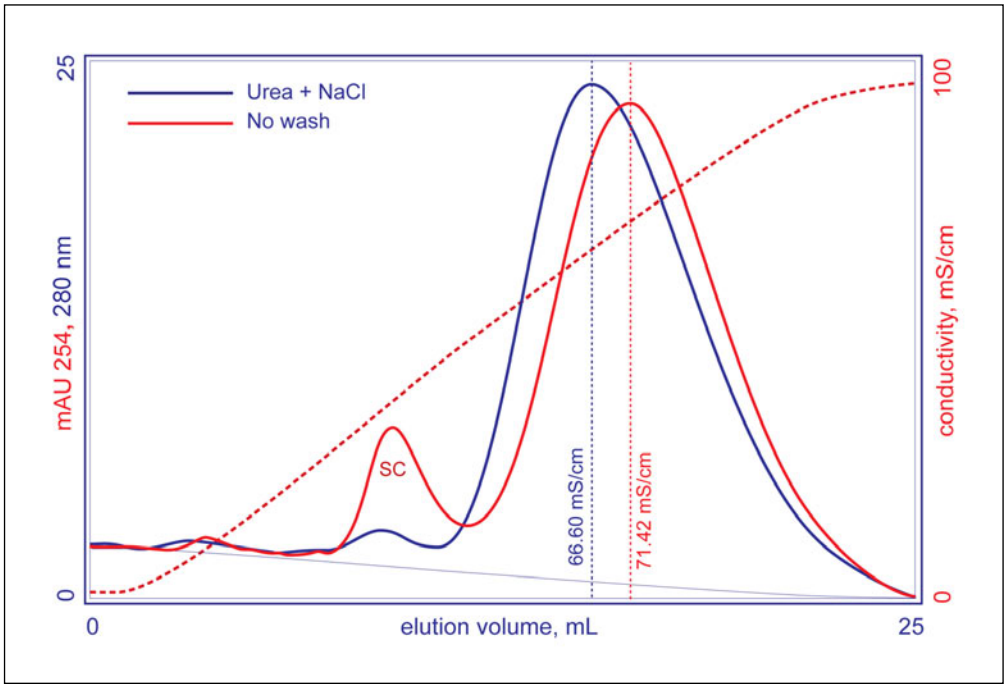

FIGURE 5. Comparison of wash treatments on HA. The red profile is the experimental control (Figure 4) eluted without a secondary wash. The blue profile illustrates elution following a secondary wash with 3.2 M urea and 1.6 M NaCl.

after loading. It reveals two important differences. The first is reduction of the contaminant peak preceding the main IgG peak. This identifies the peak in the control run as a "super-contaminant," a heteromeric contaminant aggregate. Its low 254/280 ratio (Figure 4) suggests that it comprises mostly protein contaminants which are dissociated by the decomplexing wash (Figure 5).

The second important difference is that the IgG peak elutes earlier after the urea/salt wash. The implication is that the control sample elutes later due to interaction of IgG surface-complexed DNA with HA calcium. After decomplexation by the urea/salt wash, DNA is unable to rebind to the antibody. With its native surface charge restored, the IgG elutes earlier. A similar shift of the super-contaminant peak center suggests that it too may contain a small component of DNA. These examples highlight the point that complexation elevates both contaminant and product heterogeneity, thereby complicating purification. To the extent that DNA levels vary among production lots of cell culture supernatant, for example, in relationship to cell viability at harvest, it is reasonable to expect that reproducibility of purification performance will also vary.

Figure 6 compares the 500 mM phosphate cleaning steps from the no-wash control with salt, urea, and combined washes. Note the differences in UV absorbance at 254 nm indicating relative levels of DNA. NaCl is clearly the more effective dissociation agent. At low conductivity, mutual electrostatic repulsion between HA phosphates and DNA phosphates is believed to limit the ability of DNA to bind to HA calcium.[17, 18] That repulsion is suppressed at high conductivity, allowing DNA to interact more freely with apatite calcium. In addition, mutual repulsion among DNA phosphates is reduced, making DNA more flexible and better able to conform to the HA surface.[19] The combination dramatically increases DNA binding to HA. High conductivity should meanwhile weaken electrostatic interactions between DNA and IgG. Urea is a strong hydrogen donor and acceptor that should weaken hydrogen bonds between DNA and antibody,[20] but it is non-ionic and should have no direct effect on electrostatic interactions.

HA offers yet another property that should enhance decomplexation. A recent study with IgM suggested the presence of a class of antibody-DNA complexes described as metal-bridged complexes: IgM-metal-DNA.[3] The discussion noted the ability of protein polycarboxyl domains to chelate metal ion species and simultaneously form strong coordination bonds with endotoxin or DNA phosphates.[21] Using HA as a model, it also noted in the case of calcium that such bonds survive NaCl concentrations of at least 2 M.[22] This is of interest because HA has a very strong affinity for non-calcium metal ions. Metal-contaminated protein solutions permanently discolor HA.[23] This interaction appears to be sufficiently strong to competitively dissociate non-specific metal-protein complexes. This means that with the right choice of wash conditions, HA has the potential to disrupt complex-integrity by simultaneously weakening electrostatic interactions, hydrogen bonds, metal affinity, and hydrophobic interactions; all without attenuation of the primary DNA binding mechanism. No other purification

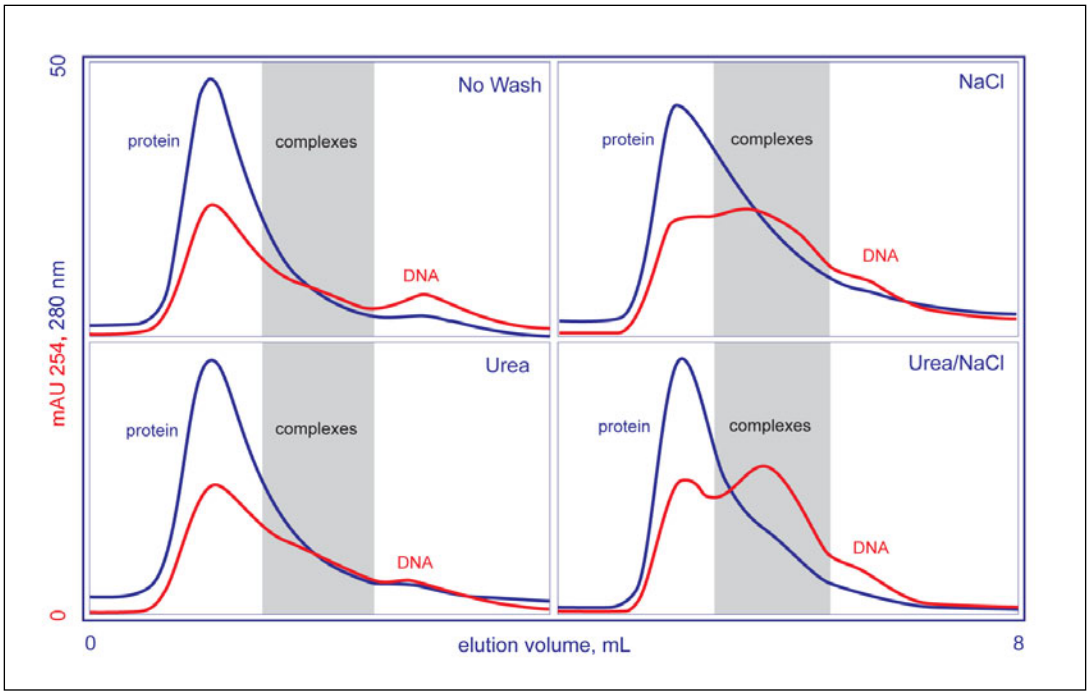

FIGURE 6. Comparison of 500 mM phosphate cleaning steps following various secondary washes and elution with NaCl at 10 mM phosphate. The profile labeled "No Wash" corresponds to Figure 4. The profile labeled "Urea" was washed with 4 M urea prior to elution. The profile labeled "NaCl" was washed with 2 M NaCl. The profile labeled "Urea/NaCl," corresponding to Figure 5, was washed with a combination of 4 M urea and 2 M NaCl.

methods except SEC and bioaffinity can support such broad-spectrum washes, but they both lack DNA- and metal-interactive surfaces to promote dissociation. AX offers a strongly DNA-interactive surface, but salt washes weaken the affinity and hence, its complex-dissociative ability.[3]

Figure 7 illustrates another benefit of aggressive decomplexation. The first SEC profile shows IgG after purification by CX, AX, and HA. The AX step was conducted in flow-through mode. The sample was diluted 1:2 with 20 mM Tris, pH 8.0, and run over a QA monolith equilibrated to the same conditions. This three-step combination normally yields low aggregate content, but in this case, the result was strikingly poor with at least 25% aggregates and a similar fragment load. The second profile illustrates the same antibody after only CX, and HA with a urea/NaCl wash. The AX step was omitted to maximize DNA content going on to the HA step. Aggregates were nearly eliminated; apparently restored to "monomeric" IgG. Fragments were similarly reduced; evidently eluted by the wash or displaced to HA and eliminated in the 500 mM phosphate cleaning step.

Figure 8 illustrates the utility of urea for dissociation of IgM-DNA complexes. The first profile illustrates elution of

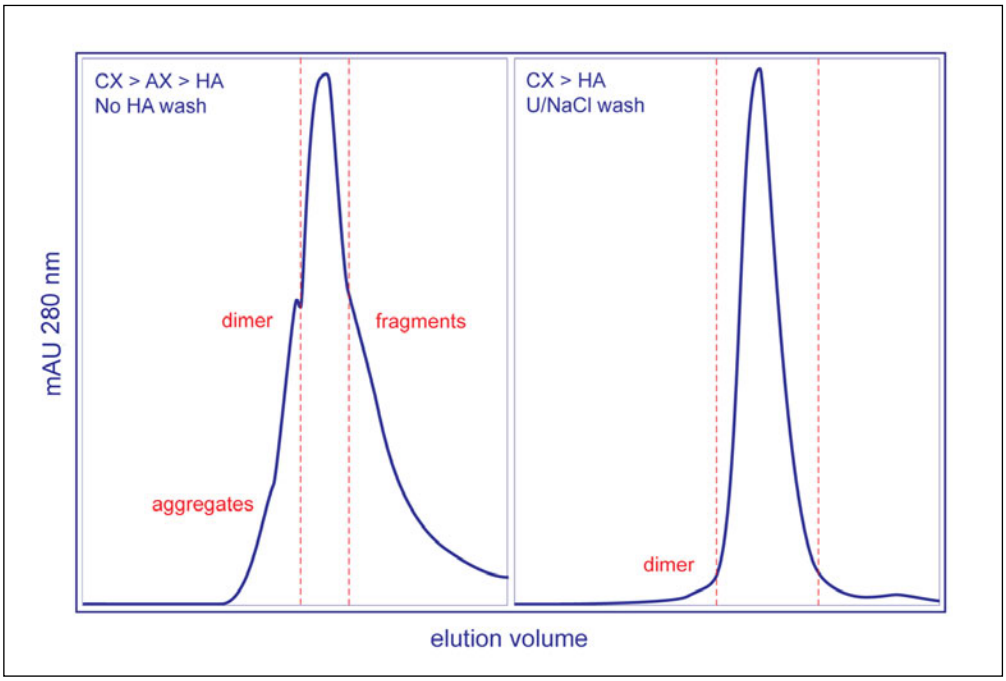

FIGURE 7. Comparison of analytical SEC profiles illustrating the benefits of a secondary wash at the HA step. The first profile shows the results of a three-step purification including HA but lacking a secondary wash. The second profile shows the results of a two-step purification where HA includes a secondary urea/NaCl wash.

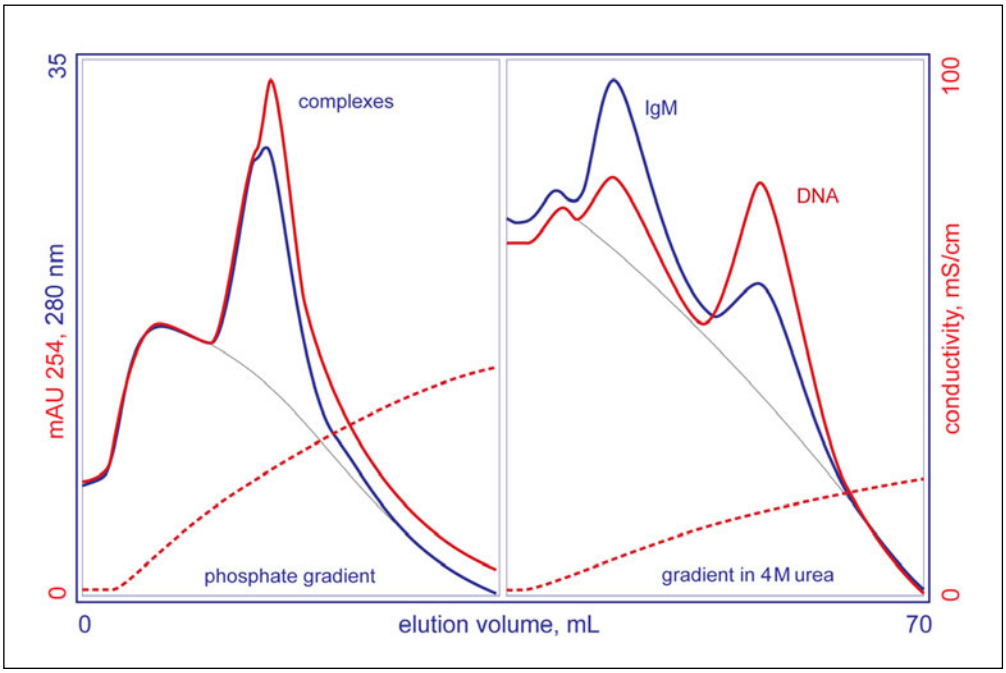

FIGURE 8. Comparison of HA profiles illustrating the ability of urea to promote dissociation of IgM-DNA complexes. The first profile illustrates phosphate gradient elution without urea. The second, level 4 M urea across the gradient. The phosphate gradients are identical. Urea depresses apparent conductivity.

complexes from HA with a conventional phosphate gradient. Decomplexation was nil. The second illustrates separation of IgM and DNA into separate populations when the gradient was carried out in the presence of 4 M urea. Figure 9 illustrates the utility of a pre-elution salt wash. In the first profile, a combined phosphate-NaCl gradient was unable to completely dissociate the components; an intermediate complex population remained. In the second, a 2 M NaCl wash was applied before elution. The components were completely dissociated.

Figures 10 and 11 highlight the benefits of decomplexation in a different way. Figure 10 illustrates IgM capture and elution on HA eluted with a conventional phosphate gradient. The peak labeled "HA1" contains most of the IgM, but the 254/280 ratio indicates that it is heavily contaminated by DNA. Figure 11 illustrates the HA capture

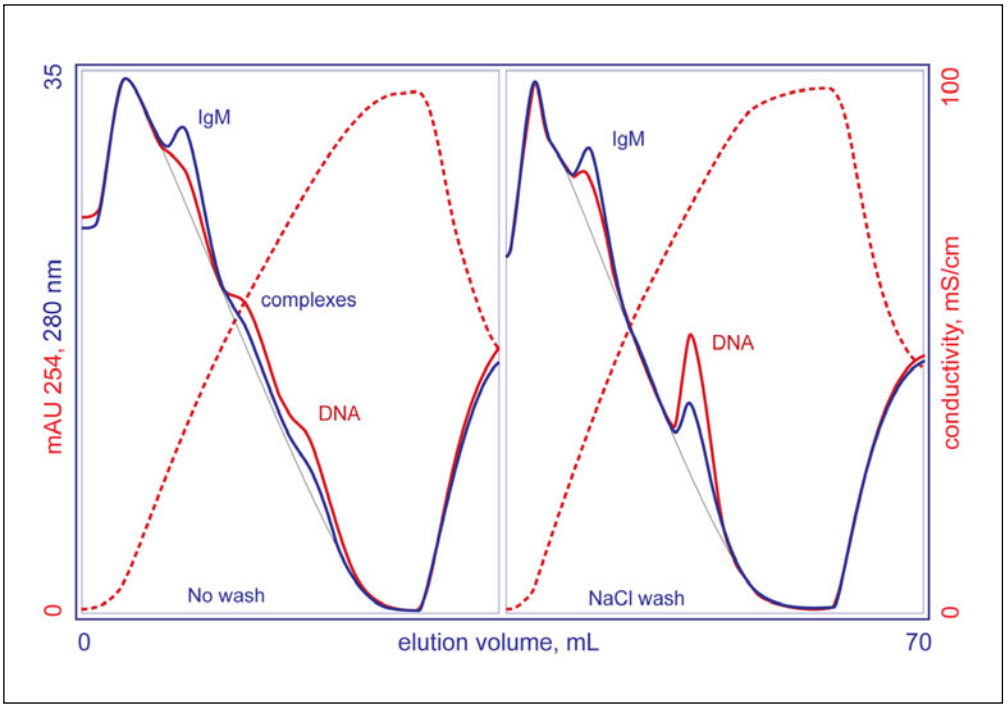

FIGURE 9. Comparison of HA profiles illustrating the ability of a pre-elution salt wash to promote dissociation of IgM-DNA complexes. The first profile illustrates phosphate-NaCl gradient elution without a previous wash. The second, a 2M NaCl wash applied before the same gradient.

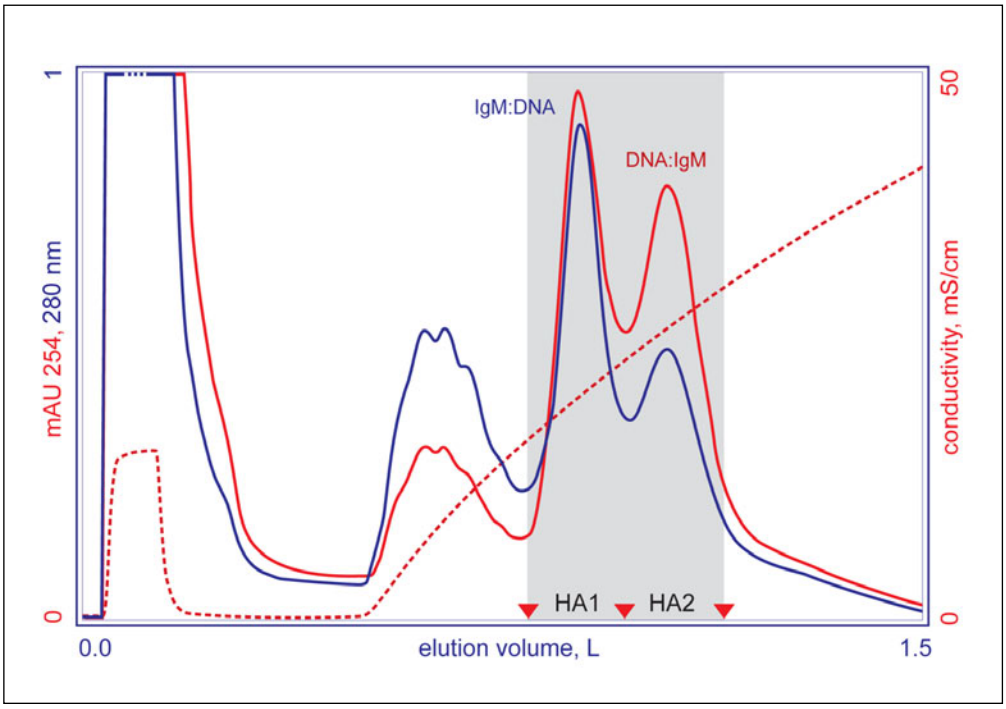

FIGURE 10. IgM capture by HA from cell culture supernatant, eluted with a linear phosphate gradient.

profile of another IgM. In this case, the column was washed with sodium chloride and urea before elution. Elution with a combined phosphate-NaCl gradient was intended to enhance the separation between IgM and DNA. The large mass of contaminants eluting before IgM (from Figure 10) are absent from Figure 11; apparently having been removed by the urea-NaCl wash. In addition, the 254/280 ratio of the main peak suggests the presence of little or no DNA, and the DNA peak is restricted to the 500 mM phosphate cleaning step. Figure 12 illustrates analytical profiles on anion exchange monoliths. The first profile is of the main IgM peak from Figure 10. The second illustrates the IgM peak, and the third, the DNA peak; both from Figure 11. The pre-elution HA wash reduces DNA contamination of the IgM peak beneath the level of chromatographic detection.

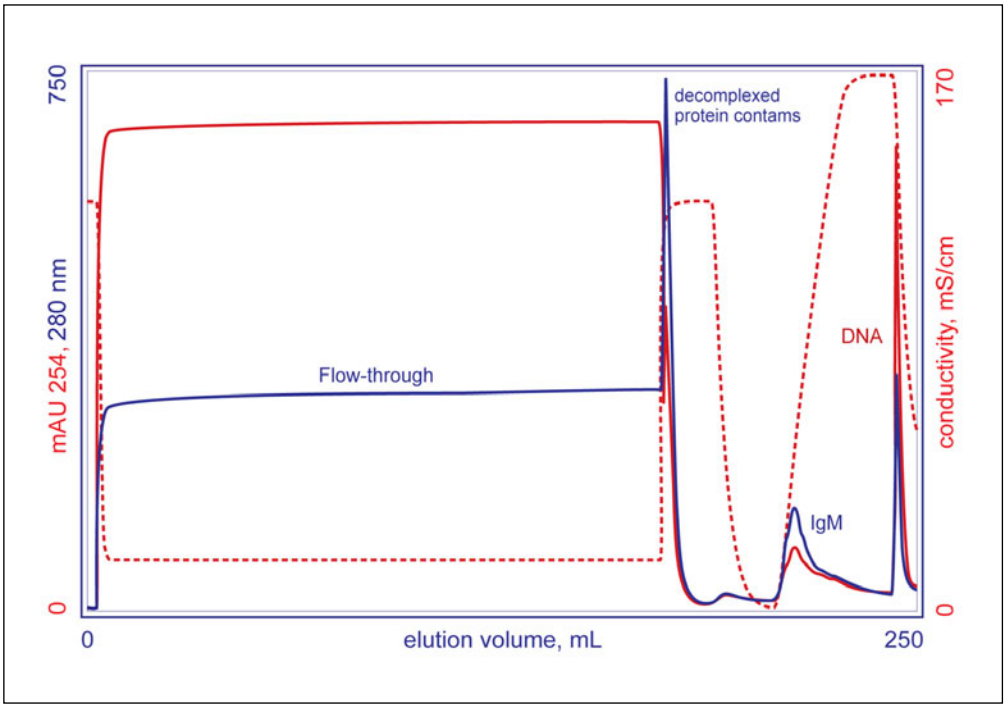

FIGURE 11. IgM capture by HA from cell culture supernatant, eluted with a phosphate/NaCl gradient following a wash with urea and NaCl.

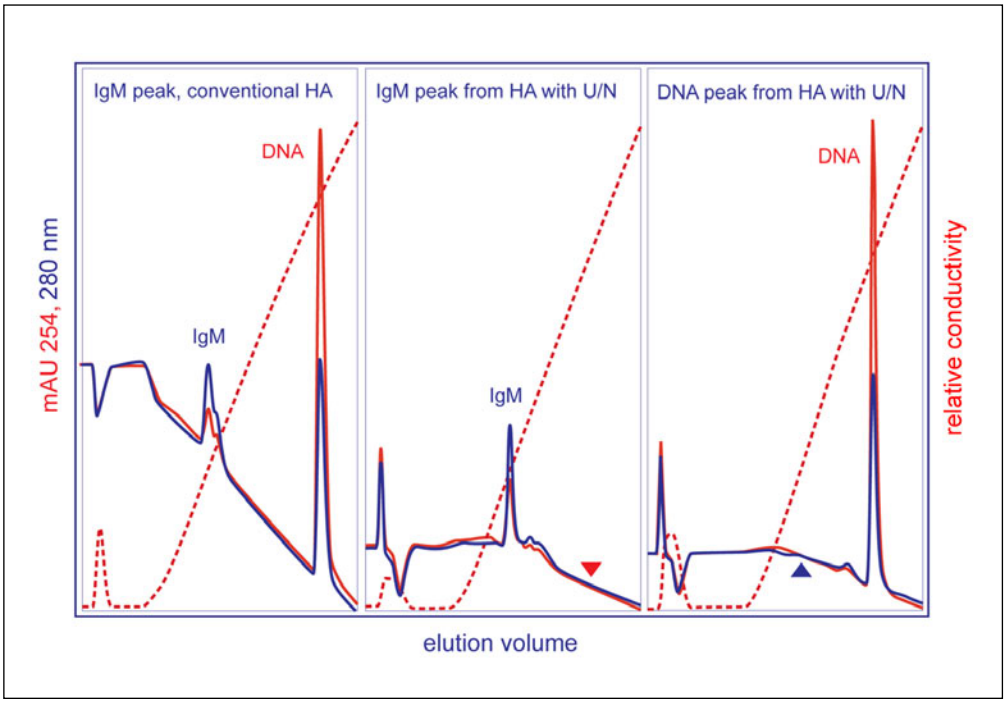

FIGURE 12. Analytical anion exchange on monolithic exchangers. The first profile shows dissociated IgM-DNA complexes from the IgM peak in Figure 10. The second shows the IgM peak from Figure 11, and the third, the DNA peak, also from Figure 11. The red triangle marks the elution point of DNA. The blue triangle marks the elution point of IgM.

Conclusions

This and previous studies have shown that complexation of contaminants with antibodies and with other contaminants creates uncontrolled product and contaminant heterogeneity that complicates purification. It appears that host cell DNA is directly involved in the formation and stabilization of these complexes. This implies that cell viability at harvest is a key determinant of complexation levels and could therefore be a significant purification process variable. It specifically suggests that the elevated aggregate levels in high-density cell cultures result from DNA fragment-mediated complexation.

Three highly effective decomplexation tools have emerged to date: 1) secondary washes at the protein A step; 2) AX on monoliths; and 3) secondary washes on HA. Secondary washes at the protein A step are now applied throughout the industry. Wash formulations vary from alcohol-detergent and alcohol-urea combinations to high-salt-urea-EDTA and will likely continue to evolve. The former are very effective for host cell protein reduction, but dissociation of DNA requires elevated salt concentrations. No wash has yet demonstrated the ability to completely remove DNA. However, the lower it can be reduced, the more realistic hopes become for effective two-step purification platforms.

Present results indicate that HA with decomplexing washes is the most powerful of the three methods. Thus far, it is the only one that has demonstrated an apparent ability to dissociate complexes to the point of restoring antibody aggregates to monomer. This logically resides with the fact that its DNA-binding mechanism is independent from complex destabilization methods. This method should have similar abilities to decomplex antibodies from other polyphosphorylated contaminants such as endotoxin and lipid-enveloped virus — not to mention host cell proteins.

Given highly effective secondary washes at the protein A step, elevated complex dissociation capabilities at later steps may seem redundant but for two exceptions: the first is recovery of monomer from aggregate. High aggregate levels in supernatants from high-titer cell cultures represent a situation of two steps forward and one step back. Teaming cell culture with purification methods capable of restoring monomer removes the backward step and increases cost-efficiency of the entire manufacturing train. The presence of covalent aggregates will impose a limit on how much monomer can be restored, but wash formulations could conceivably develop to the point of restoring those as well.

The second exception pertains to enablement of non-protein A capture methods unable to support decomplexing washes. The introduction of a variety of high capacity industrial ion exchangers has revived interest in CX capture.[24-28] The key limitation with this approach is that CX offers practically no decomplexation ability: heavily DNA-dominant antibody complexes fail to bind due to the exchanger-repellant charge on the DNA, and antibody-dominant complexes carry DNA to the next step with little or no reduction.[3, 4, 29] Subsequent purification steps with strong decomplexing ability may compensate for these limitations and substantially increase the number of antibodies that can be accommodated by CX without recourse to the expensive alternative of protein A.

Finally, the results of the present study suggest a reassessment of the importance of DNA as a contaminant. Up to the present, the main concern has been product safety, but the relative ease of achieving DNA levels suggested by regulators has caused DNA contamination to be viewed rather casually by process developers. Now, in addition, it appears that DNA may have a direct role in aggregate formation with practical consequences for both process economy and drug safety. The present work represents a step toward addressing these issues but also calls for harvest methods to be fully integrated with purification process development to support a comprehensive DNA management strategy. Some harvest practices have already made progress along these lines,[30] but making DNA a focal point will likely advance the field more rapidly.

ACKNOWLEDGEMENTS

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