Dissociation of Naturally Occurring Antibody-Contaminant Complexes with Hydroxyapatite

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Antibody-contaminant complexes

Purification process development commonly begins with an assumption that the product and contaminants are separate, wholly independent entities.

Recent publications reveal that this assumption is invalid, probably always, and often to a high degree.

Host cell protein clearance during protein A chromatography: development of an improved column wash step, A. Shukla, P. Hinckley, Biotechnol. Progr., 24 (2008) 1115-1121
Evicting hitchhiker antigens from purified antibodies, K. Luhrs, D. Harris, S. Summers, M. Parseghian, J. Chromatogr. B, 877 (2009) 1543–1552
Production of biobetter IgG with enhanced wash and elution of protein A. www.validated.com/revalbio/pdffiles/Biobetter.pdf
IgM purification: the fine print. www.validated.com/revalbio/pdffiles/MSS10B.pdf
Chromatographic behavior of IgM:DNA complexes, P. Gagnon, F. Hensel, S. Lee, S. Zaidi, J. Chromatogr. A, JCA-10-1683

Antibody-contaminant complexes

Product-contaminant complexes are Trojan Horses that can transport suites of seemingly unrelated contaminants straight through the gates of multistep purification procedures.

They form spontaneously during cell culture production. High DNA and core histone content suggest that dead cell ejecta are the primary promoters, but a diverse mix of host cell proteins and metal ions are also involved.

Antibody-contaminant complexes

Complexation creates antibody subpopulations with aberrant chromatographic characteristics. This adds a novel stratum of product heterogeneity that challenges all purification methods.

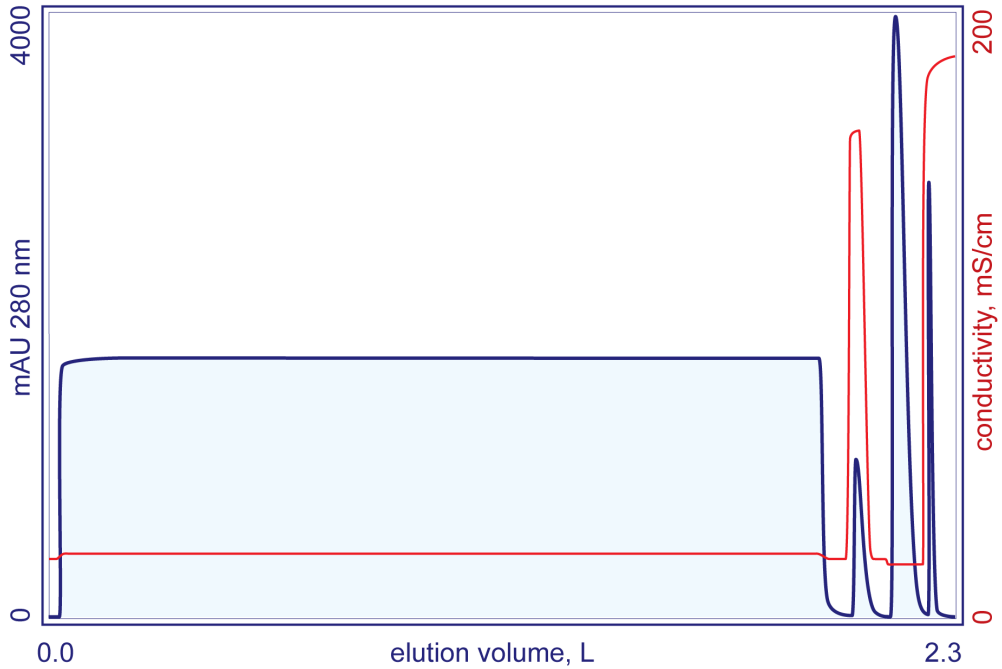
Complexation also occurs among contaminants, independent of the product, creating "super-contaminants" with poly-disperse chromatographic retention characteristics.

These phenomena complicate the task of purification process development even if the developer is aware of them; more if they are not.

Re: super-contaminants, see also, A. Tscheliessnig et al J. Chromatogr. A 1216 (2009) 7851

IgG-contaminant complexes

Protein A with a decomplexing secondary wash

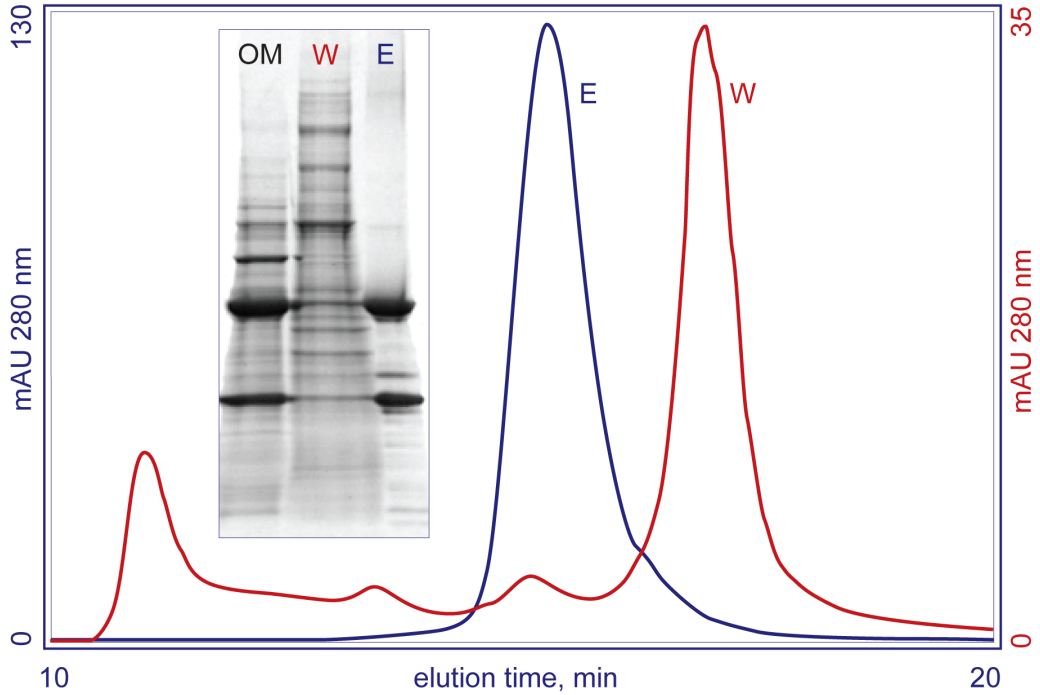


UNOsphere SuprA
1.6 x 20 cm (25 mL)
10 mL/min (300 cm/hr)
EQ: Hepes saline, pH 7
Load cell culture supe.
Wash1: Hepes Saline, pH 7
Wash2: 1.5 M NaCl, 2 M urea, 10 mM EDTA, 50 mM histidine, pH 7.
Wash3: Hepes saline, pH 7
Elute: 100 mM arginine, 100 mM acetate, pH 3.8
Clean: 2 M guanidine, pH 5

The size of the secondary wash peak is fairly consistent among individual lots of feed stream for a given antibody, but may vary with respect to the proportion of dead cells at the time of harvest (higher cell death, larger secondary wash peak). This highlights the points that the degree of cell death is an important purification process variable, and that secondary washes can control this source of variation.

IgG-contaminant complexes

PAGE and HPSEC of protein A secondary wash and eluate



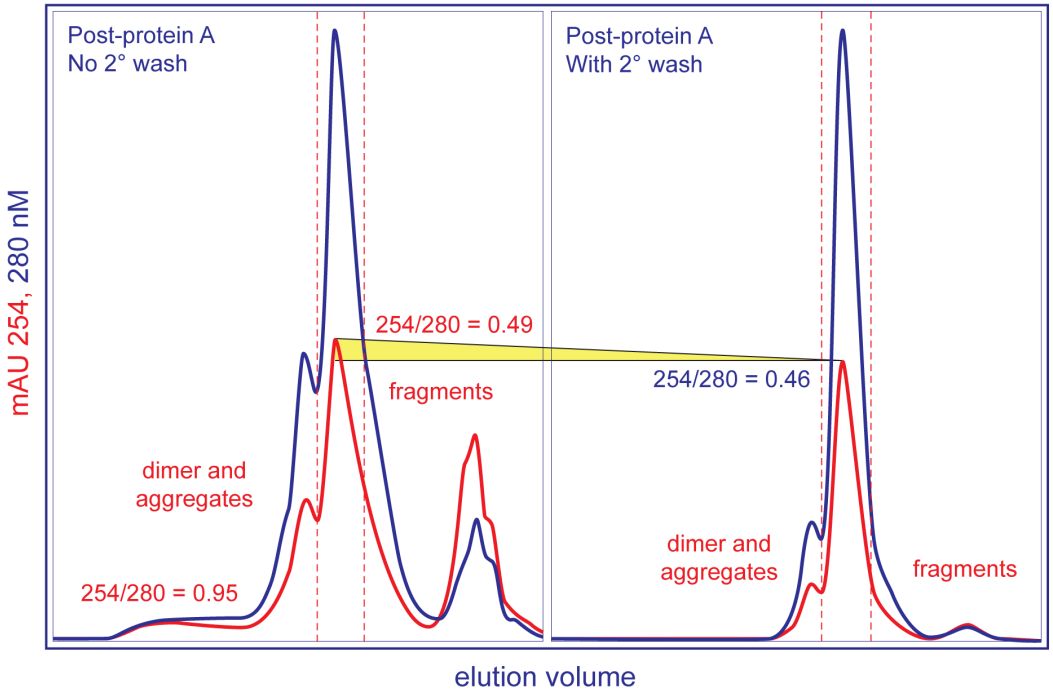
Criterion™ 4-20% gradient
Stain: Colloidal coomassie
OM: original material
W: Secondary wash
E: elution

Bio-Sil™ SEC-400-5
7.8 x 300 mm, 1 mL/min
200 mM arginine, 50 mM MES, 5 mM EDTA, pH 6.5
Inj. vol. 100 µL
E: protein A eluate
W: Secondary wash

Secondary wash peaks are usually turbid from the presence of aggregates and precipitates, and often discolored (gray/yellow–brown). In the absence of a secondary wash, these contaminants remain complexed and co-elute with the antibody, placing a greater burden on subsequent purification steps.

IgG-contaminant complexes

Protein A-purified IgG, with and without a secondary wash



TSKgel® PWXL 5000
7.8 x 300 mm, 0.25 mL/min
200 mM arginine, 50 mM MES, 5 mM EDTA, pH 6.5
Inj. vol. 100 µL
254 nm was chosen for DNA instead of 260 because it coincides with a protein absorbance minimum that permits better differentiation of DNA from protein.
The 254/280 ratio for pure IgG is about 0.45. The ratio for pure DNA is about 2.0

The 254/280 ratio of the very large aggregate peak in the no-2° wash eluate shows the presence of ~50,000 ppm DNA. Very large aggregates disproportionately increase patient risk and are a key focus of regulatory agencies. Note that even the monomer peak carries an elevated DNA load (no-2° wash).

IgG-contaminant complexes

Benefits of the decomplexing wash: ***ChoP****, ng/mg IgG (ppm)*

	w/o 2°	*w/ 2°*	*Delta*
PA wash	*------*	*9,976.0*	*------*
PA eluate	*18.3*	*10.5*	*1.74x*
CX eluate	*1.9*	*0.8*	*2.38x*
AX eluate	*1.3*	*1.1*	*0.18x*

The secondary wash roughly halves ChoP levels post-protein A and post-CX. The degree of improvement is more modest post-AX but the trend is the same: a secondary wash at the protein A step improves purification performance at every subsequent purification step.

IgG-contaminant complexes

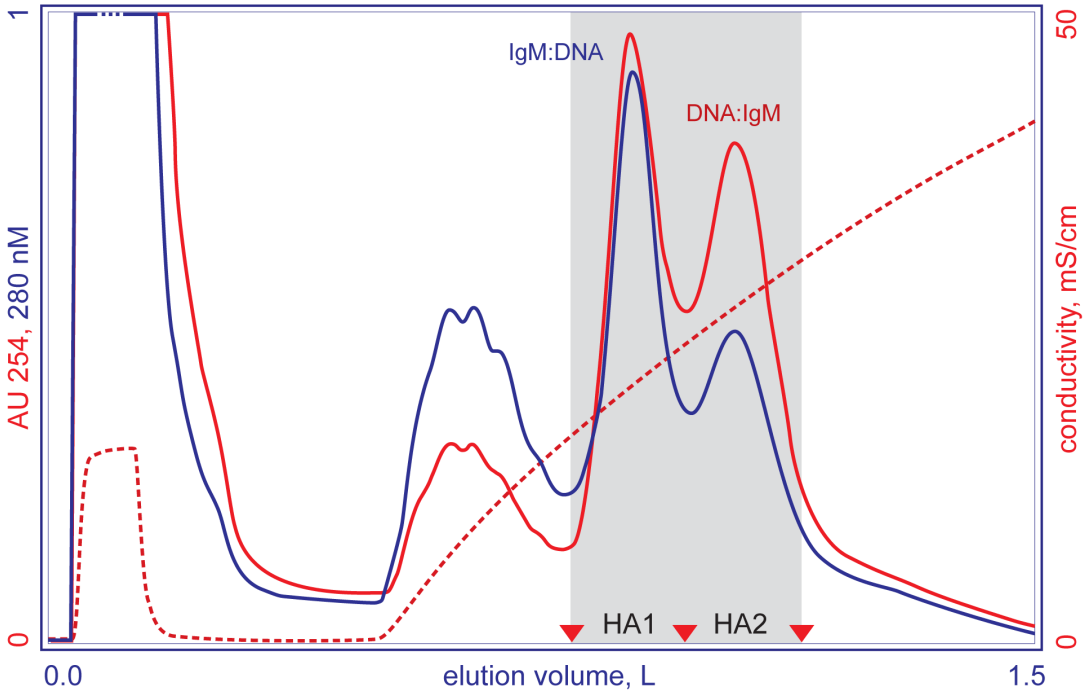
*Benefits of the decomplexing wash: **DNA**, ng/mg IgG (ppm)*

	w/o 2°	w/ 2°	Delta
PA wash	------	1,556.2	------
PA eluate	31.9	10.6	3.01x
CX eluate	32.8	10.9	3.01x
AX eluate	0.8	0.7	0.14x

The persistence of significant DNA contamination after the salt/urea/EDTA wash highlights the stability of product-contaminant complexes. Note the apparent inability of CX to dissociate complexed DNA. This means that whatever DNA protein A doesn't remove, passes directly to the AX step.

IgM-contaminant complexes

Initial capture on hydroxyapatite

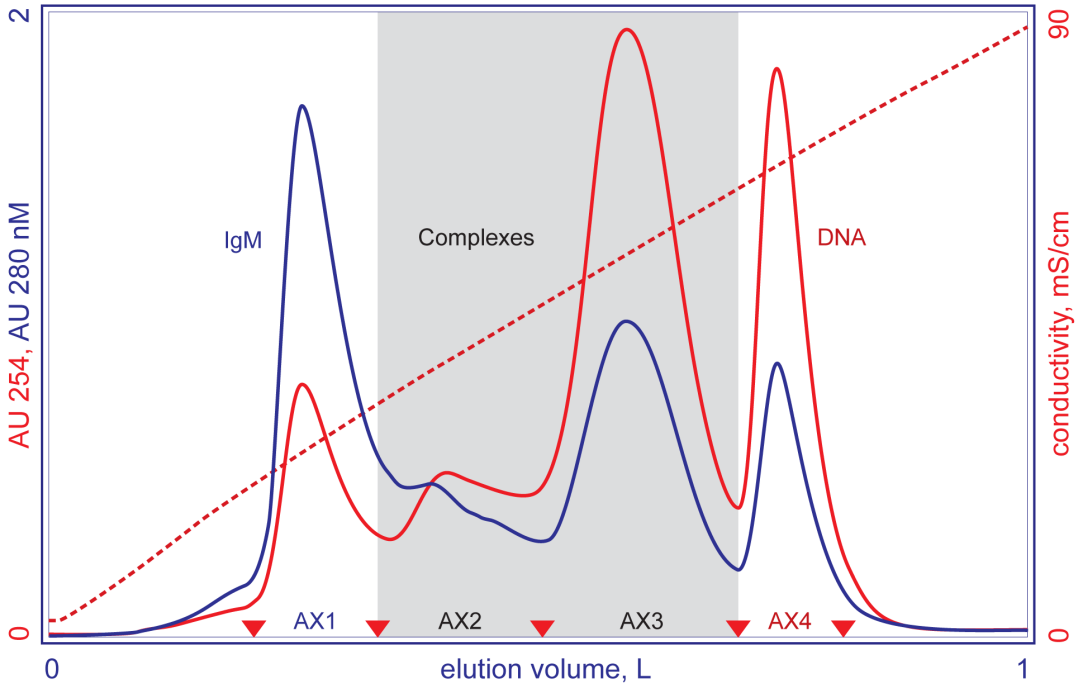


CHT™ Type II, 40 µm
426 mL, 5 x 22 cm
A: 10 mM $NaPO_4$ pH 7
B: 500 mM $NaPO_4$ pH 7
EQ: A
Load: 8 L CCS
Wash: A
Elute: 20 CVLG A > B
Clean: B
Note the break indicated during the loading phase. The indicated elution volume applies only to the illustrated interval.

One absorbance unit of DNA at 254 nm corresponds to a concentration of about 50 µg/mL. One absorbance unit of IgM at 280 nm corresponds to about 850 µg/mL. When absorbance at 280 is equivalent to absorbance at 254, as roughly in the IgM peak, about 6% of the mass is DNA. See J. Glasel, Biotechniques 18 (1995) 62 for optical proportioning of DNA and protein.

IgM-contaminant complexes

Anion exchange fractionation of pool HA1

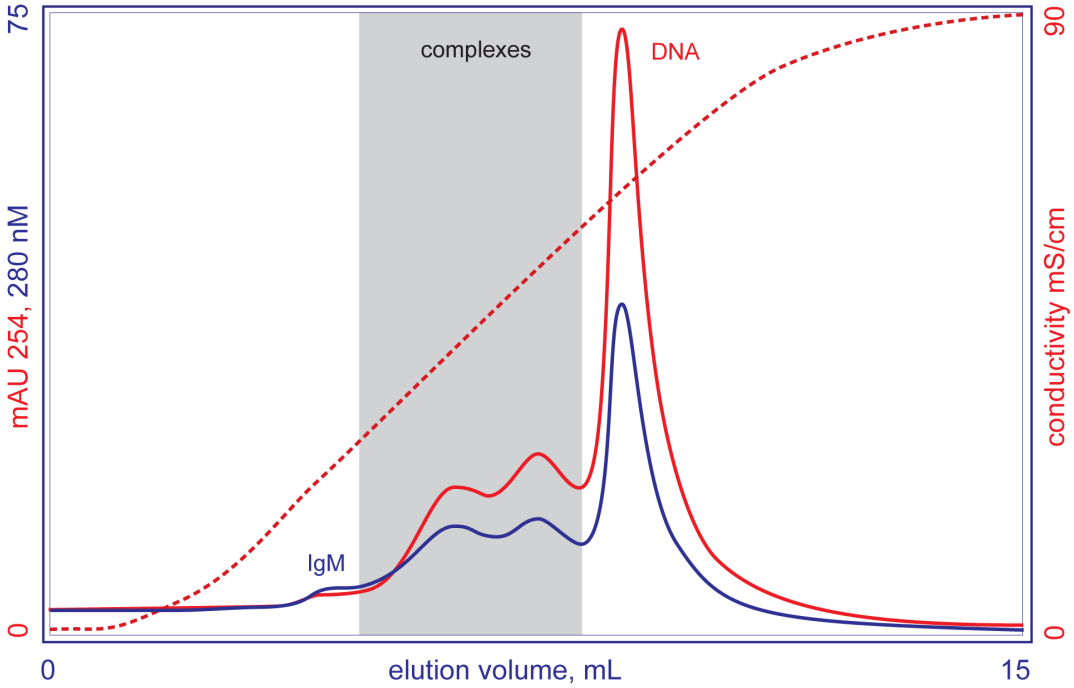


Fractogel® TMAE HiCap
118 mL, 2.6 x 22 cm
200 cm/hr
A: 25 mM Tris, pH 8
B: A + 1 M NaCl, pH 8
EQ: A
Load: 1 L HA1
Wash: A
Elute: 10 CVLG A > B

The leading shoulder contains host cell protein contaminants left over from the HA capture step. AX1 contains IgM that is relatively free of DNA, making the important point that only a small proportion of the IgM is heavily complexed with DNA. The contents of fractions AX2 and AX3 are IgM:DNA complexes. This pool was used for subsequent experiments.

IgM-contaminant complexes

Anion exchange fractionation of pool HA2

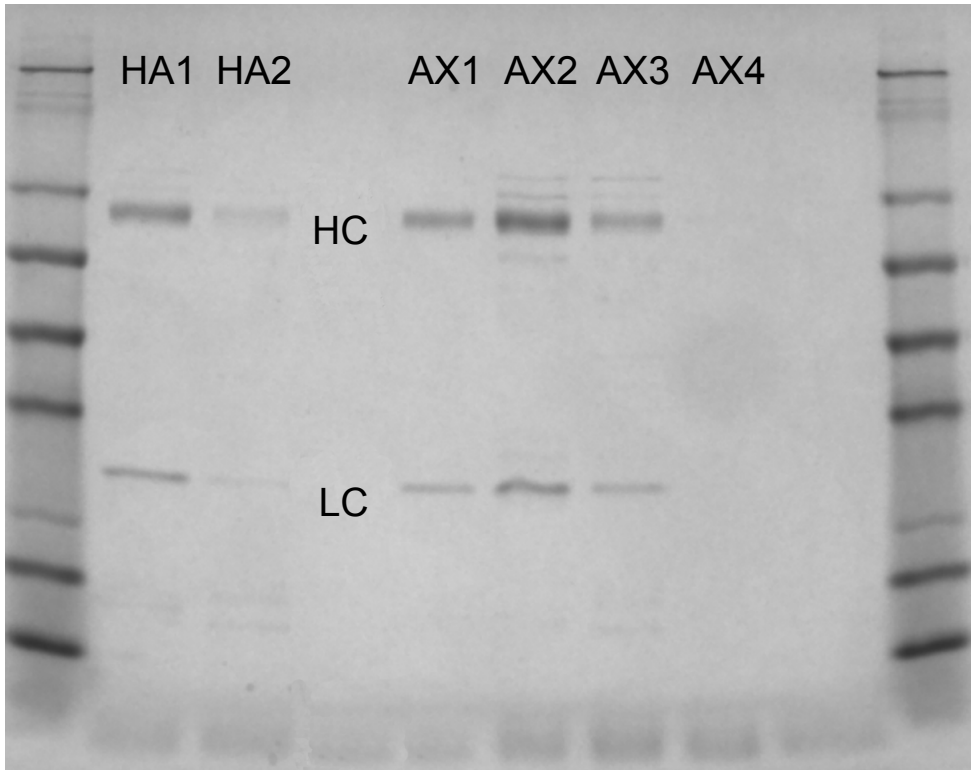


Fractogel TMAE HiCap
1 mL prepacked
200 cm/hr
A: 25 mM Tris, pH 8
B: A + 1 M NaCl
EQ: A
Load: 10 mL HA2
Wash: A
Elute: 10 CVLG A > B

Uncomplexed IgM is virtually absent from HA2. This validates exclusion of HA2 from further processing but highlights the point that complexation causes product loss. The loss however, is not as bad as it appears. Per equivalent mass unit, DNA absorbs about 10 times more UV at 280 nm than protein (assuming a protein extinction coefficient of 1).

IgM-contaminant complexes

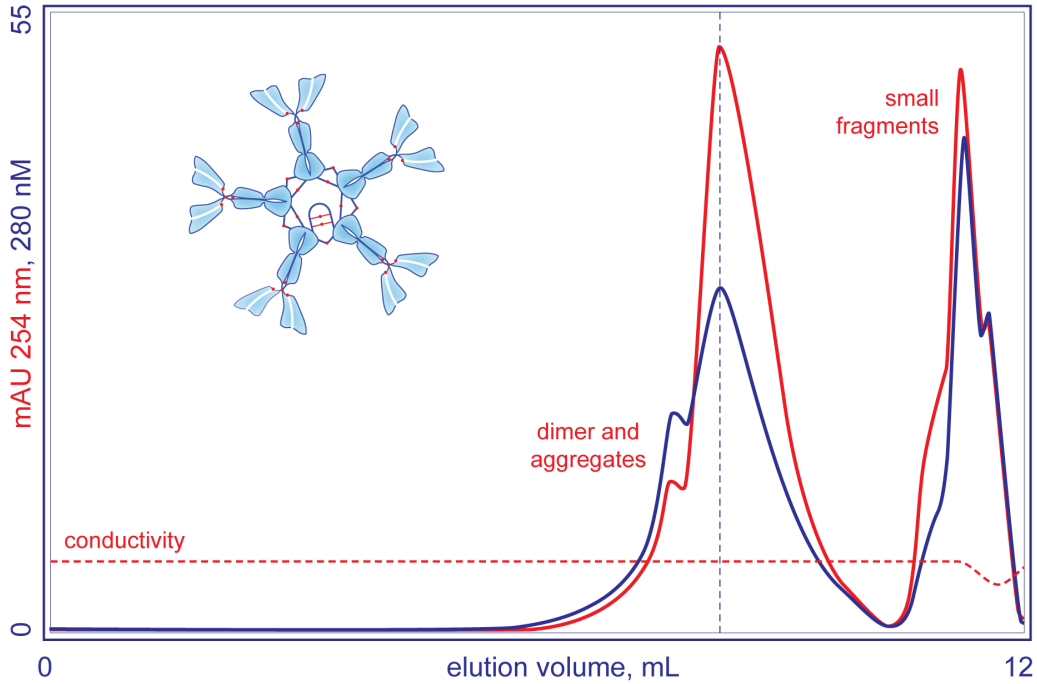
Reduced SDS-PAGE of HA and AX fractions



5-15% gradient gel Coomassie brilliant blue. All AX fractions are derived from HA1
Note the contaminating protein bands in AX2 and AX3. This cautions that complexes include more than just antibodies and DNA. Histones are likely constituents but any solute able to bind nonspecifically to IgM or DNA is a potential candidate. This elevates the priority for complex-dissociation or removal.

IgM-contaminant complexes

Size distribution of IgM:DNA complexes



TSKgel PWXL 5000
7.8 x 300 mm, 0.25 mL/min
200 mM arginine, 300 mM NaCl, 50 mM MES, pH 6.5
Inject 100 µL TMAE pool

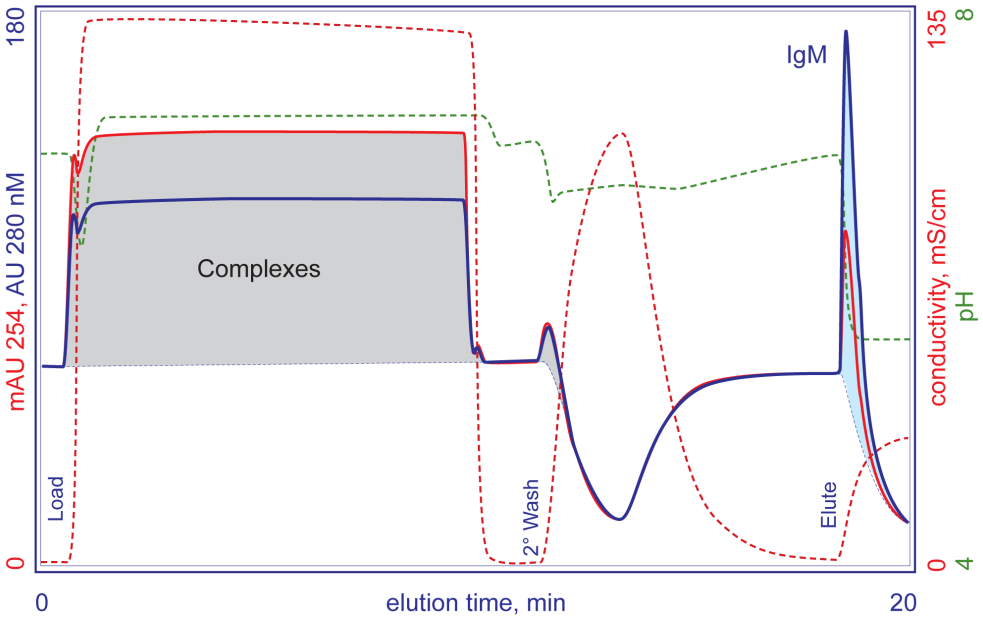
The blue vertical dashed line indicates elution volume at the peak center for DNA-free IgM.

qPCR and micro-BCA indicate that 24% of the complex mass is DNA. This amounts to about 230 kDa, or a total of about 350 base pairs.

The dominance of the 254 trace indicates that most of the DNA co-elutes with native IgM. This suggests that complexed DNA consists of small fragments. Identical elution volume with native IgM indicates that complexed DNA fragments are probably complexed between the pentamer arms and/or perpendicular to the radius of the pentamer disk.

IgM-contaminant complexes

How does complexation affect affinity chromatography?

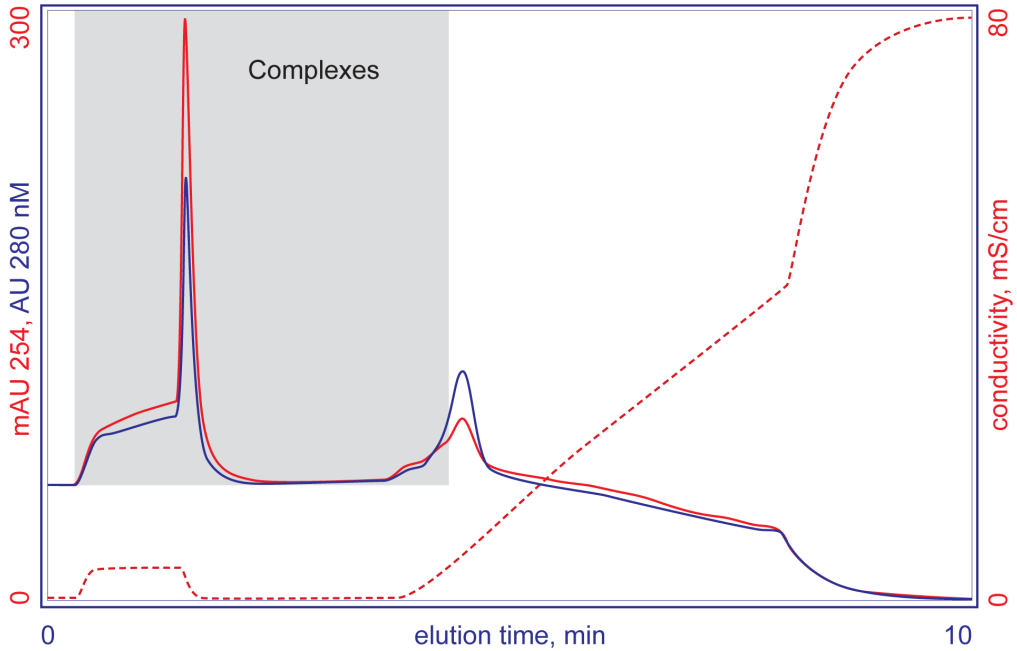


CaptureSelect® anti-lambda light chain.
EQ: 50 mM Hepes, 100 mM NaCl, pH 7 (HBS)
Load: TMAE complexes, NaCl added to ~1.5 M
Wash1: HBS
Wash2: 2M urea, 1.5 M NaCl, 10 mM EDTA, pH 7
Wash3: HBS
Elute: 500 mM arginine, 100 mM MES, pH 5.5

Complexed DNA apparently blocks immunorecognition by the affinity ligand, which means it could have similar consequences for antibody quantitation by ELISA. Experiments with the sample at 0.3 or 3.0 M NaCl produced essentially the same results. The 254/280 ratio of the eluate shows the NaCl/urea wash was able to reduce DNA to a low level, but as with 2° washes on protein A, was unable to completely eliminate it. These results also suggest that IgG losses on loading protein A could result in part from contaminant complexation.

IgM-contaminant complexes

How does complexation affect cation exchange?



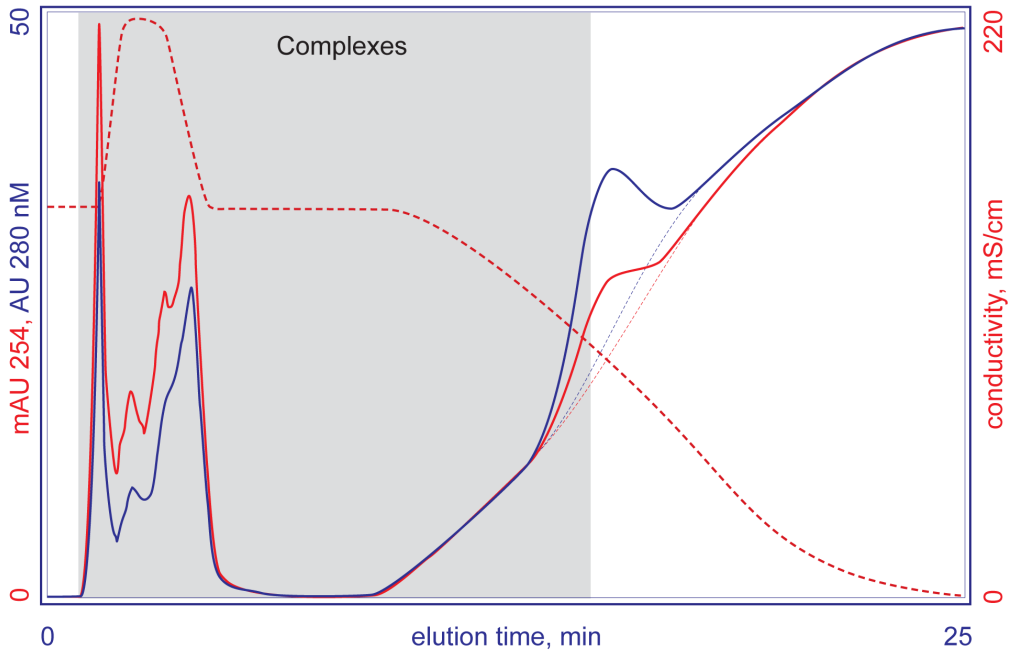
CIM SO_3, 4 mL/min
A: 20 mM Hepes, pH 7
B: A + 1 M NaCl
EQ: 100% A
Load: TMAE complexes
Wash: 100% A
Elute: 60 CVLG to 50%B
Clean: 100% B

All of the flow-through and the leading side of the elution peak are complexed.

The flow-through spike at the end of the sample load coincides with a decrease in conductivity. This suggests that the spike is caused by increased charge repulsion between exchanger SO_3 groups and negatively charged phosphates on DNA fragments complexed to the surface of the IgM. Product recovery is obviously poor.

IgM-contaminant complexes

How does complexation affect HIC?



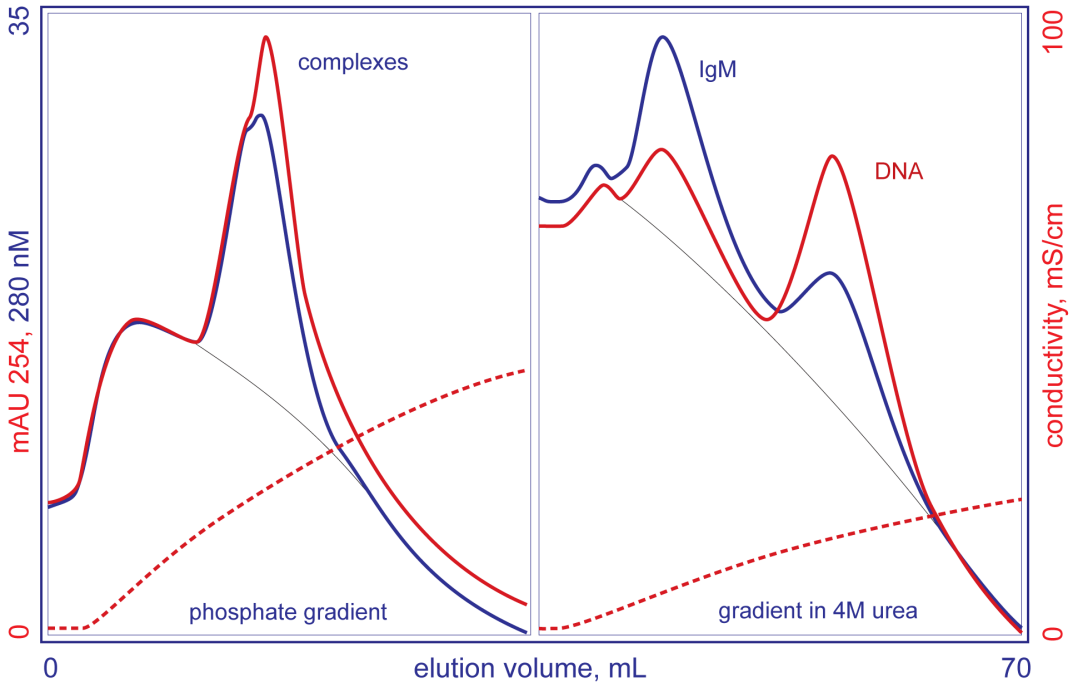
Toso PPG HW75
Post-TMAE complexes in 3.2 M NaCl
Equilibrate in 50 mM Hepes, 1 M ammonium sulfate, pH 7
Elute 10 CVLG to 50 mM Hepes, pH 7

Flow-through fractions are DNA dominant. Binding fractions appear to be IgM dominant.

Despite the sample having been applied in 3.2 M NaCl, substantial UV absorbance was unretained. Note that the 254/280 ratio diminishes across the flow-through. The 254/280 ratio of the leading shoulder on the elution peak also indicates the presence of complexes. DNA apparently reduces surface hydrophobicity of complexes, which is consistent with the inability of DNA to bind weakly hydrophobic media like PPG.

IgM-DNA decomplexation by HA

Decomplexation on HA by relaxing hydrogen bonds



CHT™ Type II, 40 µm
5 x50 mM, 0.67 mL/min
A: 10 mM $NaPO_4$ pH 7
B: 500 mM PO_4 pH 7
C: 10 mM PO_4 4 M urea
D: 500 mM PO_4 4 M urea

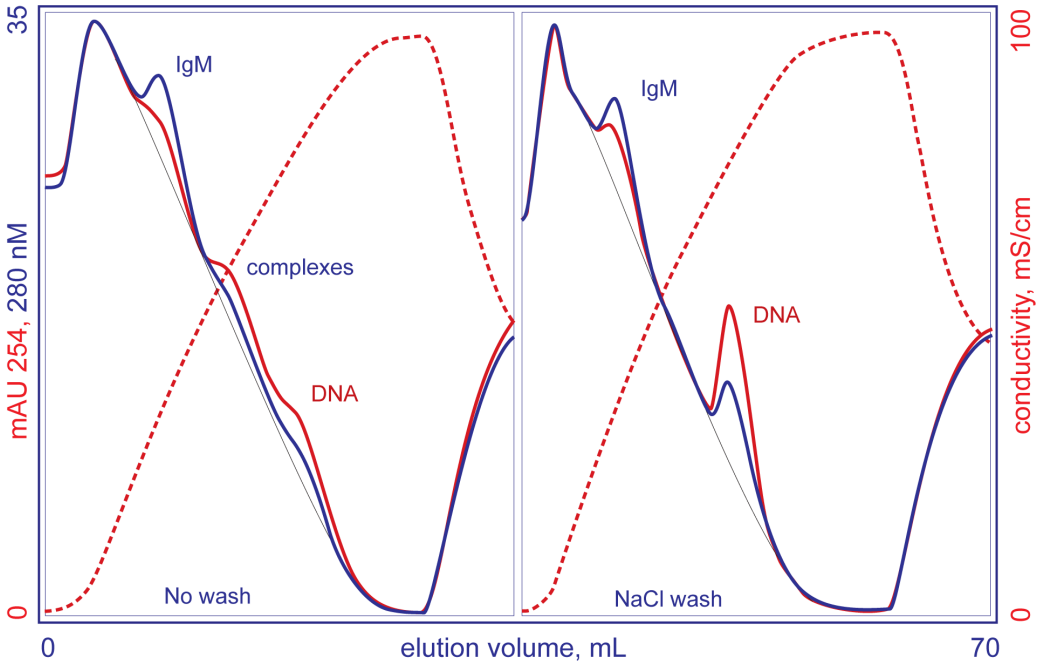
Profile 1:
EQ: A. Inject. Wash: A
Elute: 20 CVLG A > B

Profile 2:
EQ: C. Inject. Wash: C
Elute: 20 CVLG C > D

The sample for both runs was the TMAE IgM:DNA complex pool (AX2 + AX3, slide 11). Urea causes both components to elute earlier, indicating that hydrogen bonding contributes to retention of both but affects IgM more. Urea meanwhile destabilizes complexes sufficiently in the presence of HA to produce a respectable separation.

IgM-DNA decomplexation by HA

Decomplexation on HA by relaxing electrostatic interactions



CHT™ Type II, 40 µm
5 x50 mM, 0.67 mL/min
A: 10 mM $NaPO_4$ pH 7
B: 500 mM PO_4 1 M NaCl
C: 10 mM PO_4 2 M NaCl

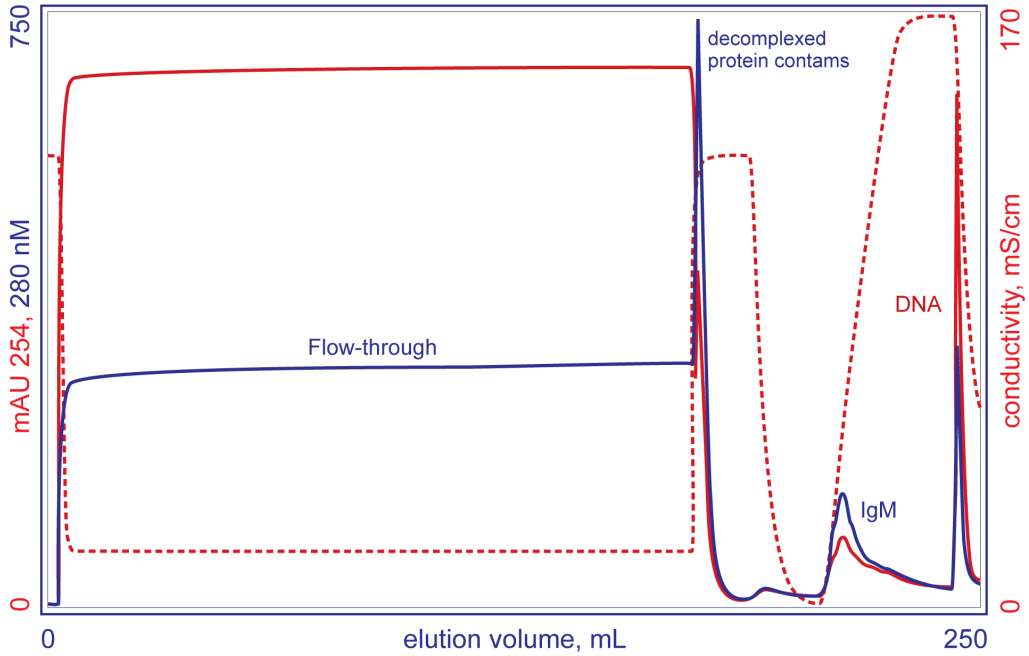
Profile 1:
EQ: A. Inject. Wash: A
Elute: 20 CVLG A > B

Profile 2:
EQ: A. Inject. Wash: A
Wash: C Wash: A
Elute: 20 CVLG A > B

Elution with phosphate in the presence of NaCl enhances DNA binding, but not enough to suspend complexation, as shown in the first profile. The 2 M NaCl wash dissociates those complexes prior to elution. This reveals that dissociating agents need not be present during elution to achieve decomplexation, but can be if they if they enhance the separation.

IgM-DNA decomplexation by HA

Simultaneous relaxation of H-bonds and charge interactions

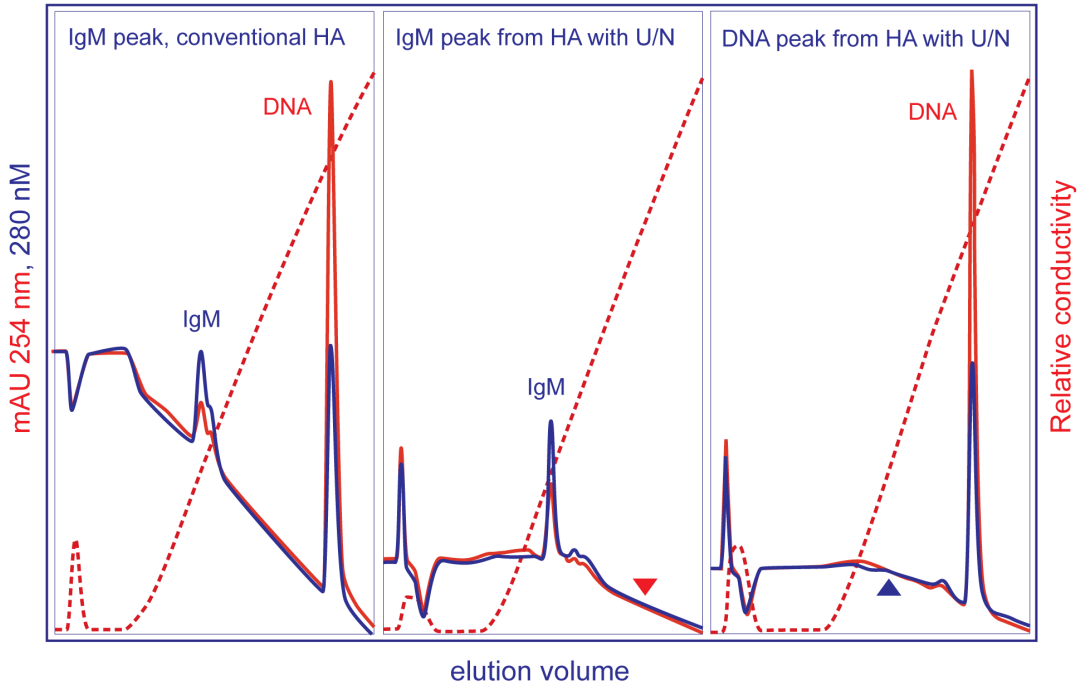


CHT type II 40 µm
5 x 50 mm, 200 cm/hr
EQ: 3.2 M urea, 1.6 M NaCl, 10 mM $NaPO_4$ pH 7
Load filtered supernatant
Wash1 with EQ buffer
Wash2 with 10 mM $NaPO_4$
4 M urea, pH 7
Elute 15CV LG to 200 mM $NaPO_4$ 2 M NaCl, pH 7
Clean: 500 mM PO_4 pH 7
Note that urea and NaCl cross during the gradient.

Compare this profile with conventional HA capture in slide 10. Here, the protein peak preceding the IgM peak is absent, and the 254/280 ratio of the elution peak suggests that DNA is either very low or absent. The high concentration of urea early in the gradient causes the IgM to elute at lower conductivity (compare with slide 19) which means that the eluate can be prepared for a subsequent ion exchange step by dilution.

IgM-DNA decomplexation by HA

Comparison of DNA contamination, w/ and w/o decomplexation

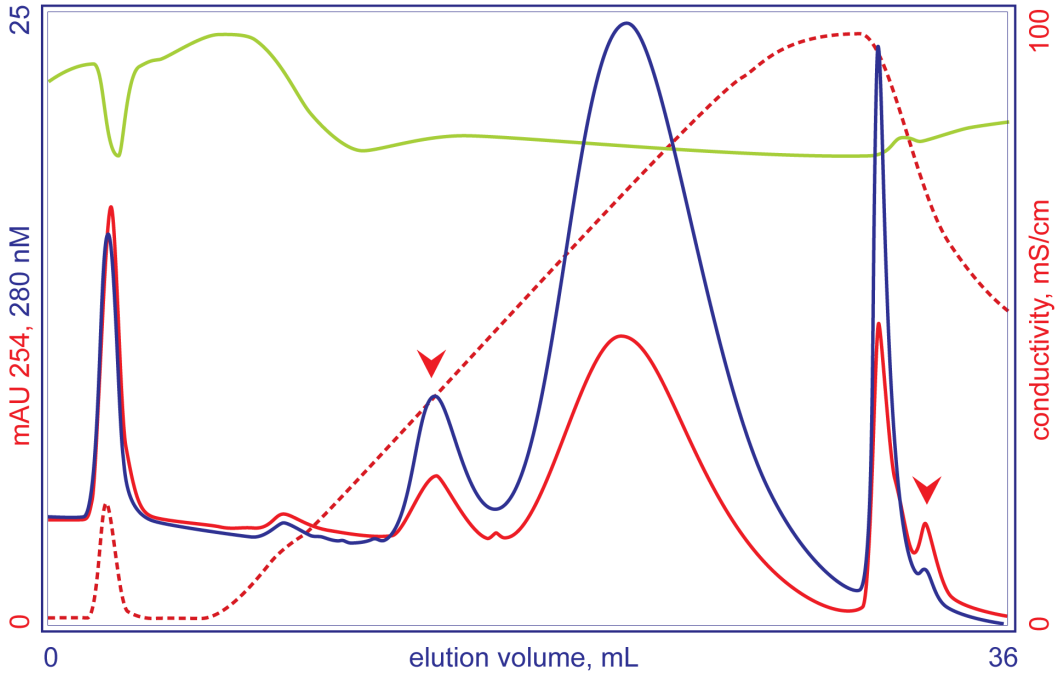


Profile 1: CIM® EDA
Profiles 2,3: CIM QA
4 mL (12 CV)/min
EQ: 20 mM Hepes, pH 7
Inject
Wash: equilibration buffer
Elute: 10 minute linear gradient to 20 mM Hepes, 1 M NaCl, pH 7 (2 M NaCl for EDA)
Red triangle: elution conductivity of DNA.
Blue triangle: elution conductivity of IgM.

Monolithic anion exchangers dissociate IgM:DNA complexes to a large degree. The IgM peak from HA eluted with a conventional phosphate gradient is heavily contaminated with DNA. Exposure to the NaCl/urea wash destabilizes complexes while HA calcium outcompetes the antibody for DNA. This leaves the IgM free of DNA — and aggregates. It also leaves the DNA free of IgM, which translates into higher product recovery (compare with slides 11,12).

IgG-DNA decomplexation by HA

Experimental control: HA eluted with NaCl at 10 mM phosphate

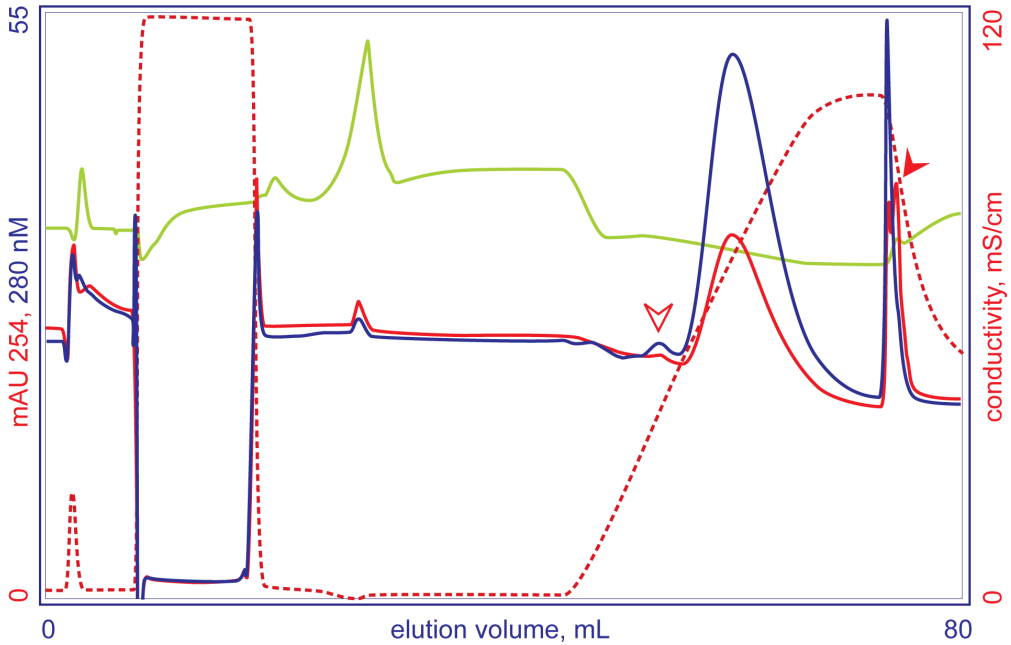


CHT type I 40 µm, 5 x 50 mm, 200 cm/hr (0.67 mL/min
EQ: 50 mM Hepes, 5 mM $CaCl_2$, pH 7
Inject: CX-purified IgG, 1010 ppm DNA
Re-EQ: 10 mM $NaPO_4$ pH 7
Elute: 20 CVLG to 10 mM PO_4 1 M NaCl, pH 7
Clean: 500 mM $NaPO_4$ pH 7
The DNA level of CX-captured IgG is 25x higher than pA w/o 2° wash; 100x pA w/ 2°wash (see slide 9).

Note the marked peak immediately before the main IgG peak. Also note the cross-over of UV absorbance at 254 and 280 nm during the phosphate cleaning step. The marked peak with higher 254 is DNA. This is consistent with data showing the ability of this elution format to remove greater than 3.5 logs of DNA – even without decomplexing washes.

IgG-DNA decomplexation by HA

The effect of a urea/NaCl wash after sample loading

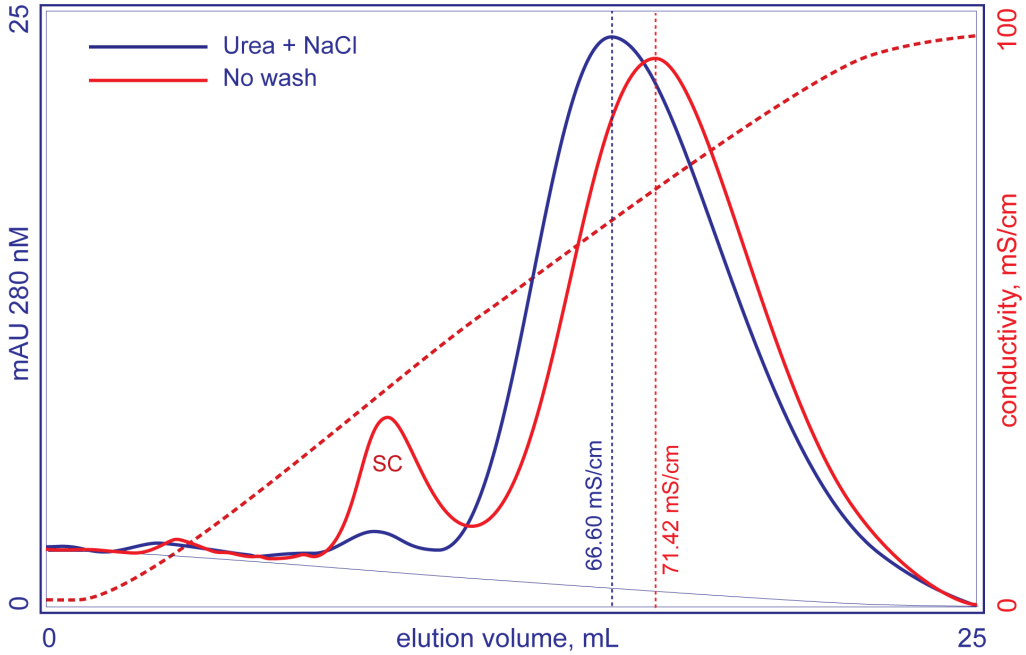


CHT type I 40 µm, 5 x 50 mm, 200 cm/hr (0.67 mL/min
EQ: 50 mM Hepes, 5 mM $CaCl_2$, pH 7
Inject CX-purified IgG, 1010 ppm DNA
Wash: 50 mM Hepes, 2 M NaCl, 4 M urea, pH 7
Re-EQ: 10 mM $NaPO_4$ pH 7
Elute: 20 CVLG to 10 mM PO_4 1 M NaCl, pH 7
Clean: 500 mM $NaPO_4$ pH 7

The initial conversion to the calcium-derivatized form of HA is so that the IgG will remain bound at NaCl concentrations that would elute IgG on underivatized HA in low-phosphate buffers. Conversion to CaHA also increases calcium density on HA and increases its DNA complex-dissociative potential. Note that the peak eluting before IgG is much smaller than in the control run, and the DNA peak much bigger.

IgG-DNA decomplexation by HA

Comparison of control with urea/NaCl wash

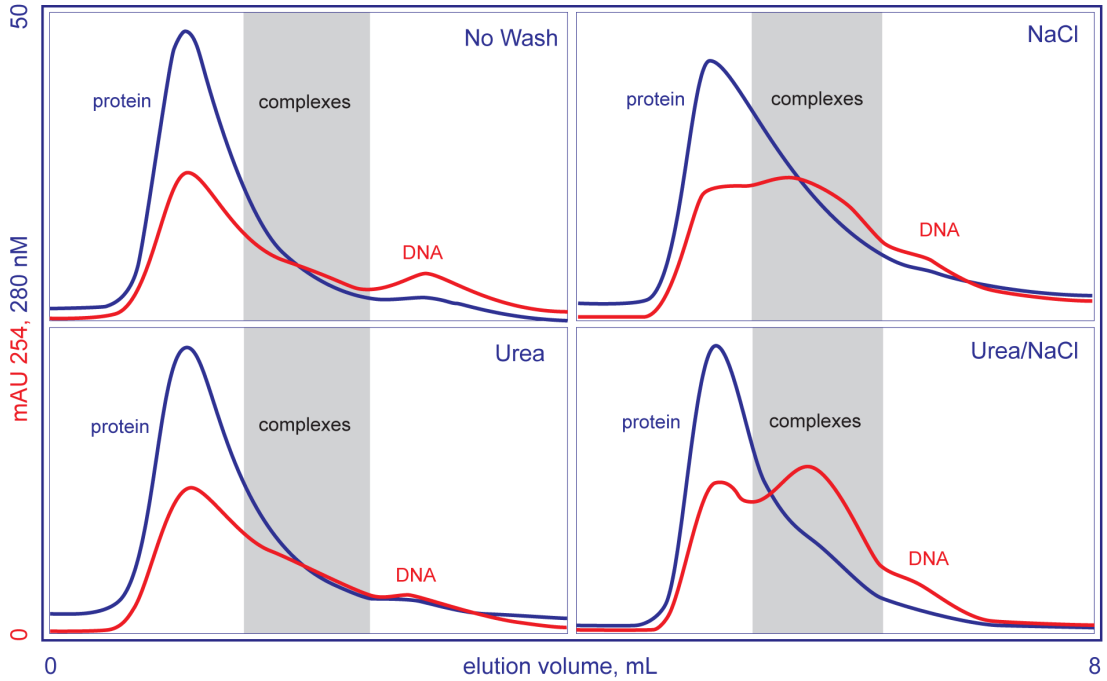


Comparison of gradient profiles from the previous two slides reveals the peak preceding the IgG in the control run to be a super-contaminant, a complex of multiple contaminant species. Dissociation into its constituents reduces the contaminant load close to the IgG and improves purification performance: recovery, resolution, reproducibility.

Note the offset in elution conductivites of the main IgG peaks. The implication is that the control sample elutes later due to interaction of IgG surface-complexed DNA to HA calcium, under conditions that are unable to entirely decomplex the DNA from the antibody. Once displaced to HA by the wash, DNA is unable to rebind to the antibody. With its native surface charge restored, the IgG elutes earlier.

IgG-DNA decomplexation by HA

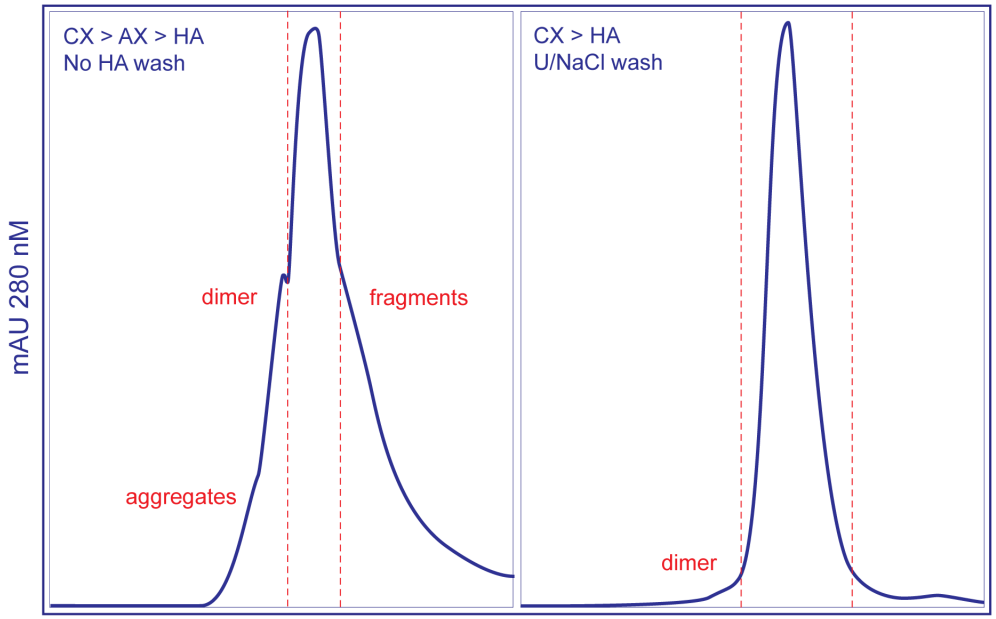
Relative DNA removal efficiency of different washes



These profiles illustrate the 500 mM phosphate cleaning steps following various washes and elution with the same NaCl gradient at 10 mM phosphate. NaCl is the stronger decomplexing agent because it disrupts complex integrity at the same time it increases affinity between DNA and HA calcium. Even though urea appears not to significantly enhance DNA removal, it contributes to dissociation of super-contaminants (not shown).

IgG-DNA decomplexation by HA

Comparison of aggregate levels, w/ and w/o decomplexation



Profile 1:
Bio-Sil™ SEC-400-5
7.8 x 300 mm, 1 mL/min
200 mM arginine, 50 mM MES, 5 mM EDTA, pH 6.5
Inj. vol. 100 µL

Profile 2:
Superdex® 200HR 10/30
0.5 mL/min
200 mM arginine, 50 mM MES, 5 mM EDTA, pH 6.5
Inj. vol. 100 µL

These profiles illustrate three important points: 1) DNA carried by IgG following capture by cation exchange can result in highly elevated aggregate and fragment levels even after usually effective purification steps, 2) Decomplexing washes at the HA step dramatically improve product quality, even with an abbreviated purification process, and 3) Decomplexation contributes to higher product recovery by restoring aggregates to monomer.

Conclusions

Hydroxyapatite with complex-dissociating washes offers unique and powerful abilities to manage complexation in purification process development.

It simplifies contaminant profiles by dissociating super-contaminants, yielding better purity, recovery, and reproducibility.

It supports DNA reduction to levels probably never attained before; likely the same for other phosphorylated contaminants like endotoxin and lipid-enveloped virus.

And it dissociates non-covalent aggregates, apparently by removing the complexants that form and maintain them. Fragmented host cell DNA seems to be the primary promoter.

Conclusions

These abilities reside with the strong coordination of acidic and phosphorylated contaminants with HA calcium. High salt enhances these affinities by suppressing electrostatic repulsion between negatively charged solutes and HA phosphate groups. Salt simultaneously disrupts complex-integrity by weakening charge interactions. HA calcium then outcompetes the antibody for DNA and prevents re-association.

Urea disrupts complex-integrity by weakening hydrogen bonds, enhancing the ability of HA calcium to dissociate complexes.

HA also binds non-calcium metal ions, eliminating metal affinity as a potential source of salt-resistant complex-stabilization.

Conclusions

Beyond reducing levels of important contaminants, complex dissociation on HA offers compelling economic benefits:

Restoration of monomer from complexes and aggregates improves product recovery in direct proportion. This becomes increasingly valuable as higher density cell cultures produce antibodies with higher aggregate levels.

Contaminant decomplexation by HA also suspends the Achilles Heel of IgG capture by cation exchange: low pH, low salt binding conditions that favor complexation. HA thereby enables cation exchange capture for a larger diversity of antibodies, with the benefit of eliminating the expensive alternative of protein A.

Endnotes

Look beneath the surface; let not the several quality of a thing nor its worth escape thee.

—Marcus Aurelius

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