Production of Biobetter IgG with Enhanced Wash and Elution of Protein A

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The challenge of biosimilars

Biosimilars need to be equivalent to established products to achieve regulatory approval:

at least equivalent therapeutic profile,

at least equivalent product quality,

at least equivalent immunogenicity.

*They need to be **better** than established products to be competitive.*

Opportunities for biobetter IgG

Simple changes in the use of protein A buffers offer:

- *Higher IgG purity*
- *Better IgG quality*
- *Lower immunogenicity*
- *Better process reproducibility*

Limitations with protein A

It has been known since the 1980s that protein A columns bind many contaminants in addition to IgG. This is inevitable, since protein A itself is essentially a hydrophobic cation exchanger.

Contaminants can also bind to the chromatography support by nonspecific interactions.

Limitations with protein A

Recent studies have shown in addition, that many contaminants co-elute with IgG because they form stable complexes with antibodies during the cell culture process, and fail to be decomplexed prior to elution of protein A.

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

Nonspecific contaminant binding

Mechanism:	***Dissociate with:***
Electrostatic interactions	*salts*
Hydrophobic interactions	*urea, arginine, guanidine*
	detergents, organic solvents
	chaotropic salts
Hydrogen bonding	*urea, arginine, guanidine*
Metal affinity interactions	*chelating agents, amino acids*

Why is metal contamination important?

A wide variety of metals are added to cell culture media used to produce monoclonal antibodies. Fe, Ca, Cu, Zn, Mg, and Mn, among others. These metals can bind to IgG during cell culture production.

The high concentration of carboxyl and histidyl residues on protein A cause it to concentrate metals from cell culture supernatant at the same time it concentrates IgG.

When IgG is eluted from protein A at low pH, metals co-elute with the IgG and create an environment that promotes the maximum degree of IgG:metal complex formation.

Why is metal contamination important?

Metal complexation can affect purification performance, product recovery, solubility, stability, pharmacokinetics, pharmacodynamics, and immunogenicity.

If metal complexation is not controlled as part of a purification process, it is certain to cause uncontrolled process variation, and uncontrolled variation of product quality.

Pharmacodynamics refers to drug physiological effect, mechanism of action.
Pharmacokinetics refers to duration of drug effect, clearance time/pathway.

Why is metal contamination important?

Metal:protein coordination bonds can survive a wide range of pH values, and salt concentrations over 5.0 M NaCl.

Thus the conditions to dissociate bound metals may not occur in the normal course of affinity, ion exchange, or hydrophobic interaction chromatography.

This can result in complexed metals being carried through an entire purification process.

Why is metal contamination important?

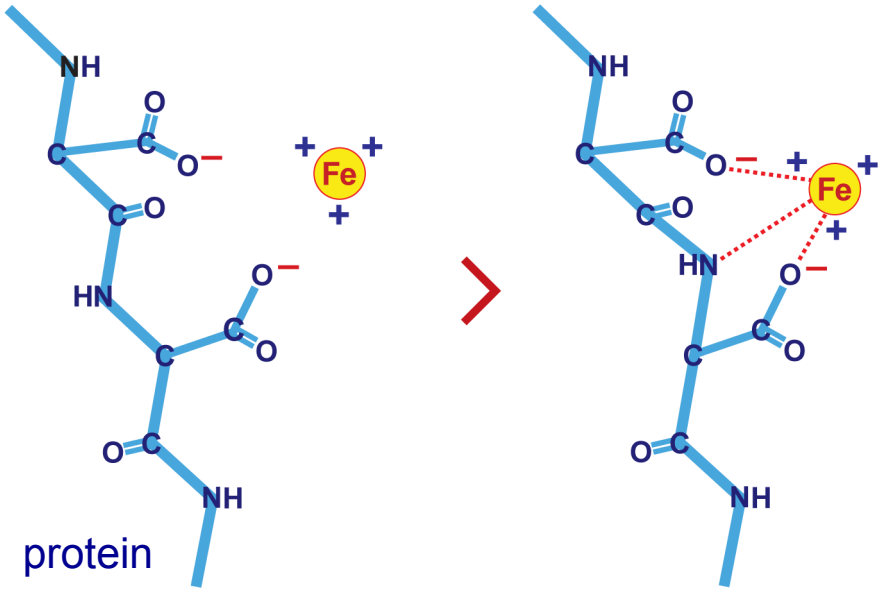
In addition to altering antibody charge and hydrophobicity, metals can cause local conformational distortions that create novel antigenic domains.

Metals also have the potential to form secondary complexes between IgG and a wide range of contaminants, including but not limited to metalloproteases, DNA, endotoxin, and virus.

Besides reducing IgG purity and elevating the risk of proteolysis, these contaminants can act as adjuvants, and increase product immunogenicity.

Metal modification of protein surfaces

Protein charge modification

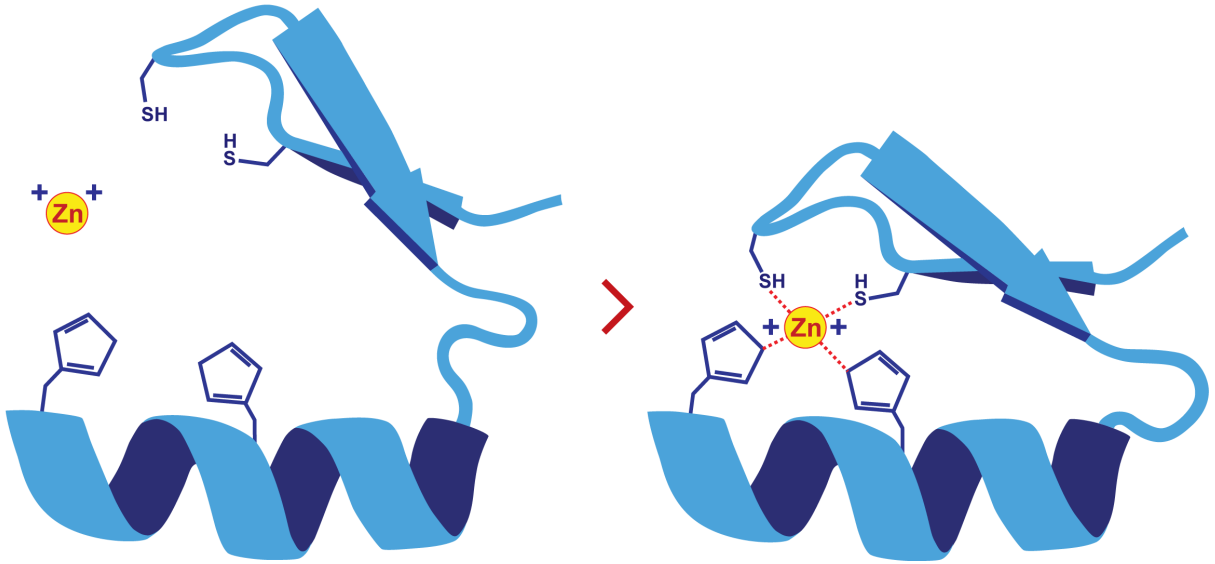


Protein charge shifts from 2- to 1+ at this one complexation site, a change of 3 charge units. With divalent metals, the charge shift would be 2 units, from 2- to zero. If multiple sites become complexed, each will shift protein charge by the same increment. Coordinated water molecules not shown.

For more information, see Metal Coordination Sites in Proteins: http://tanna.bch.ed.ac.uk/

Metal modification of protein surfaces

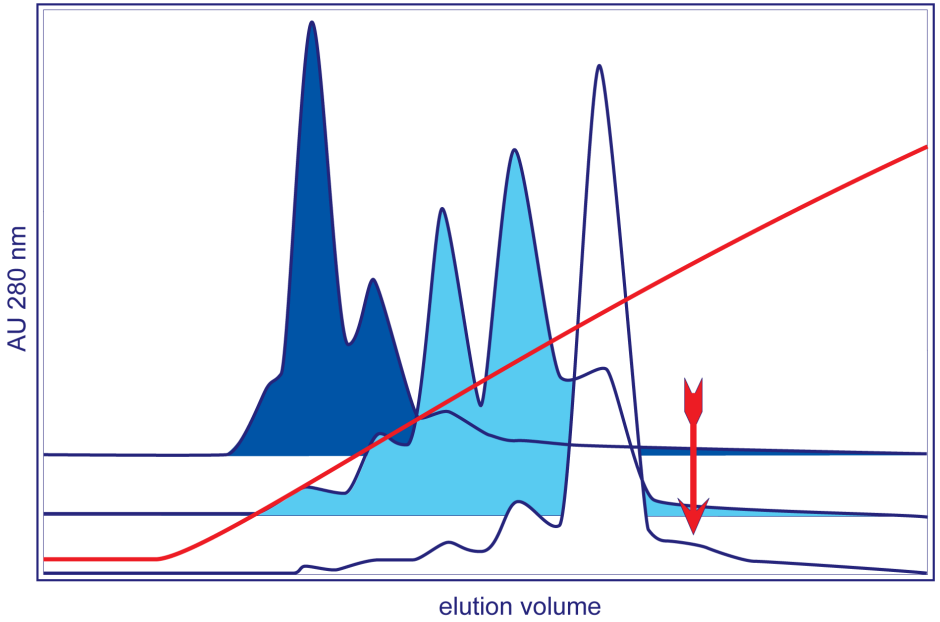
Protein charge modification and conformational change



In this case, zinc forms coordination bonds with histidine and cysteine, which are uncharged at neutral to alkaline pH. Protein charge increases from zero to 2+. Protein conformation is also strongly affected. This figure makes the important point that coordination bonds are charge independent. Overall however, metal interactions may involve electrostatic interactions and hydrogen bonding (via coordinated water molecules) in addition to coordination bonds.

Metal modification of protein surfaces

Metal complexation affects IgG retention



Monoclonal mouse IgG_1

White: Dialyzed in EDTA

Lt. blue: + 1 mM $ZnCl_2$

Dk. Blue: + 5 mM $ZnCl_2$

Anion exchange

Mono-Q™ HR 5/5

A: 50 mM Tris, pH 8

B: A + 125 mM NaCl

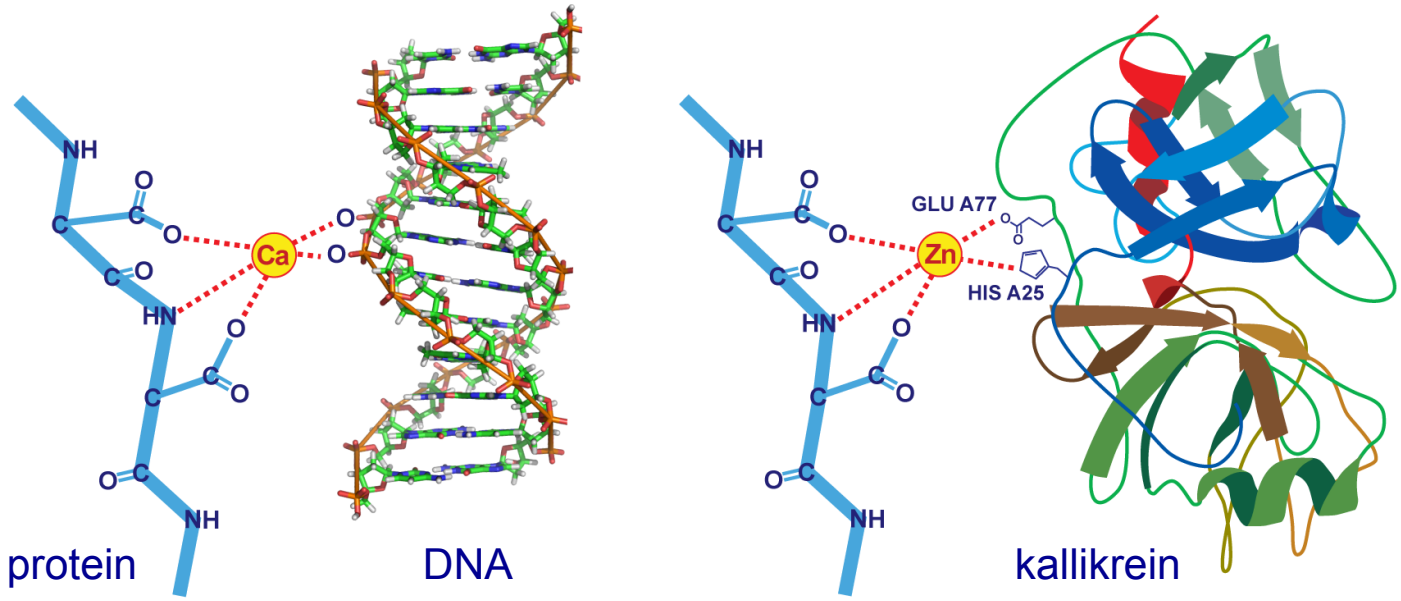
Elute: 20 CVLG, A > B

Arrow: apo-metallic form

The earliest peak represents the most highly complexed form. Complexation is seldom this dramatic but the pattern is always the same. Due to their higher net-positive charge, metal-complexed forms are retained more weakly on anion exchangers; more strongly on cation exchangers. Metal complexed forms bind more strongly to HIC columns than native proteins. Hydroxyapatite removes non-calcium metals from proteins by competitive displacement.

Metal modification of protein surfaces

Metal-mediated IgG complexation with other contaminants



Calcium and iron are abundant in cell culture media. Both can form stable chelation complexes with protein carboxy clusters, and simultaneous coordination complexes with DNA phosphates (or with endotoxin or virus envelope phosphates). Kallikrein is one of the serine proteases, all of which are metalloproteins. Coordinated water not shown.

DNA image modified from Wikipedia. Kallikrein redrawn from RCSB Protein Data Bank www.rcsb.org/pdb/

Evolution of protein A wash buffers

*1980: **PBS**: 20-50 mM phosphate, 100-150 mM NaCl, pH 7.0-7.2*

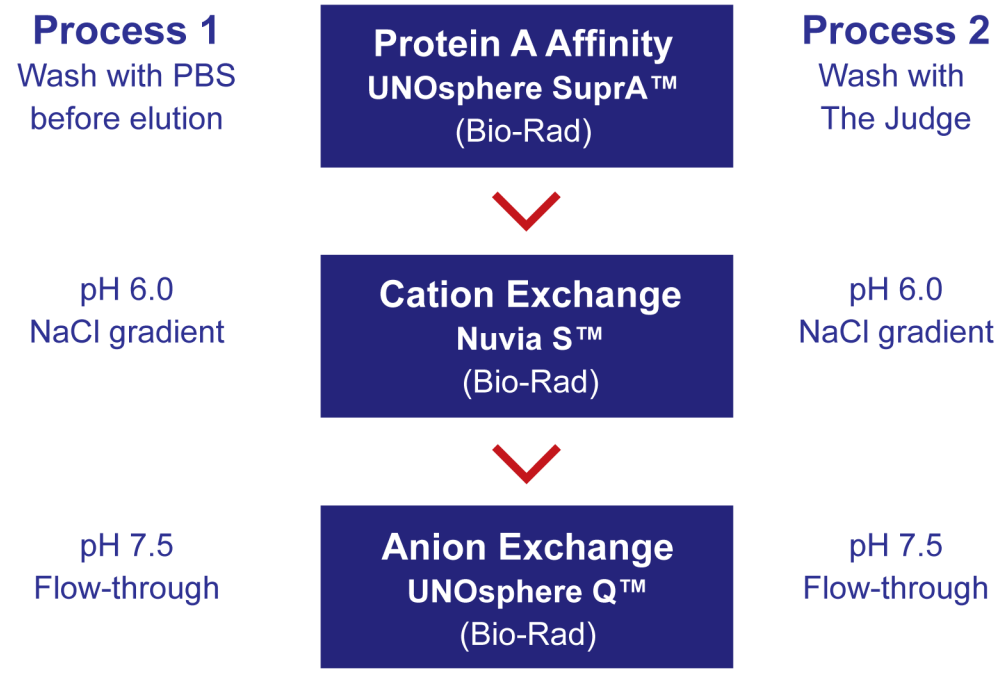
*2004: **The Kitchen Sink**: 1 M NaCl, 5-10 mM EDTA, 2 M urea, 50 mM phosphate, pH 7.0-7.2*

*2007: **The Judge**: 1.5 M NaCl, 5-10 mM EDTA, 2 M urea, 50 mM histidine or phosphate, pH 6.5 to 7.0*

*2010: Coming soon…**The Terminator***

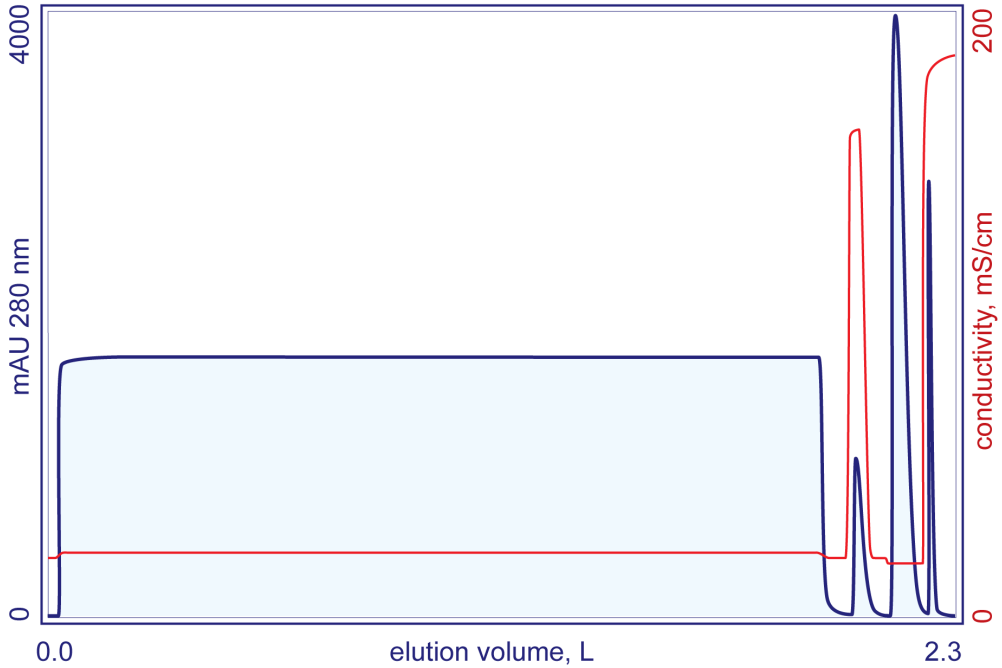
Effects of protein A wash buffers

Lab scale process comparison



Effects of protein A wash buffers

Protein A elution profile



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: The Judge

Wash3: Hepes saline, pH 7

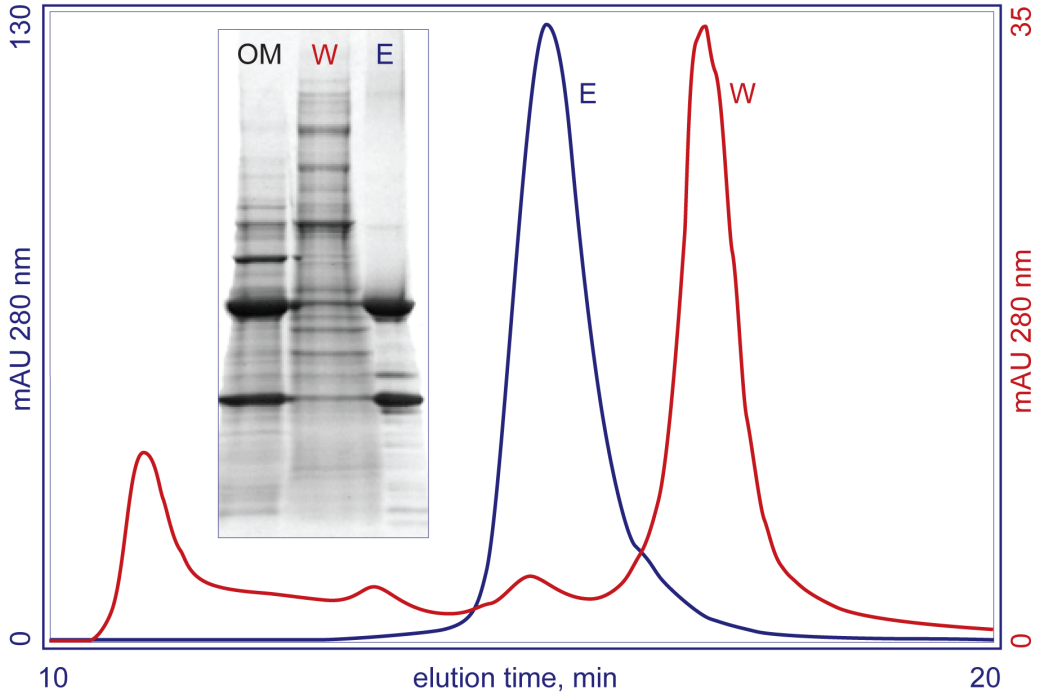
Elute: 100 mM arginine, 100 mM acetate, pH 3.8

Clean: 2 M guanidine, pH 5

The size of the secondary wash peak is fairly consistent among lots of a given feed stream, 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 point that the degree of cell death is an important purification process variable, and that secondary washes can suspend this source of variation.

Effects of protein A wash buffers

PAGE and HPSEC of protein A secondary wash and elution



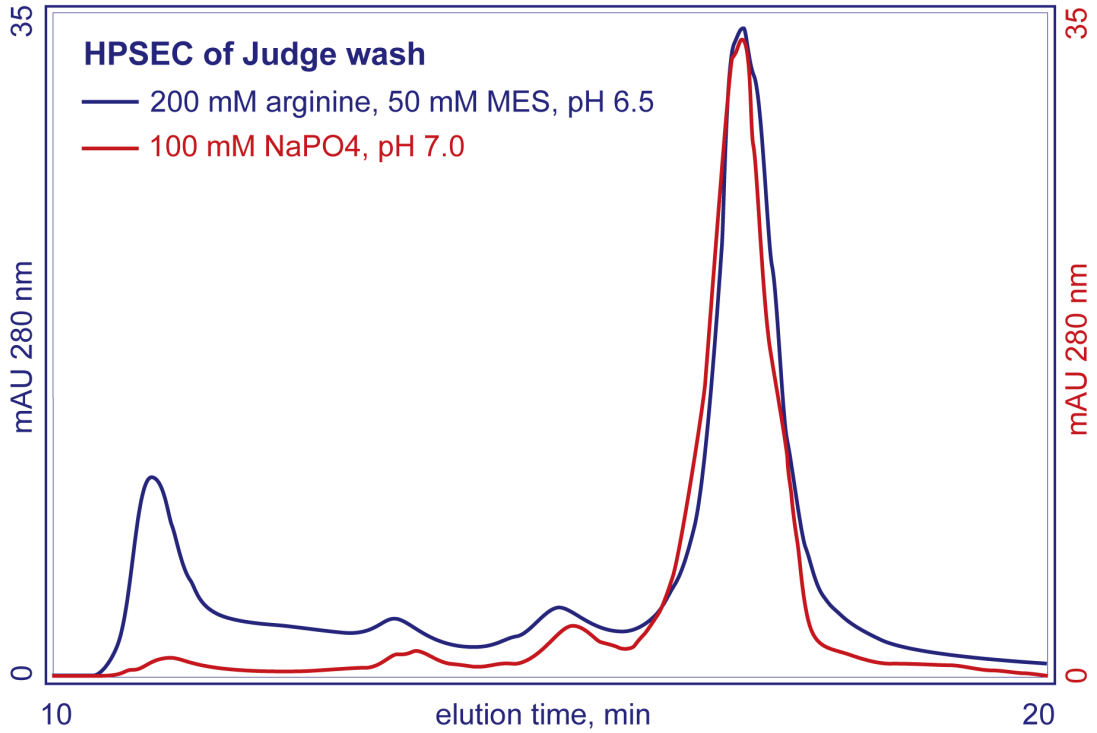
Reduced SDS PAGE
Criterion™ 4-20% gradient
Stain: Colloidal coomassie
OM: original material
W: Judge wash
E: elution

HPSEC
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: elution
W: Judge wash

Secondary wash peaks are usually turbid, from the presence of aggregates and precipitates. They are often discolored as well (gray/yellow–brown). In the absence of a secondary wash, these contaminants co-elute with the antibody, and place a burden on later purification steps. Note the small amount of IgG in the wash (<5%).

Effects of protein A wash buffers

Decomplexing buffers are important for HPSEC too!



Contaminants that bind non-specifically to protein A columns are also likely to bind non-specifically to SEC columns. Phosphate buffer is not able to fully suspend these interactions. This causes aggregate levels to be under-estimated.

The Critical Role of Mobile Phase Composition in Size Exclusion Chromatography of Protein Pharmaceuticals, T. Arakawa, D. Ejima, T. Li, J. Philo, J. Pharm. Sci., 99(4), 2009, 1674-1692

Effects of protein A wash buffers

Benefits of the Secondary Wash: ***ChoP****, ng/mg IgG (ppm)*

	w/o 2°	*w/ 2°*	*improvement*
PA wash	*------*	*9,976.0*	*------*
PA eluate	*18.3*	*10.5*	*74%*
CX eluate	*1.9*	*0.8*	*238%*
AX eluate	*1.3*	*1.1*	*18%*

Effects of protein A wash buffers

*Benefits of the Secondary Wash: **DNA**, ng/mg IgG (ppm)*

	w/o 2°	*w/ 2°*	*improvement*
PA wash	*------*	*1,556.2*	*------*
PA eluate	*31.9*	*10.6*	*301%*
CX eluate	*32.8*	*10.9*	*301%*
AX eluate	*0.8*	*0.7*	*14%*

Effects of protein A wash buffers

Benefits of the Secondary Wash: ***leached protein A*** *(ppm)*

	w/o 2°	*w/ 2°*	*improvement*
PA wash	*------*	*12.0*	*------*
PA eluate	*4.8*	*1.2*	*400%*
CX eluate	*1.3*	*1.0*	*30%*
AX eluate	*0.3*	*0.1*	*300%*

The high concentration of leached protein A in the secondary wash suggests why a small percentage of IgG is lost at this step: it probably represents IgG that has leached protein A fragments bound on both sides of the Fc, which therefore cannot bind to available immobilized ligand.

Protein A elution buffers

"Traditional" protein A elution buffers include agents such as citrate or glycine, usually at pH values ranging from 3.0 to 3.5, most commonly 3.2-3.3.

*Exposure to low pH is documented to alter the conformation of IgG. This is part of the mechanism of protein A affinity elution. Low pH alters antibody structure to the point that it disables binding.**

**Mutual charge repellency between opposing histidine triplets in the binding site makes a contribution to dissociation of protein A and IgG at acidic pH, but this is insufficient by itself to accomplish elution.*

Protein A elution buffers

The danger of inducing conformational changes is that they may not be fully reversible. Thus the eluted IgG population may contain subpopulations of mis-folded forms.

Misfolded forms represent antigenically novel structures that may enhance immunogenicity.

Misfolds can also act as nucleation centers for aggregate formation. Aggregates are well known to promote formation of therapy-neutralizing antibodies. The need for aggregate removal burdens later purification steps.

Aggregate detection and removal from biopharmaceutical proteins, P. Gagnon and T. Arakawa, eds. Current Pharmaceutical Biotechnology, 2009, 10(4) (16 articles).
Effects of protein aggregates: an immunological perspective, A. Rosenburg (CEDER), The AAPS Journal 2006; 8 (3) Article 59 (http://www.aapsj.org).

Protein A elution buffers

Another symptom of conformationally modified IgG with traditional elution methods is the frequent observation of turbidity or precipitation when the pH of eluted antibody is raised into the range of 6-7.

This rarely occurs with IgG purified by non-affinity methods.

Enhanced elution of protein A

Arginine

Relaxes hydrogen bonds, hydrophobic interactions, and electrostatic interactions.

USP. FDA approved injectable.

Used extensively up to 600 mM for refolding of recombinant proteins produced in inclusion bodies.

Enhanced elution of protein A

Arginine

One frequently used formulation: 100 mM arginine, 100 mM acetate, pH 3.8. Antibodies with very high isoelectric points may require addition of NaCl to overcome cation exchange interactions with protein A.

These milder conditions eliminate the problem of antibody precipitation upon pH neutralization. They also reduce aggregate levels by an average of 1-2%.

Arakawa, T., Philo, J.S., Tsumoto, K., Yumioka, R. and Ejima, D. (2004) Elution of antibodies from a Protein-A column by aqueous arginine solutions. Pro. Purif. Exp. 36, 244-248.

Ejima, D., Yumioka, R., Tsumoto, K., and Arakawa, T. (2005) Effective elution of antibodies by arginine and arginine derivatives in affinity chromatography. Anal. Biochem., 345, 250-257.

Arakawa, T., Kita, Y., Tsumoto, K., Ejima, D., and Fukada, H. (2006) Aggregation suppression of proteins by arginine during thermal unfolding. Protein Pept. Lett., 13, 921-927.

Enhanced elution of protein A

Virus inactivation at pH 3.8 requires 45-60 minutes.

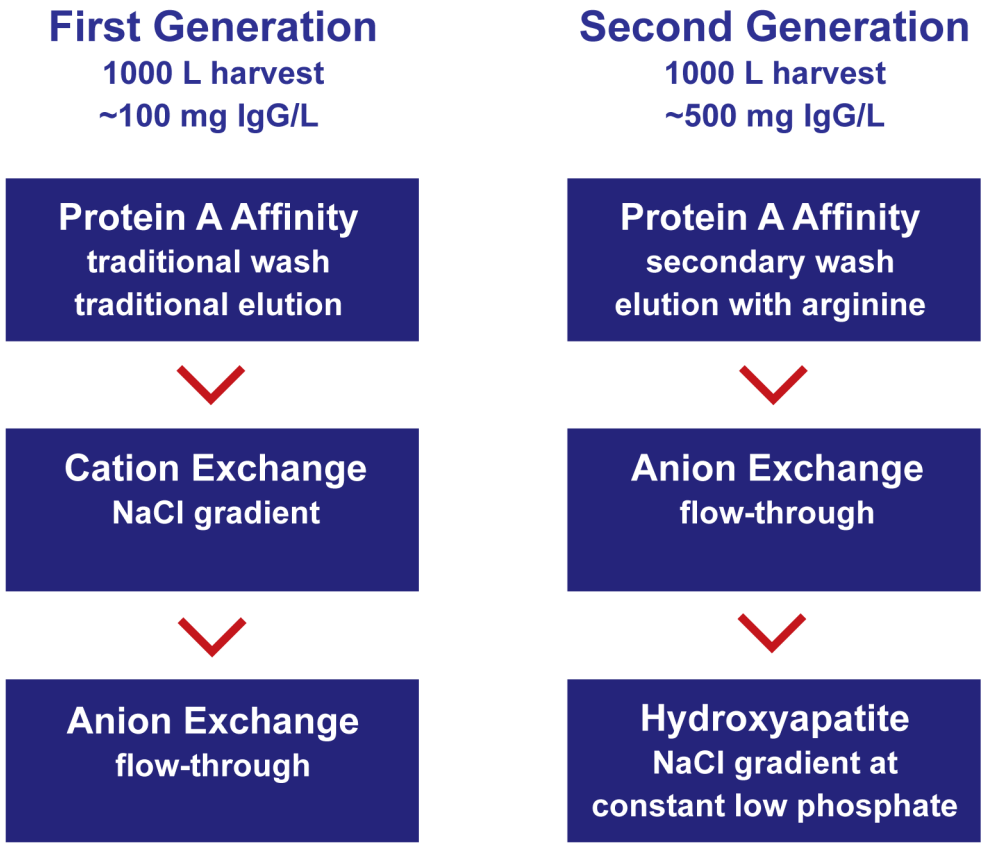
Arginine itself is antiviral, thereby enhancing inactivation.

Synergistic virus inactivation effects of arginine: Arakawa, T., Kita, Y., and Koyama, A.H. Biotechnol. J. 4, 174-178, 2009.

Co-operative thermal inactivation of herpes simplex virus and influenza virus by arginine and NaCl: Utsunomiya, H., Ichinose, M., Tsujimoto, K., Katsuyama, Y., Yamasaki, H., Koyama, A.H,, Ejima, D., and Arakawa, T. Int. J. Pharm. 366, 99-102, 2009.

Arginine facilitates inactivation of enveloped viruses: Yamasaki, H., Tsujimoto, K., Koyama, A.H., Ejima, D., and Arakawa, T. J. Pharm. Sci. 97, 3067-3073, 2008.

Clinical Process Comparison



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Clinical Process Comparison

Second generation, process summary (initial volume 1029 L)

Step	End vol. (L)	IgG (g)	Recovery (%)
Protein A	38	438	
Anion exchange	41	439	100
Hydroxyapatite	158	428	98
Difiltration	14	397	93
Virus filtration	16	386	98
Overall			**79**

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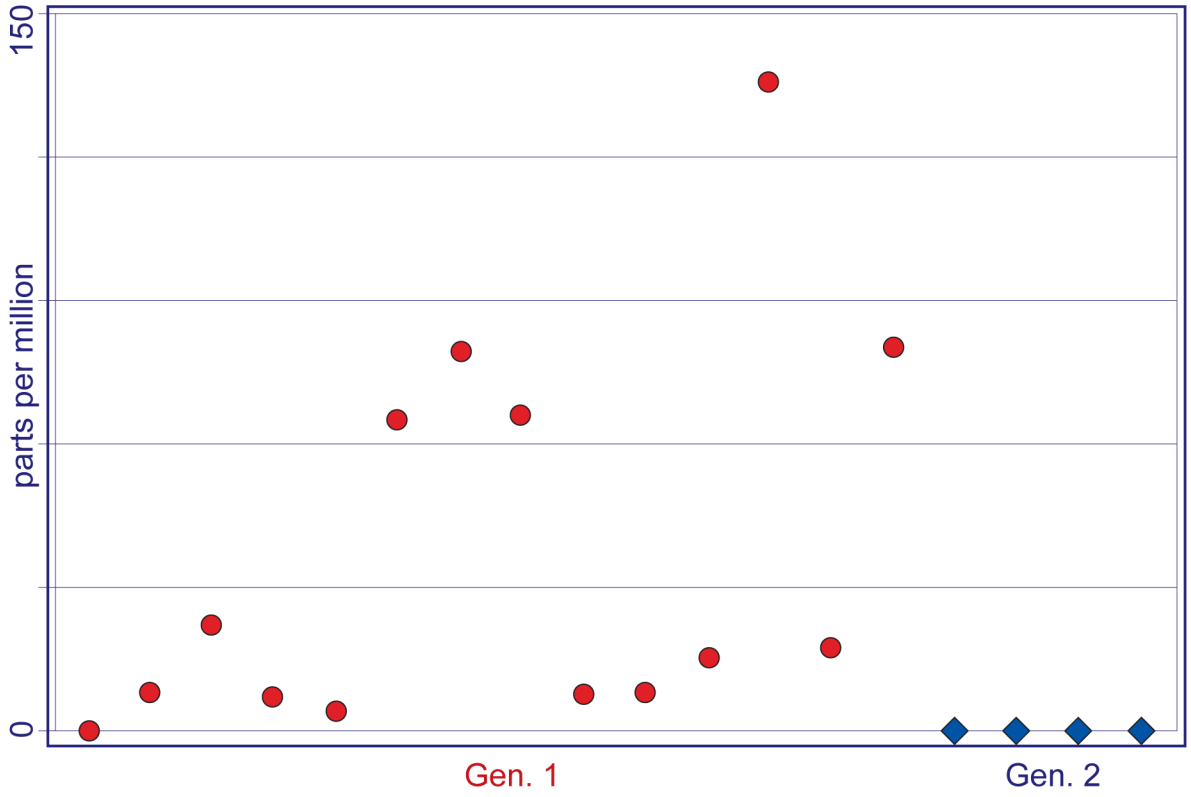
Clinical Process Comparison

Leached protein A content



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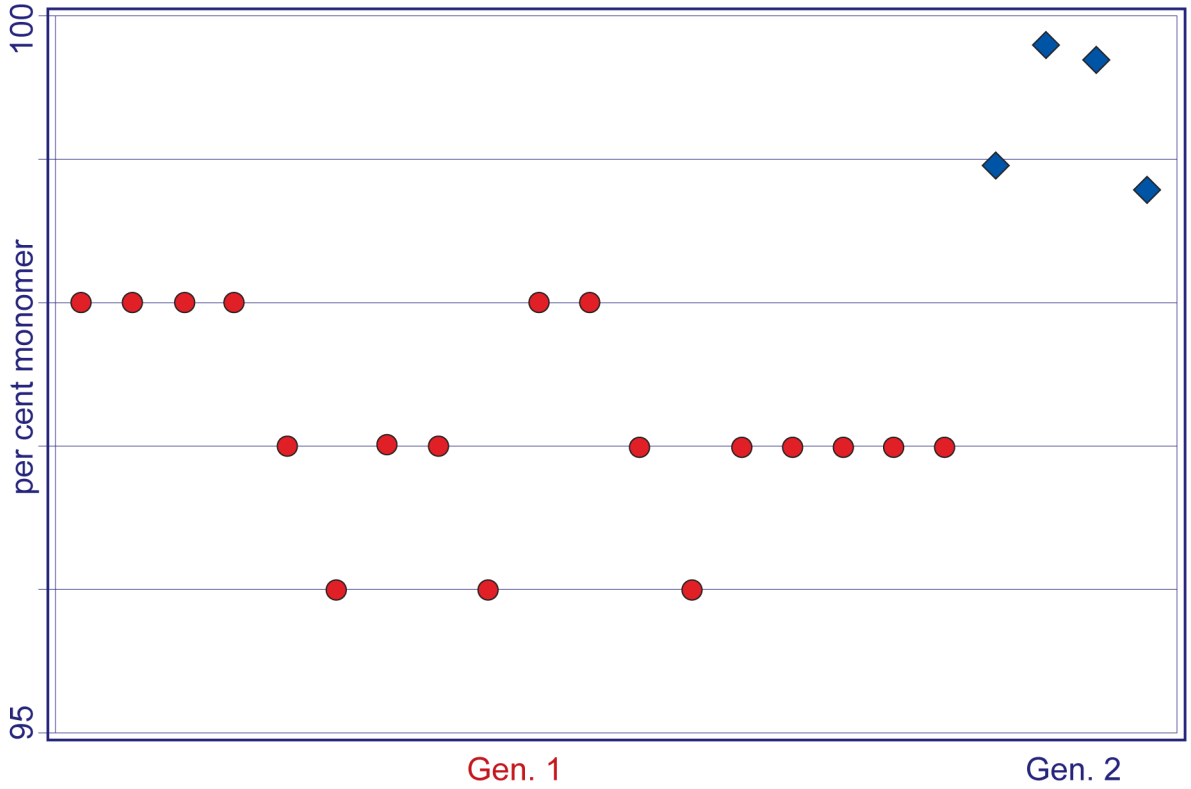
Clinical Process Comparison

Monomer content



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Clinical Process Comparison

Second generation purification, ChoP and particulates

ChoP, ppm	Lot 1	Lot 2	Lot 3	Lot 4
	< 1	< 1	< 1	< 1

Particulates per vial of formulated product*

>10 µm	10	23	28	35
>25 µm	1	8	5	2

**USP particulate target values: fewer than 6,000 particles > 10 µm and fewer than 600 particles >25 µm*

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Summary

Metals are potentially the most dangerous of all contaminant classes because they can affect process performance and product quality in so many ways —even more because they are so easy to overlook.

At best, they represent a source of uncontrolled variation in the purification process, and in product quality.

At worst, they have potential to interfere directly with the therapeutic action of an antibody, and to elevate patient immune response against it.

Summary

Secondary washes at the protein A step measurably improve the performance of every downstream process step, ensuring the highest purity of IgG.

This makes the entire purification process more robust, ensuring the highest level of process reproducibility and product quality.

Lower contaminant loads may also increase the loading capacity of intermediate and final purification steps, reducing column volume, buffer volume, eluted product volume, and process time, all of which can improve process economics.

Summary

Perhaps most important of all, the combination of secondary washes and improved elution conditions offers significant potential to reduce product immunogenicity;

by producing IgG that is conformationally more native,

by producing IgG with lower aggregate content,

by producing IgG with a lower nonspecific adjuvant load.

Better purity, better reproducibility, and lower immunogenicity all translate to safer more effective use, and maximize the probability that a given biobetter will be approved and compete successfully in the market.

Acknowledgements

Very special thanks to Avid BioServices of Tustin, California for making Phase I process and product data available from more than twenty 1000 L lots of cell culture supernatant, to illustrate the benefits of protein A method improvements.

Copies of this presentation can be downloaded at www.validated.com