Dissection of the Separation Mechanisms and Enhancement of Aggregate Removal by Charged-Hydrophobic Mixed-Mode Chromatography

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Validated Biosystems

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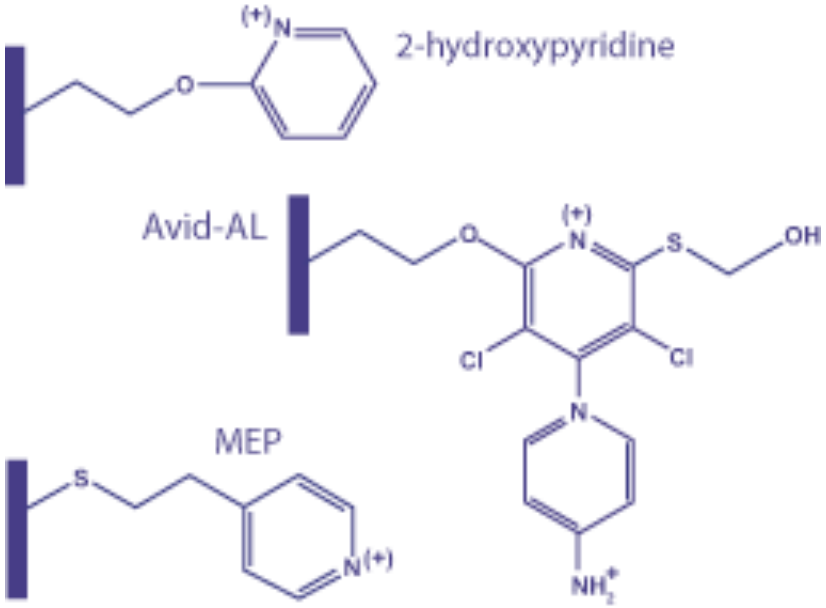
Charged-hydrophobic mixed-modes

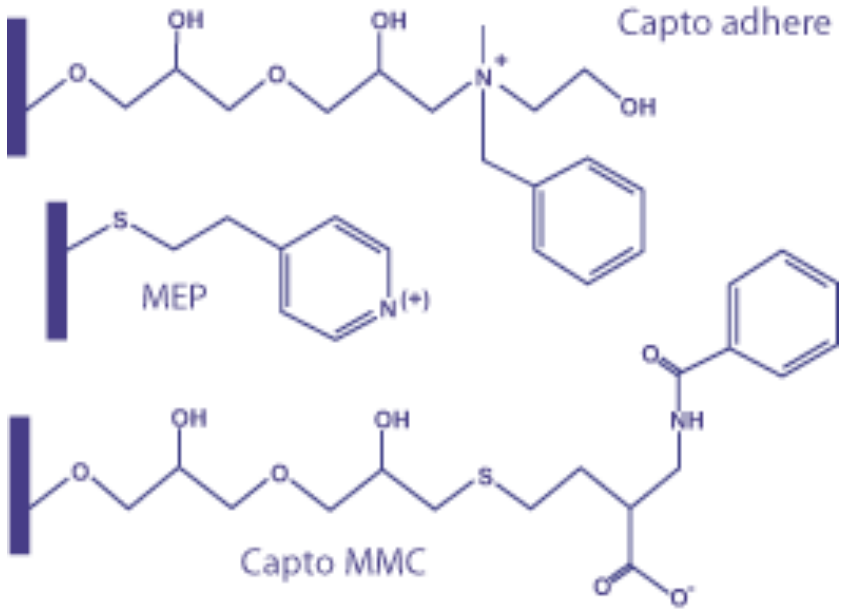
Charged-hydrophobic mixed-modes were introduced in the early 1990s, primarily as alternatives to bioaffinity chromatography for capture-purification of IgG monoclonal antibodies.



Sulfur or oxygen moieties in the spacer arms relate to thiophilic chromatography theory, which suggests that IgG binding is enhanced by their presence.

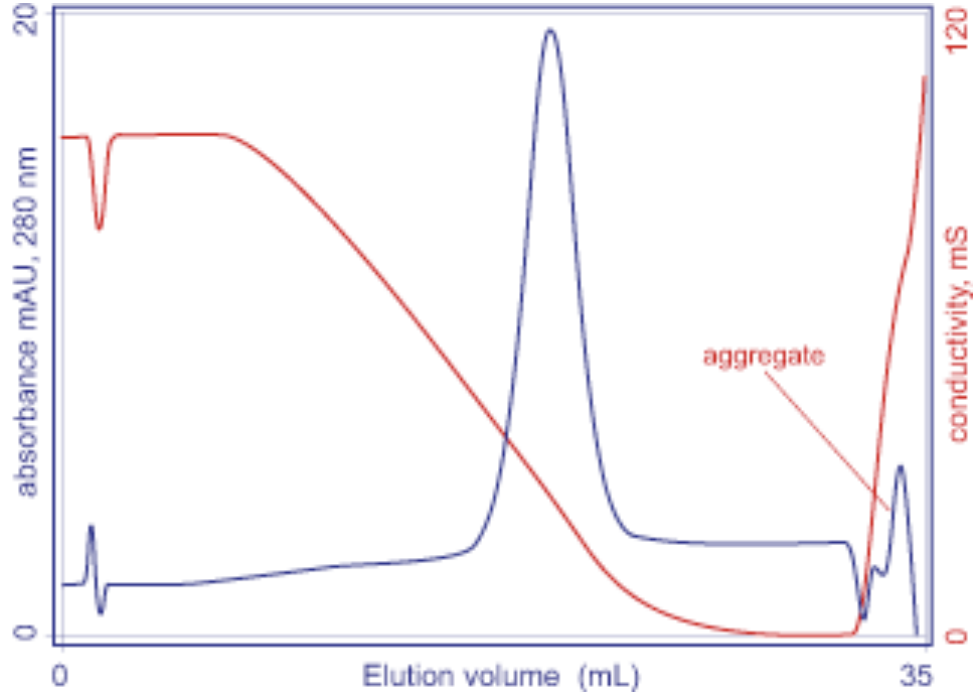
Charged-hydrophobic mixed modes

More recent introductions have focused on polishing applications, particularly for aggregate removal from monoclonal IgG.



Protein:mixed-mode interactions

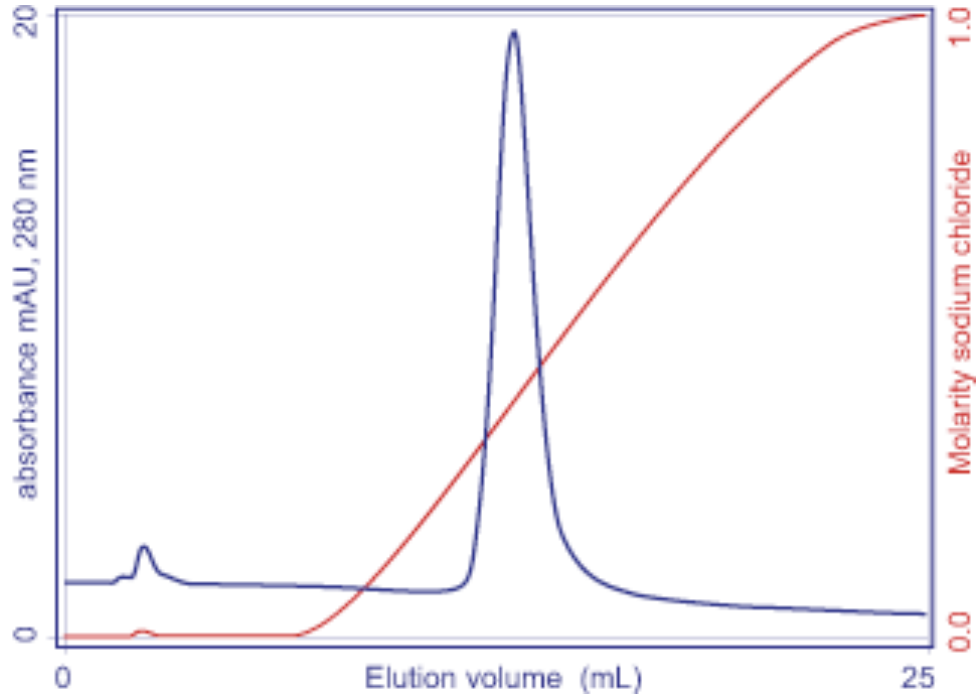
Hydrophobic binding. Descending salt gradient elution.
Positively charged hydrophobic mixed-mode.



Capto adhere™, 1 mL HiTrap
1 mL/min
A: 50 mM MES, 1 M NaCl, pH 5.5
B: 50 mM MES, pH 5.5
C: 6 M guanidine, pH 5
Equilibrate: A
Inj: 50 µL protein A purified hIgG$_1$ Mab
Wash: A
Elute: 20 CV linear gradient to B
Clean: C

Protein:mixed-mode interactions

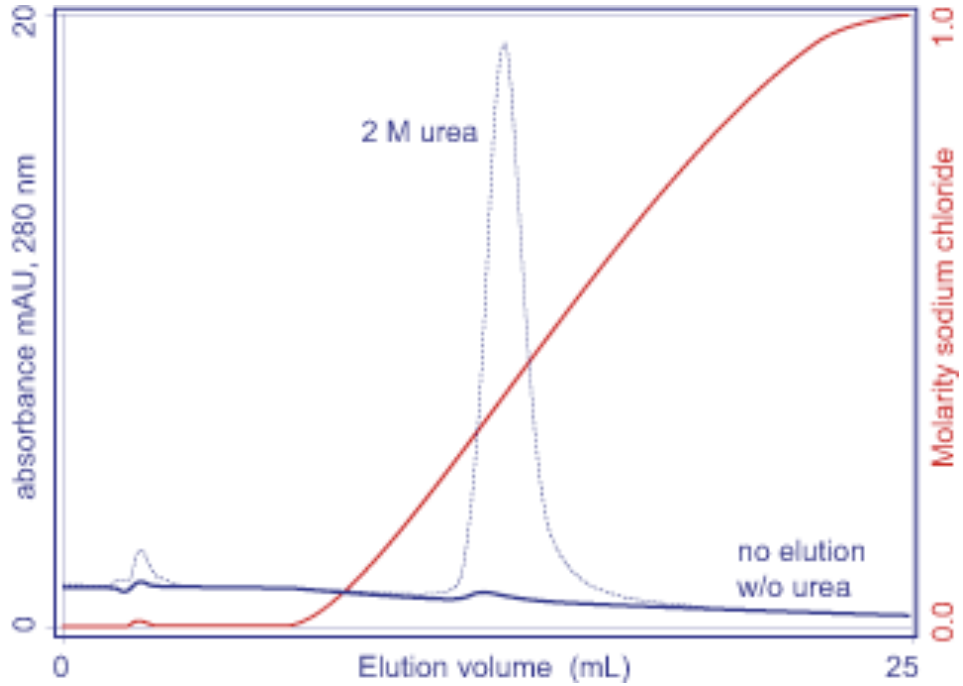
Electrostatic binding. Ascending salt gradient elution.
Positively charged hydrophobic mixed-mode.



Capto adhere, 1 mL HiTrap
1 mL/min
A: 50 mM Tris, 2 M urea, pH 8.5
B: 50 mM Tris, 2 M urea, 1 M NaCl pH 8.5
C: 6 M guanidine, pH 5
Equilibrate: A
Inj: 50 µL protein A purified hIgG$_1$ Mab
Wash: A
Elute: 20 CV linear gradient to B
Clean: C

Protein:mixed-mode interactions

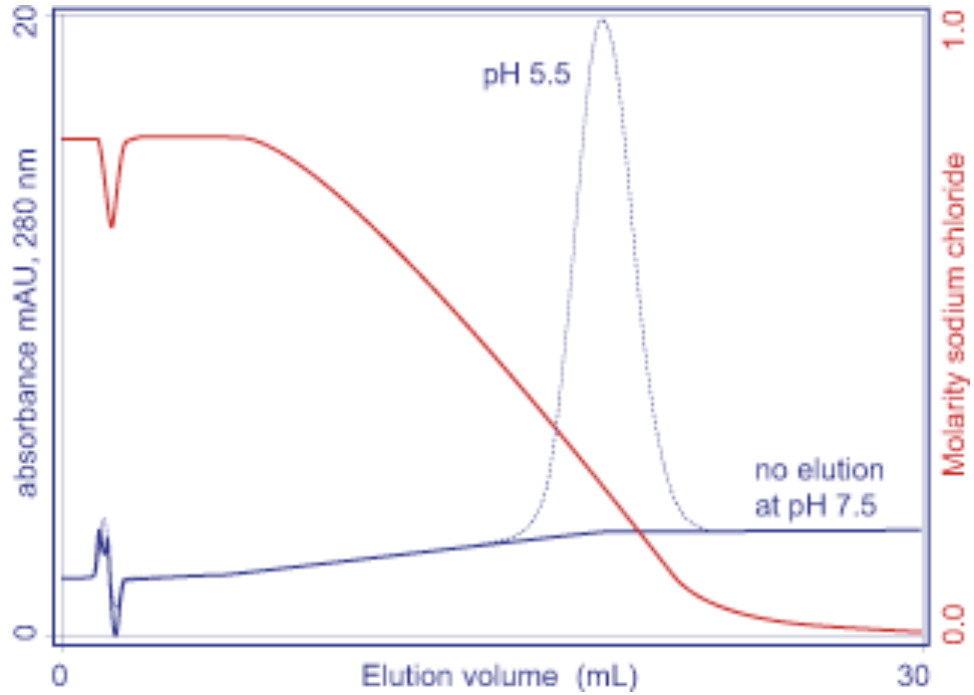
Cooperativity of hydrophobic and electrostatic binding. Ascending salt gradient elution. Positively charged hydrophobic mixed-mode.



Capto adhere, 1 mL HiTrap
1 mL/min
A1: 50 mM Tris, pH 8.5
A2: 50 mM Tris, 2 M urea, pH 8.5
B1: 50 mM Tris, 1 M NaCl, pH 8.5
B2: 50 mM Tris, 2 M urea, 1 M NaCl pH 8.5
C: 6 M guanidine, pH 5
Equilibrate: A
Inj: 50 µL protein A purified hIgG$_1$ Mab
Wash: A
Elute: 20 CV linear gradient to B
Clean: C
These results illustrate the salt tolerance of mixed-mode binding.

Protein:mixed-mode interactions

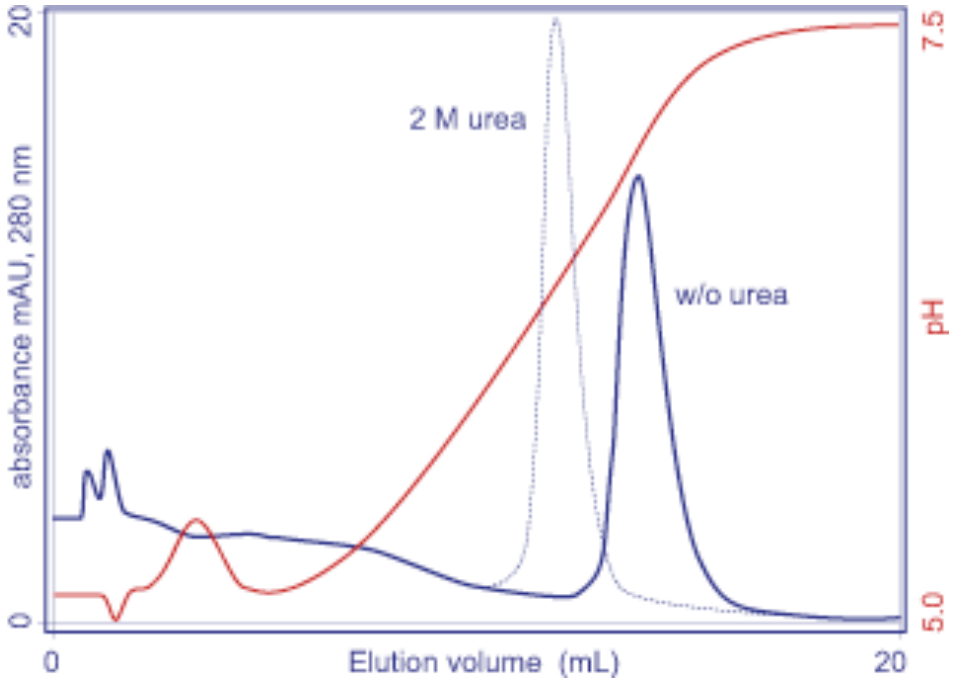
Cooperativity of hydrophobic and electrostatic interactions. Descending salt gradient elution. Positively charged hydrophobic mixed-mode.



Capto adhere, 1 mL HiTrap
1 mL/min
A1: 50 mM MES, 1 M NaCl, pH 5.5
A2: 50 mM Hepes, 1 M NaCl, pH 7.5
B1: 50 mM MES, pH 5.5
B2: 50 mM Hepes, pH 7.5
C: 6 M guanidine, pH 5
Equilibrate: A
Inj: 50 µL protein A purified hIgG$_1$ Mab
Wash: A
Elute: 20 CV linear gradient to B
Clean: C
Antibody pI is about 8.3. Elution at pH 5.5 but failure to elute at 7.5 demonstrates the contribution of electrostatic repulsion.

Protein:mixed-mode interactions

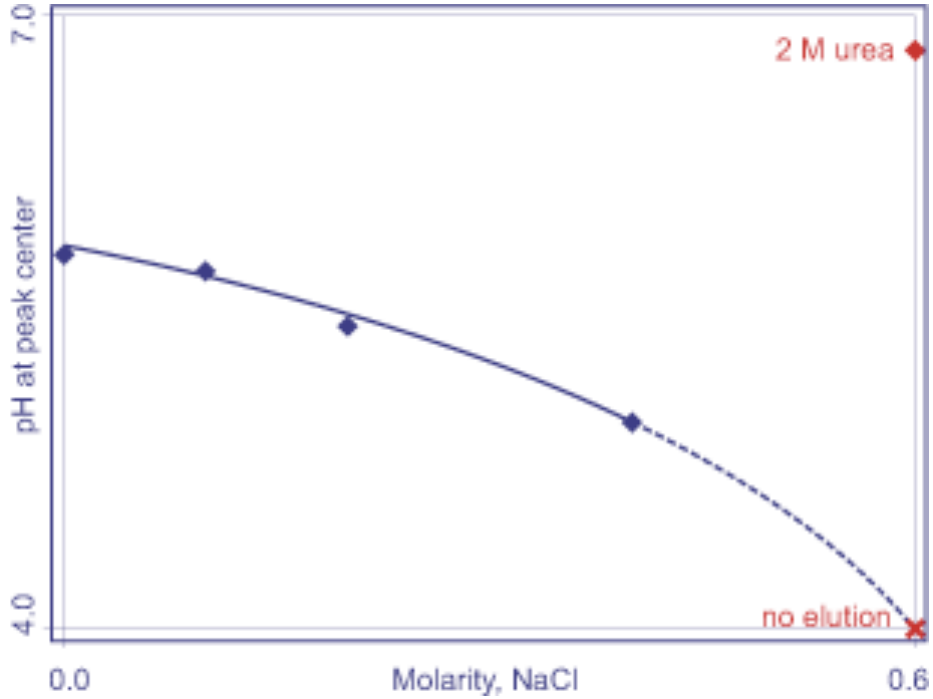
Cooperativity of hydrophobic and electrostatic interactions. Increasing pH gradient elution. Negatively charged hydrophobic mixed-mode.



Capto MMC, 1 mL HiTrap
1 mL/min
A1: 10 mM Na citrate, 10 mM Na phosphate, pH 5.0
A2: 10 mM Na citrate, 10 mM Na phosphate, 2 M urea, pH 5.0
B1: 10 mM Na citrate, 10 mM Na phosphate, pH 7.5
B2: 10 mM Na citrate, 10 mM Na phosphate, 2 M urea, pH 7.5
C: 6 M guanidine, pH 5
Equilibrate: A
Inj: 50 µL protein A purified hIgG$_1$ Mab
Wash: A
Elute: 20 CV linear gradient to B
Clean: C

Protein:mixed-mode interactions

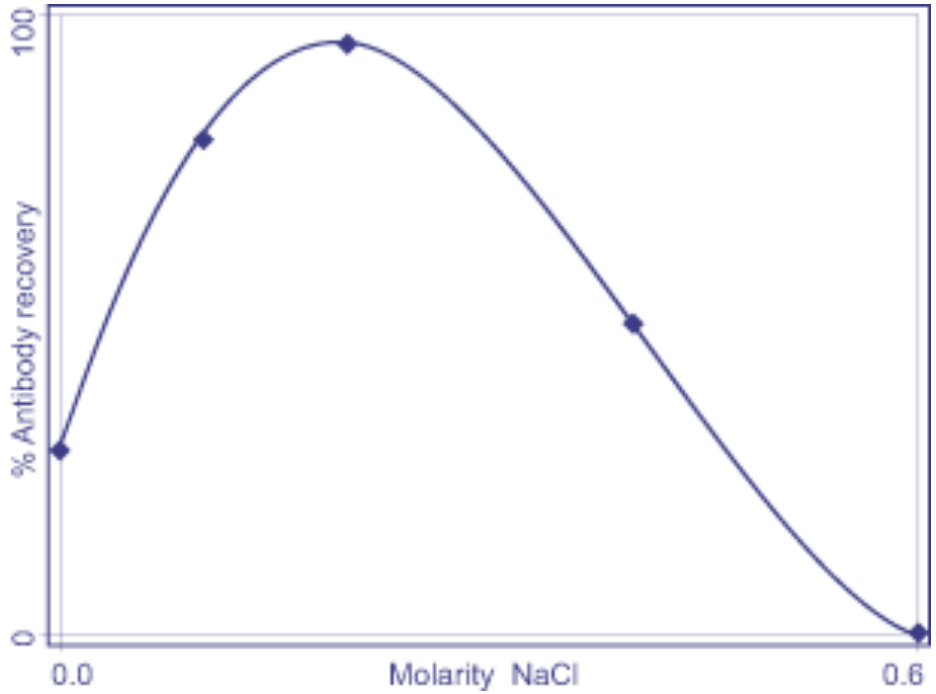
Cooperativity of hydrophobic and electrostatic interactions.
Elution in a pH gradient as a function of NaCl concentration.
Positively charged hydrophobic mixed-mode.



Capto Adhere, 1 mL HiTrap
1 mL/min
A: 20 mM MES, 20 mM Hepes, 20 mM Tris, pH 8.0
B: 20 mM MES, 20 mM Hepes, 20 mM Tris, pH 5.0
A,B subsequent runs: plus NaCl at indicated concentrations
C: 6 M guanidine, pH 5
Equilibrate: A
Inj: 50 µL protein A purified hIgG$_1$ Mab
Wash: A
Elute: 20 CV linear gradient to B
Clean: C
These results emphasize the futility of using pI as a predictor of retention.

Protein:mixed-mode interactions

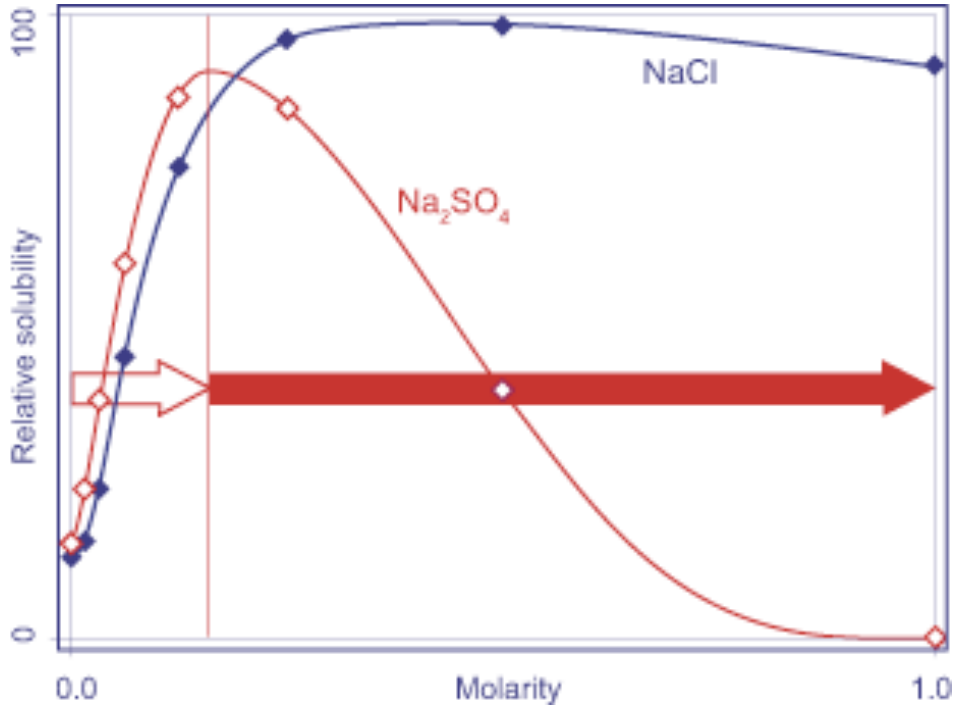
Cooperativity of hydrophobic and electrostatic interactions.
IgG recovery as function of salt concentration in pH gradients.
Positively charged hydrophobic mixed-mode.



Capto Adhere, 1 mL HiTrap
1 mL/min
A: 20 mM MES, 20 mM Hepes, 20 mM Tris, pH 8.0
B: 20 mM MES, 20 mM Hepes, 20 mM Tris, pH 5.0
A,B subsequent runs: plus NaCl at indicated concentrations
C: 6 M guanidine, pH 5
Equilibrate: A
Inj: 50 µL protein A purified hIgG$_1$ Mab
Wash: A
Elute: 20 CV linear gradient to B
Clean: C
These results emphasize the value of a guanidine (or similarly effective) cleaning step at the end of each run.

Protein:solvent interactions

Protein solubility: salting-in and salting-out.



Relative solubility of purified mouse IgG_{2a} as a function of salt concentration at pH 7.0.
Salting-in indicated by open arrows.
Salting-out indicated by closed arrows.
Redrawn from Gagnon, P., Mayes, T., Danielsson, A.,1997, An adaptation of hydrophobic interaction chromatography for estimation of protein solubility optima, *J. Pharm., Biomed. Anal.,* **16** 587-592.

Protein:solvent interactions

Deficiency of solution counter-ions intensifies electrostatic interactions within protein structural domains. This causes conformational changes that expose hydrophobic residues which are buried under physiological conditions. Proteins simultaneously become macro-ions and associate electrostatically, with secondary stabilization by hydrogen bonding and hydrophobic interactions. Solubility diminishes.

Salting-in refers to the ability of increasing salt concentration to bring proteins back into solution by restoration of physiological charge equilibrium. Chaotropic and kosmotropic salts both promote salting-in, essentially in proportion with conductivity.

Cohn, E., Edsall, J. (1943) Proteins, peptides and amino acids as ions and dipolar ions, Rheinhold, New York

Protein:solvent interactions

Salting-out refers to the ability of kosmotropic salts to depress protein solubility, eventually leading to precipitation.

Kosmotropic salts typically embody high molar surface tension increments and are preferentially excluded from protein surfaces.

Increasing kosmotropic effect

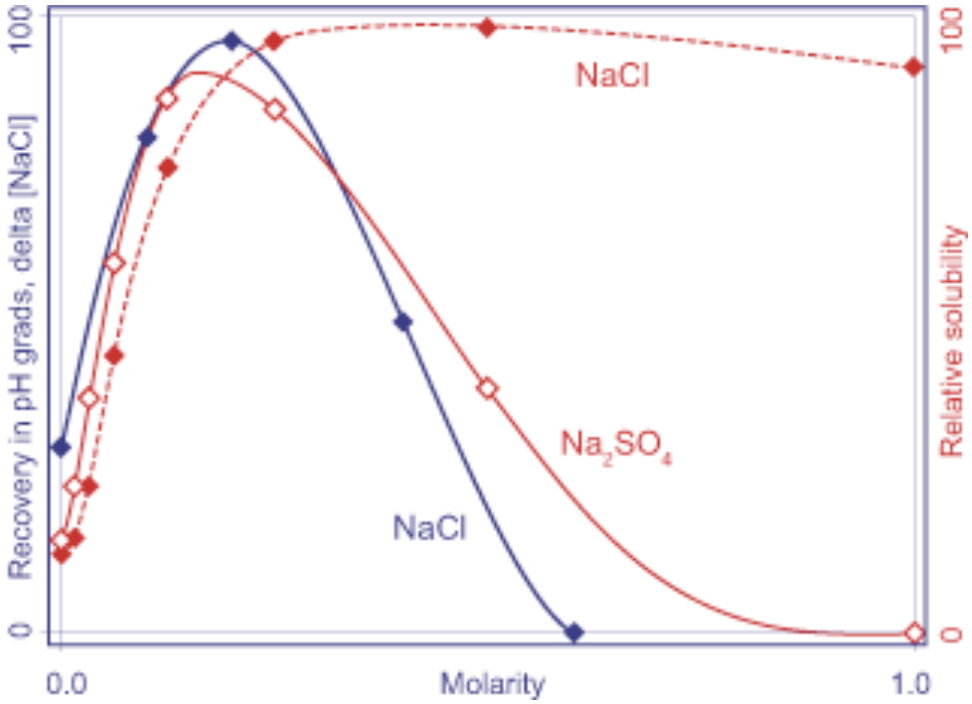
SCN^- ClO_4^- NO_3^- Br^- Cl^- COO^- SO_4^{2-} PO_4^{2-}

Ba^{2+} Ca^{2+} Mg^{2+} Li^+ Cs^+ Na^+ K^+ Rb^+ NH_4^+

Increasing chaotropic effect

Protein:solvent:ligand interactions

Parallel response of IgG solubility and recovery from a positively charged hydrophobic mixed-mode



This slide overlaps the recovery plot from slide 10 (Capto adhere) with the solubility plots from slide 11, at the same scales.

The key to reconciling conformance of the NaCl recovery curve with the Na_2SO_4 solubility curve is to factor in the hydrophobic contribution of the ligand, which acts as a surrogate for precipitating salts for enhancement of hydrophobic interactions. Poor recovery is seen at very low conductivity values due to exposure of more hydrophobic residues.

This phenomenon also occurs with traditional HIC.

Pluripotency of eluents

Pluripotency, in the context of eluents for mixed-mode chromatography, refers to the ability of a single mobile phase component to simultaneously affect two or more kinds of interactions between a solute and a solid phase.

Pluripotency of eluents

pH: *Determines protein charge, which affects electrostatic attraction, electrostatic repulsion, protein surface hydrophobicity, and protein solubility. It may also affect the charge and/or hydrophobicity of mixed modes with weak ion exchange groups.*

NaCl: *Suppresses electrostatic attraction*

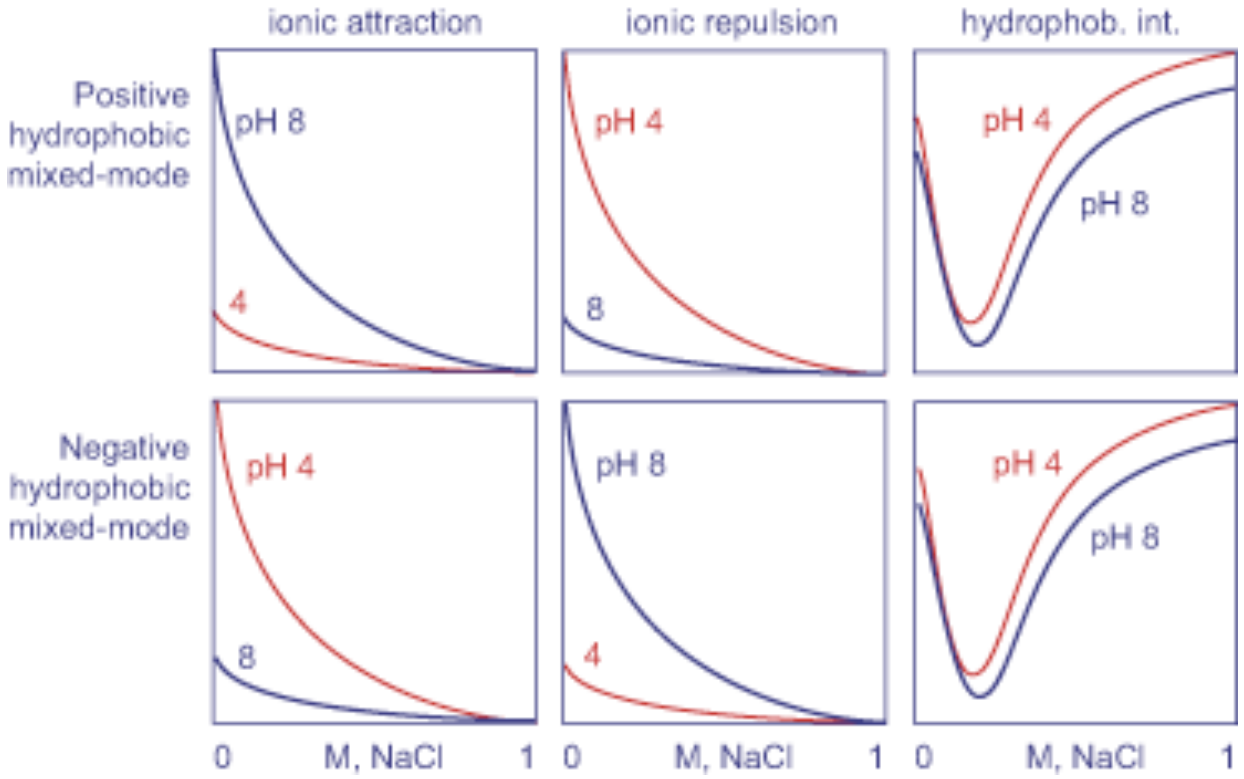
Suppresses electrostatic repulsion

Increases protein solubility, concurrently reducing the effects of protein surface hydrophobicity

Promotes hydrophobic interactions between protein hydrophobic residues and a hydrophobic solid phase

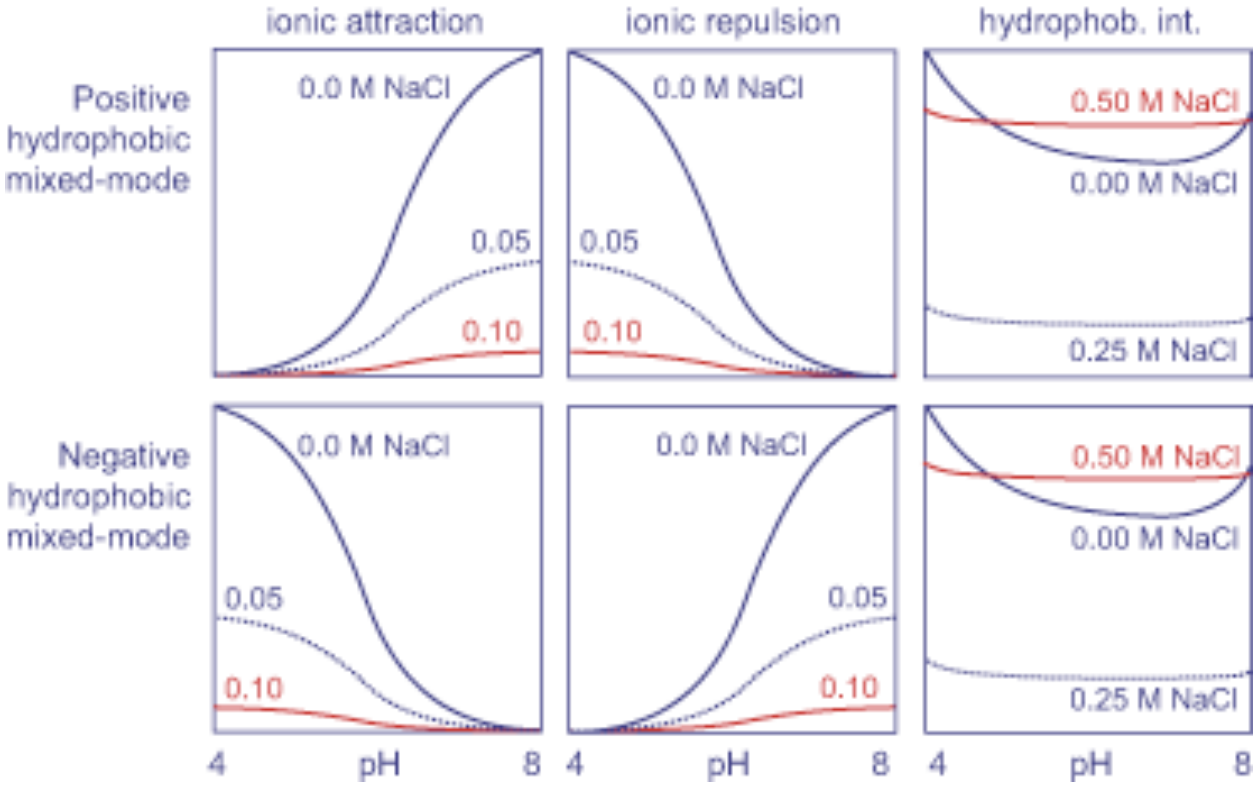
Pluripotency of eluents

Representative effects of NaCl



Pluripotency of eluents

Representative effects of pH



Pluripotency of eluents

Other salts:

Strong kosmotropes should enhance hydrophobic interactions at lower concentrations but also suppress electrostatic interactions slightly more (due to the higher molar conductivity of multivalent ions). Chaotropes will simultaneously depress both electrostatic and hydrophobic interactions.

Polyethylene glycol (PEG):

PEG enhances protein retention by electrostatic attraction and metal affinity but its mild hydrophobicity weakens hydrophobic interactions. It also depresses protein solubility and increases mobile phase viscosity.

Pluripotency of eluents

Ethylene glycol, propylene glycol

These nonionic compounds are protein stabilizing and suppress hydrophobic interactions by reducing the mobile phase dielectric constant.

Urea

Also nonionic, it weakens hydrophobic interactions and also breaks hydrogen bonds. Negligible risk of denaturation at 1-2 M.

Arginine

The guanido side group suppresses hydrogen bonds and hydrophobic interactions, but arginine augments conductivity and thereby suppresses electrostatic interactions as well.

Pluripotency of eluents

Zwitterionic amino acids

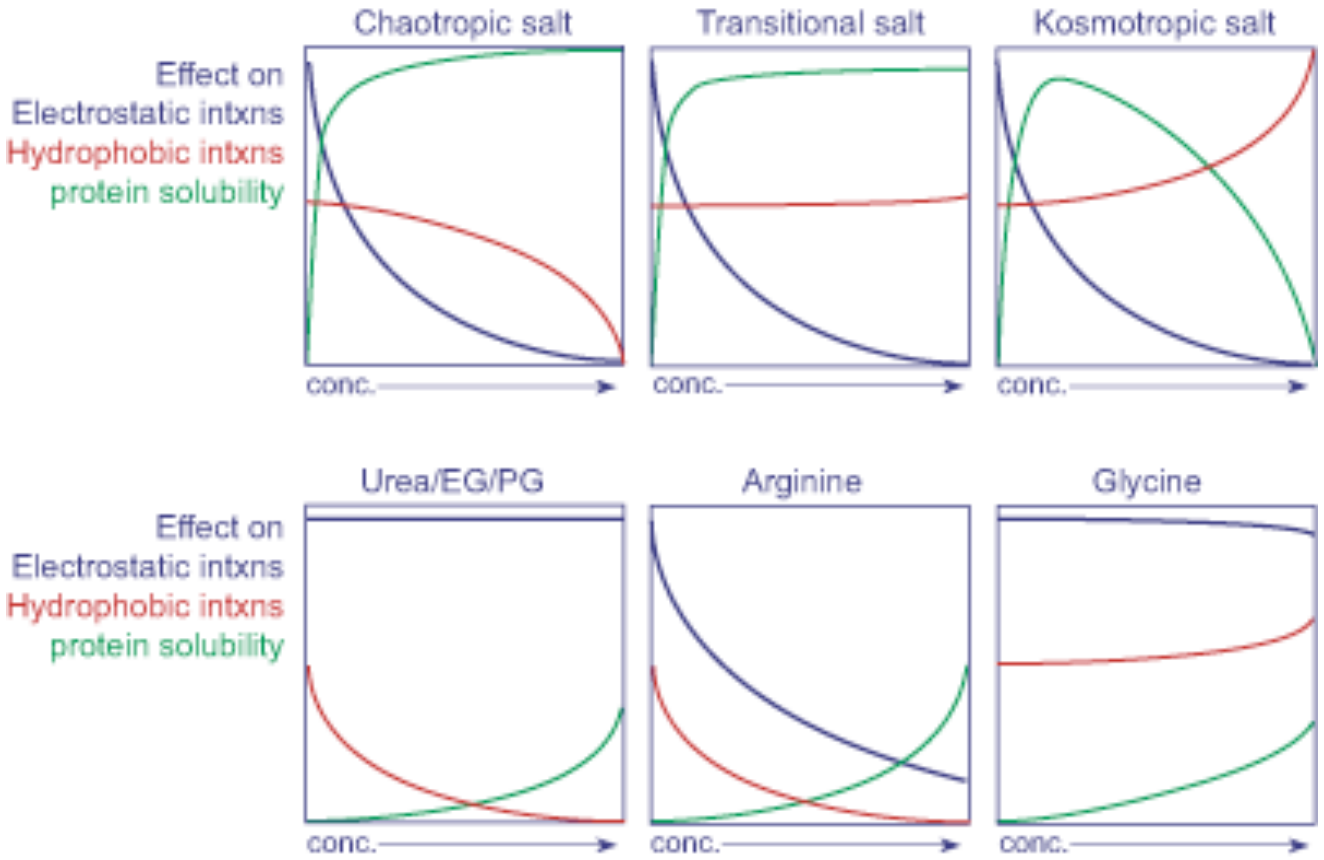
Some zwitterions, such as glycine, have high molar dielectric increments. They increase the polarity of aqueous solvents, which increases the interactivity of water with charged residues on proteins. This increases protein solubility. It also mildly weakens electrostatic interactions among solutes, or between solutes and a charged solid phase.

Glycine also has a positive surface tension increment, and is preferentially excluded from protein surfaces, which means that it simultaneously enhances retention by electrostatic, hydrophobic, or other protein:solid phase interactions.

In its zwitterionic form, from about pH 4 to 8, glycine makes no contribution to conductivity and –except as noted above– does not interfere with electrostatic interactions.

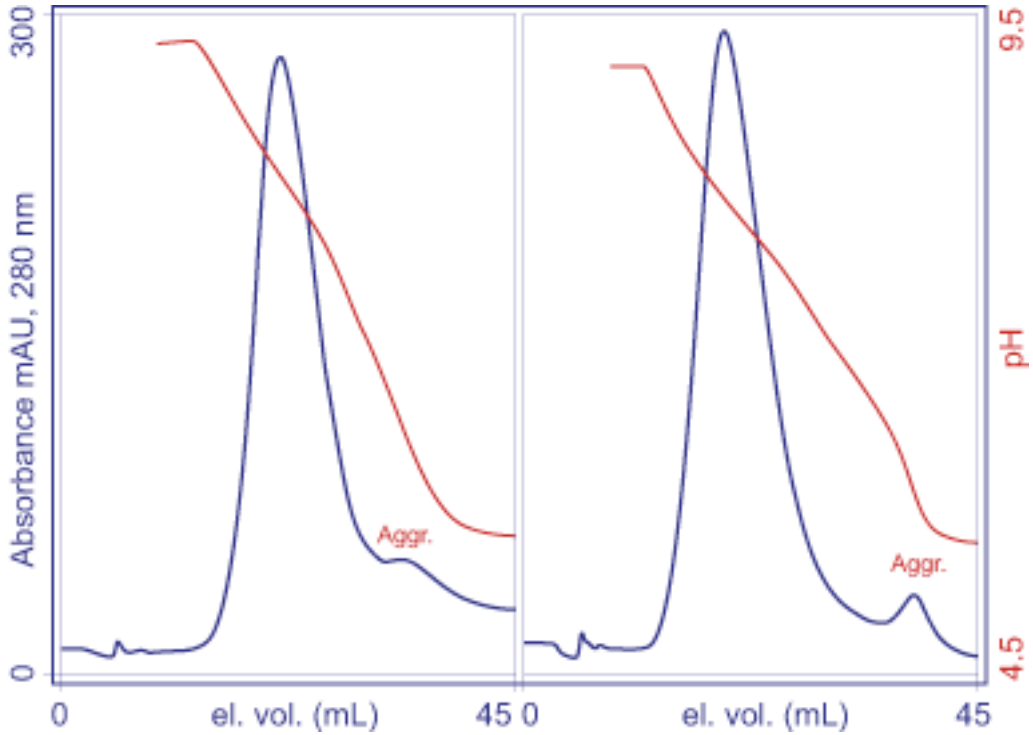
Pluripotency of eluents

The Mixed-Mode Toolbox (absent pH)



Enhancement of aggregate removal

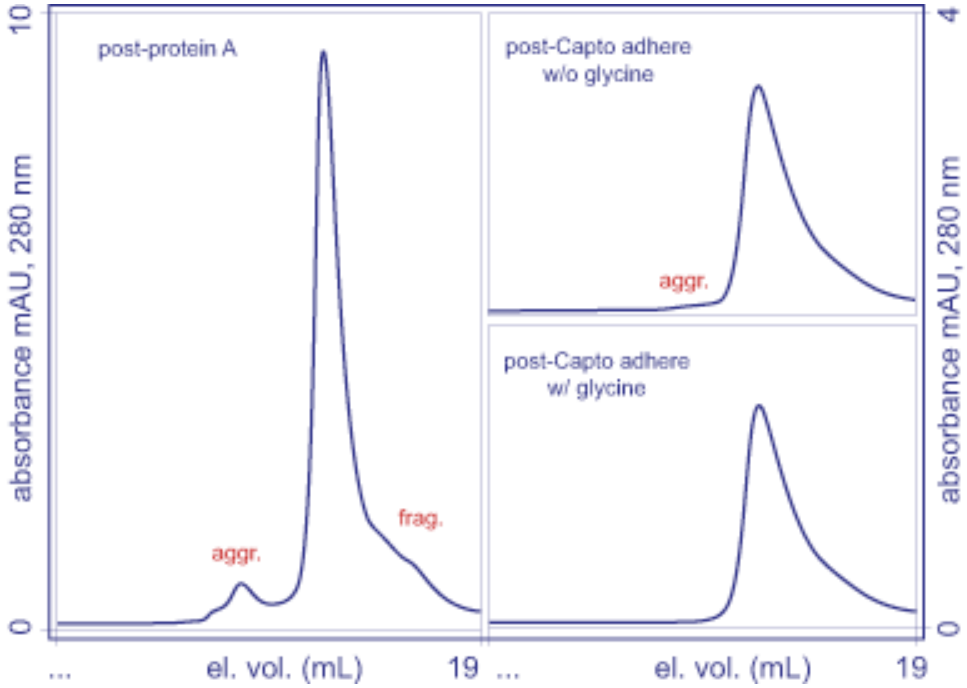
Preferential enhancement of IgG aggregate retention by glycine



Capto adhere, 1 mL HiTrap
1 mL/min
A1: 20 mM Tris, 20 mM Hepes, 20 mM MES, pH 9.0
A2: 20 mM Tris, 20 mM Hepes, 20 mM MES, 1 M glycine, pH 9.0
B1: 20 mM Tris, 20 mM Hepes, 20 mM MES, pH 5.0
B2: 20 mM Tris, 20 mM Hepes, 20 mM MES, 1 M glycine, pH 5.0
C: 6 M guanidine, pH 5
Equilibrate: A
Inj: 2 mL protein A purified $hIgG_1$ Mab (~5 mg)
Wash: A
Elute: 20 CV linear gradient to B
Clean: C

Enhancement of aggregate removal

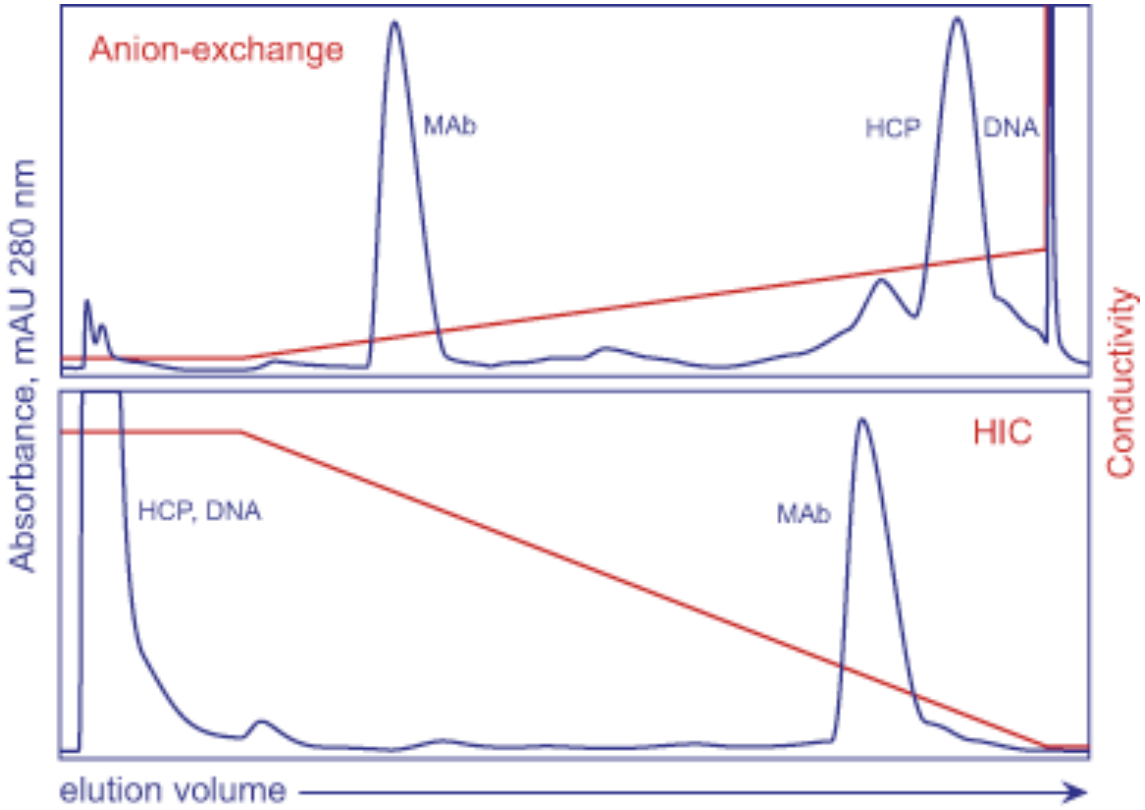
Analytical size exclusion chromatography



Superdex™ 200 HR 10/30
0.5 mL/min
20 mM Hepes, 100 mM arginine, 200 mM NaCl, pH 7.0
Inj: 50 µL protein A purified hIgG$_1$ Mab

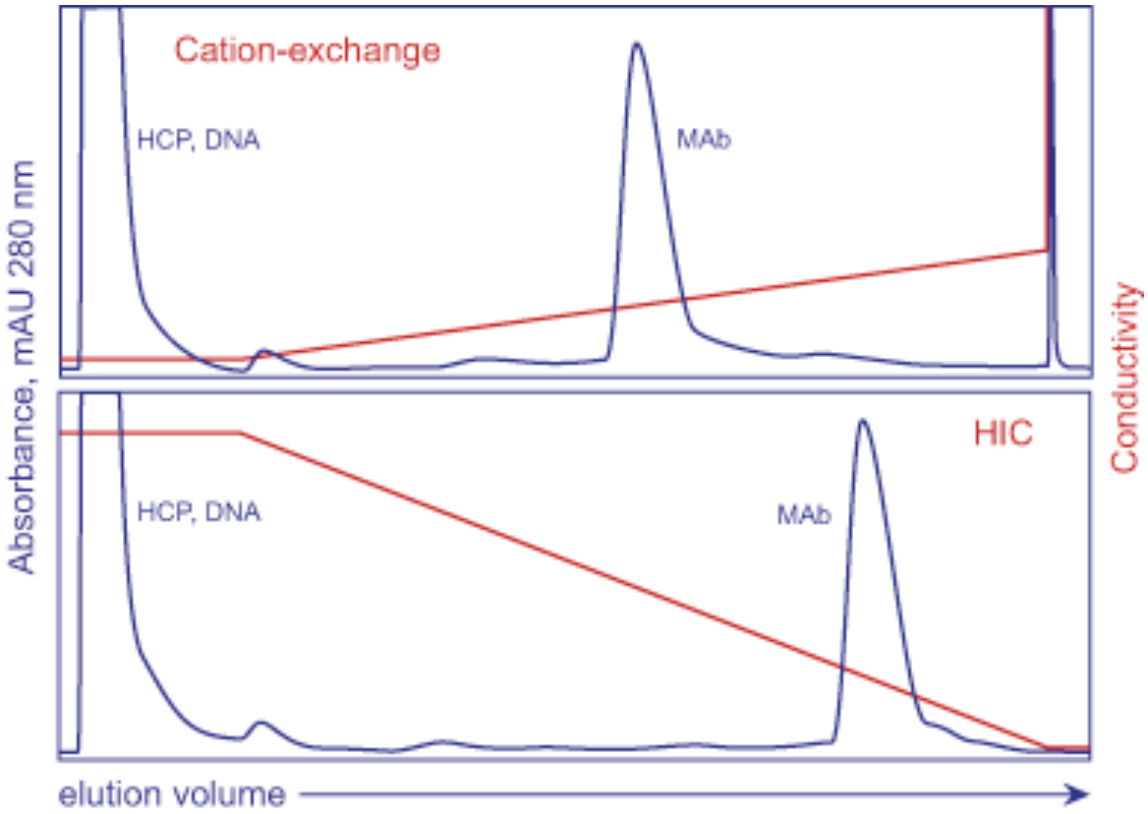
Which mix of modes?

Positively charged, hydrophobic



Which mix of modes?

Negatively charged, hydrophobic



Conclusions

Mixed-modes represent more than just a simple combination of selectivities.

Cooperative interactions between solutes and mixed-modes are compounded by the influence of protein solubility, creating novel selectivities.

Although the individual influences of the parent selectivities (electrostatic interactions, hydrophobic interactions) can be detected in the retention behavior of a given solute, neither HIC nor ion exchange behavior provide useful predictions of net selectivity.

Conclusions

Method development is further challenged by the ability of any process variable to affect multiple kinds of interactions.

The more mobile phase components the more complex the system, especially since the effects of a given component may not be linear.

Gradual accumulation of experimental results (industry-wide) may reveal general trends for what to expect from a given class of solutes under a relatively defined range of conditions, but until then –and probably even after– mixed modes will require screening over a broader range of conditions than is customary for traditional methods.

Conclusions

Each mixed-mode represents a distinct window of selectivity.

Preliminary results show the real practical utility of these media, but also demonstrate that no single mixed-mode can accommodate all antibodies.

Commercial introduction of mixed-modes with different balances of charge and hydrophobicity can be expected. This will add yet another layer to initial screening, but will also have the effect of making mixed-modes more broadly applicable and more competitive with traditional methods.

Acknowledgements

Thanks to GE Healthcare for providing Capto Adhere and Capto MMC.

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