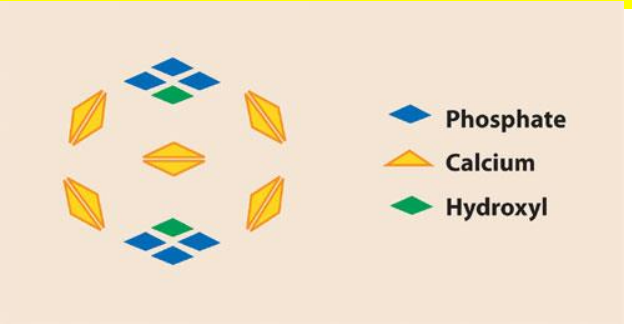
Mixed Mode (MMC) and other Chromatography Procedures

Hydroxyapatite Chromatography

“Mixed-mode" ion exchange separation: cation exchange and calcium metal affinity

- Complementary to more traditional IEX and HIC techniques.
- Formula is : $Ca_{10}(PO_4)_6(OH)_2$

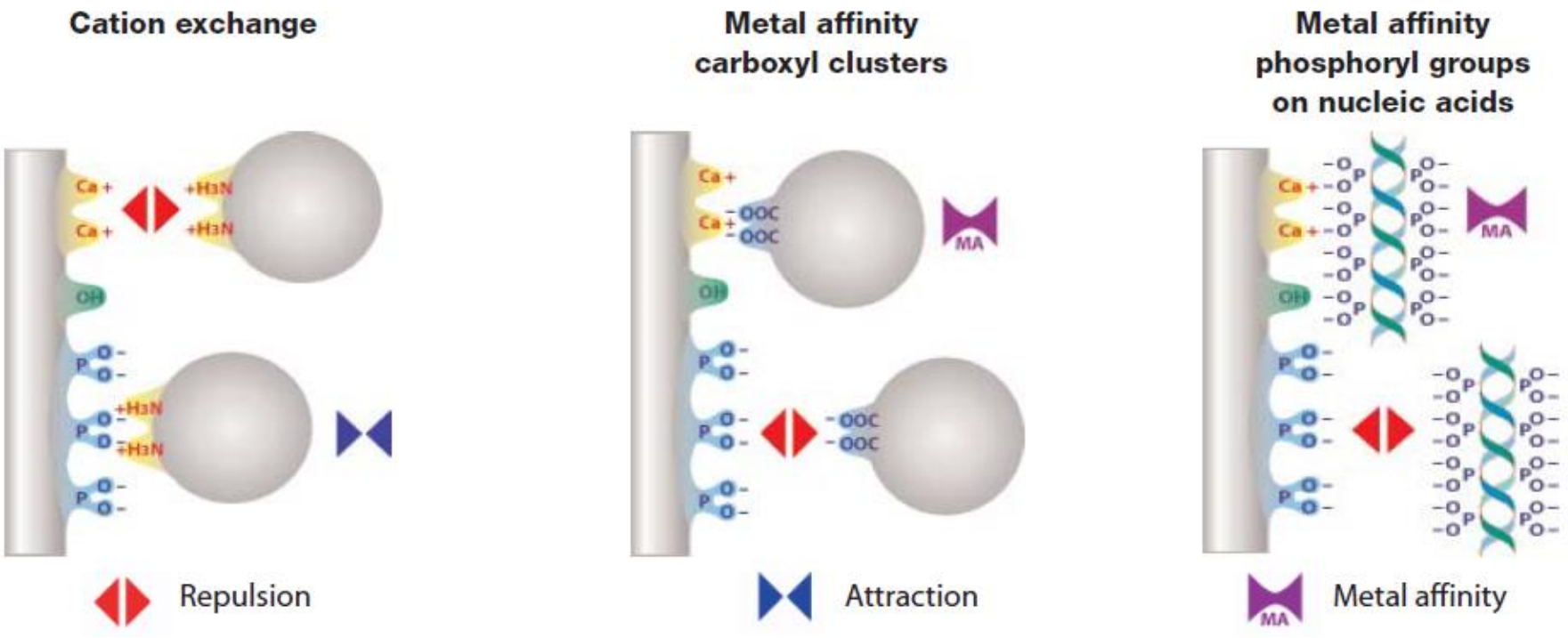
Functional groups comprise positively charged pairs of crystal calcium ions and clusters of six negatively charged oxygen atoms associated with triplets of crystal phosphates.



- CHT ceramic hydroxyapatite is a spherical, macroporous form of hydroxyapatite. Produced at high temperatures to modify it from a crystalline to a ceramic form. The ceramic material overcomes many of the limitations of traditional crystalline hydroxyapatite that prevent its use in industrial-scale applications.
- Growing popularity from the last years: Ability to remove aggregates, endotoxin and Protein A from IgG preparations
- Virus purification and viral clearance
- Separates single- and double-stranded DNA and discriminates among DNA molecules according to size
- Binds RNA less strongly than DNA
- IgM purification **Pete Gagnon et al., *BioProcess International 12(2) February 2014***

Hydroxyapatite Chromatography
Mode of Interaction

BIO-RAD



Most large proteins bind by a combination of mechanisms

Hydroxyapatite Chromatography Calcium Interaction

✓**Calcium affinity** occurs via interactions with carboxyl clusters and/o phosphoryl groups on proteins or nucleic acids

✓These groups are repelled by the negative PO_4 groups.

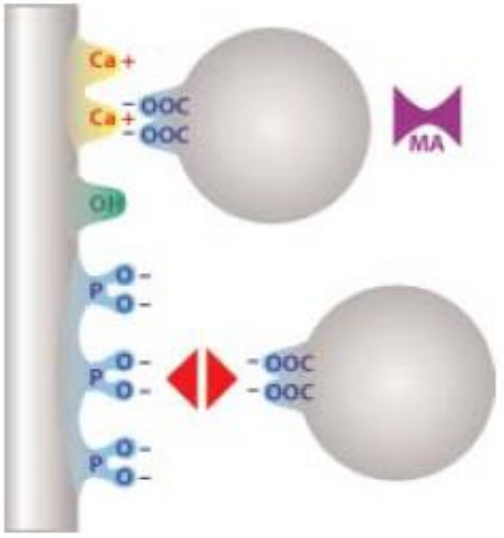
✓The affinity interaction is stronger than ionic interactions and is not affected by increasing ionic strength

✓Species binding through calcium affinity may adsorb more strongly as the ionic strength increases due to ionic shielding of the charge repulsion from the CHT phosphate sites.

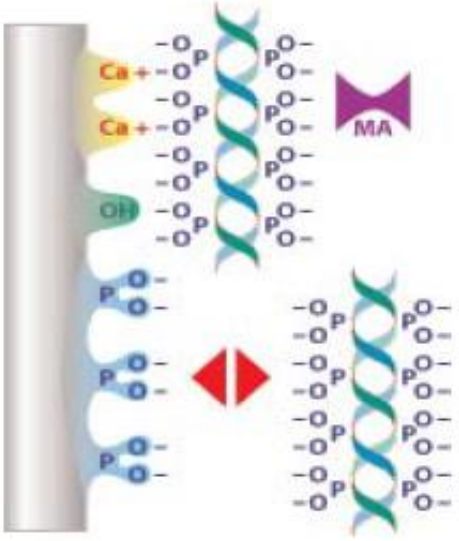
✓**Metal affinity elution: PO_4 gradient**

✓Acidic proteins, such as albumin use this mechanism, and the presence of high NaCl almost not affect the binding

Metal affinity carboxyl clusters



Metal affinity phosphoryl groups on nucleic acids



BIO-RAD

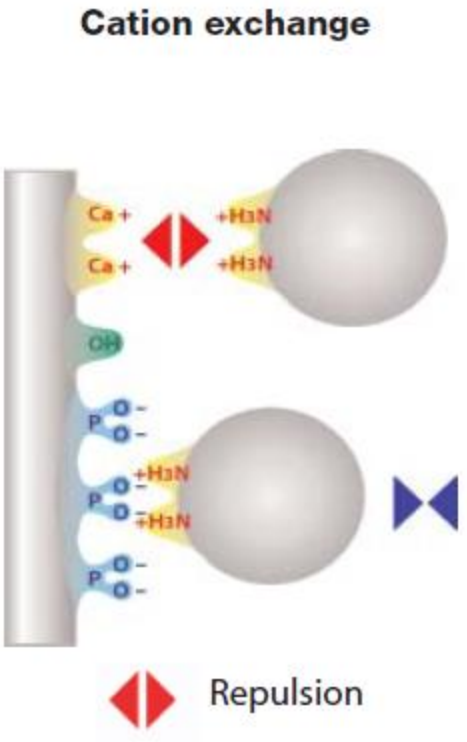
Hydroxyapatite Chromatography
Cation exchange Interaction

✓**Cation exchange**: protein amino groups interact with the negatively charged PO_4

✓**Elution by increasing salts as NaCl, increasing phosphate concentration or increasing pH.**

✓The anion exchange interactions with calcium, does not make a significant contribution.

✓Basic proteins, such as IgG, bind mainly by cation interactions and may be selectively eluted with either phosphate or salts



✓**Type I:** higher protein binding capacity and better capacity for acidic proteins

✓**Type II**: lower protein binding capacity. Better resolution of nucleic acids and certain proteins. Low affinity for albumin and is particularly well-suited for the purification of immunoglobulin's

✓Available as 20, 40 & 80µ

BIO-RAD

Protocol I
IgG

CHT™ ceramic hydroxyapatite

Equilibrate: 10 column volumes buffer A–10 mM $NaP0_4$, pH 6.5

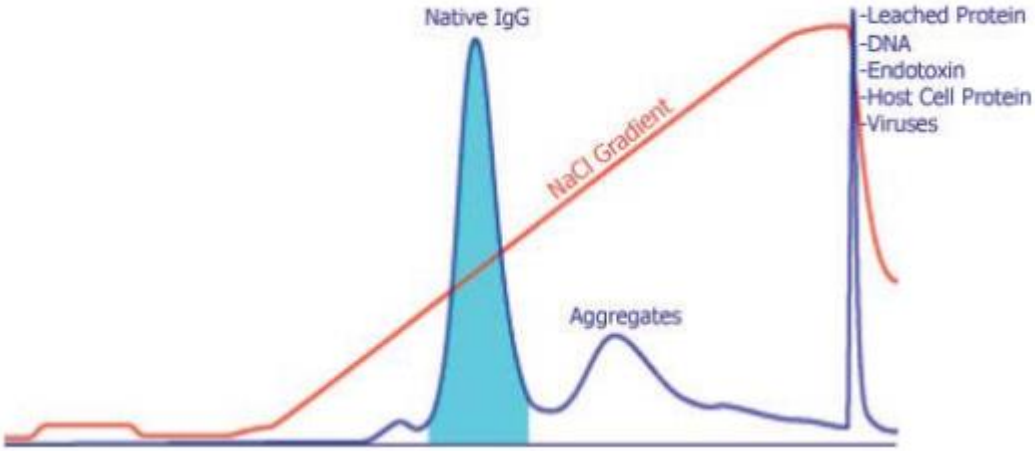
Load: clarified sample with buffer containing 5 mM $NaPO_4$, pH 6.5

Wash: 5 column volumes of buffer A–10 mM $NaPO_4$, pH 6.5

Elute: 20 column volume buffer B—linear gradient 10 mM $NaPO_4$, 0–2 M NaCl, pH 6.5

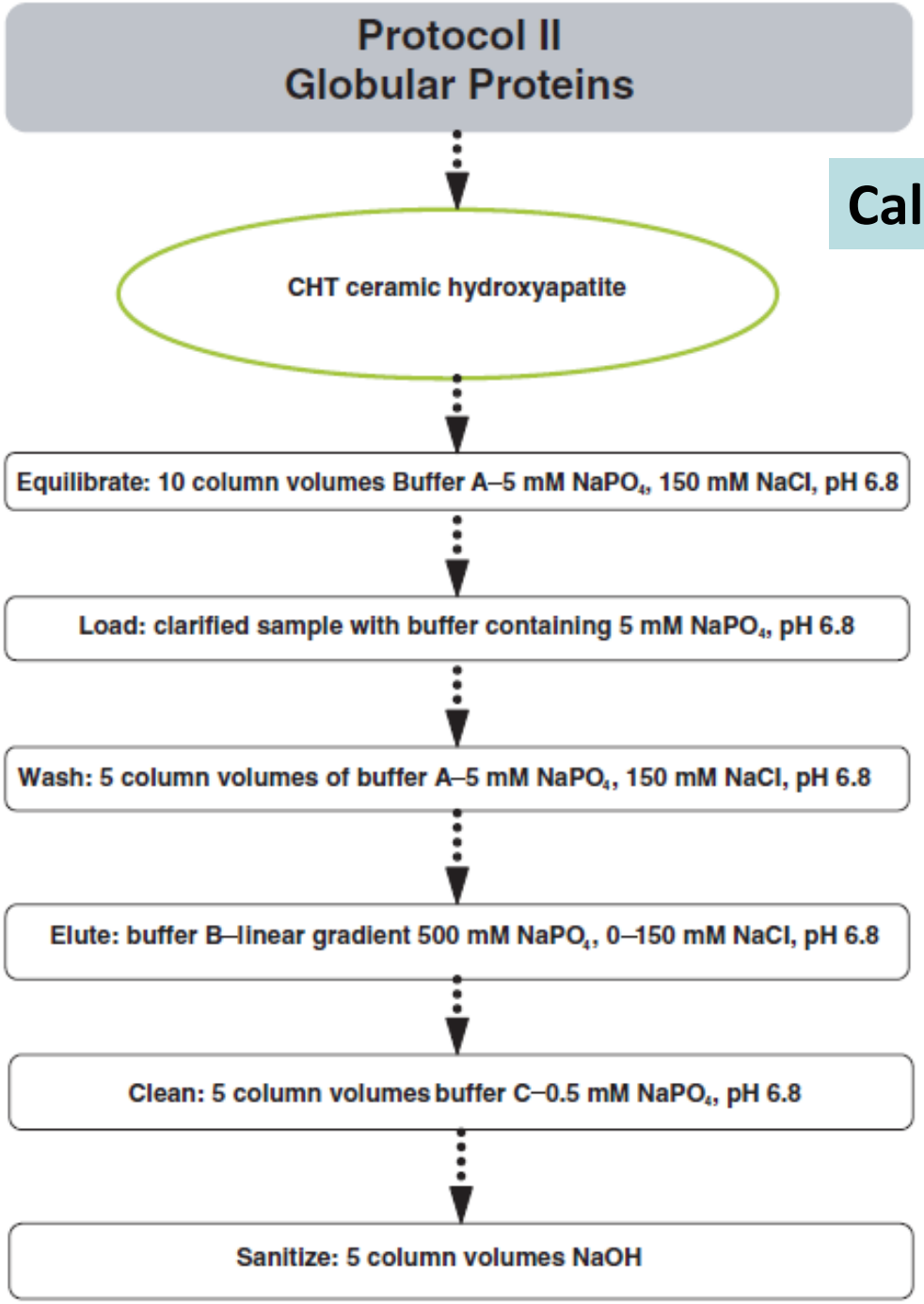
Clean: 5 column volumes buffer C—500 mM $NaPO_4$, pH 6.5

Sanitize: 5 column volumes NaOH



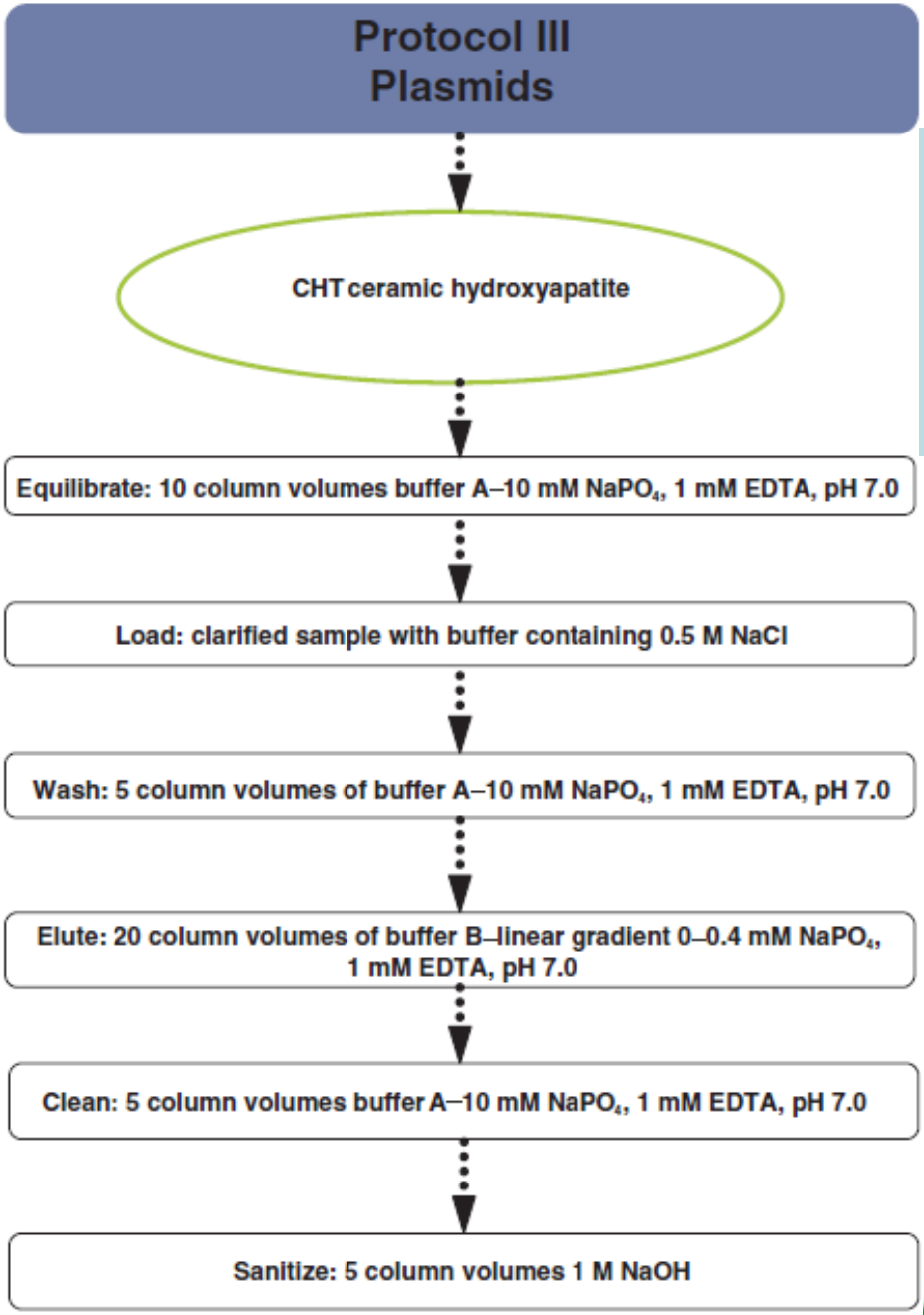
Cation exchange Interaction

BIO-RAD



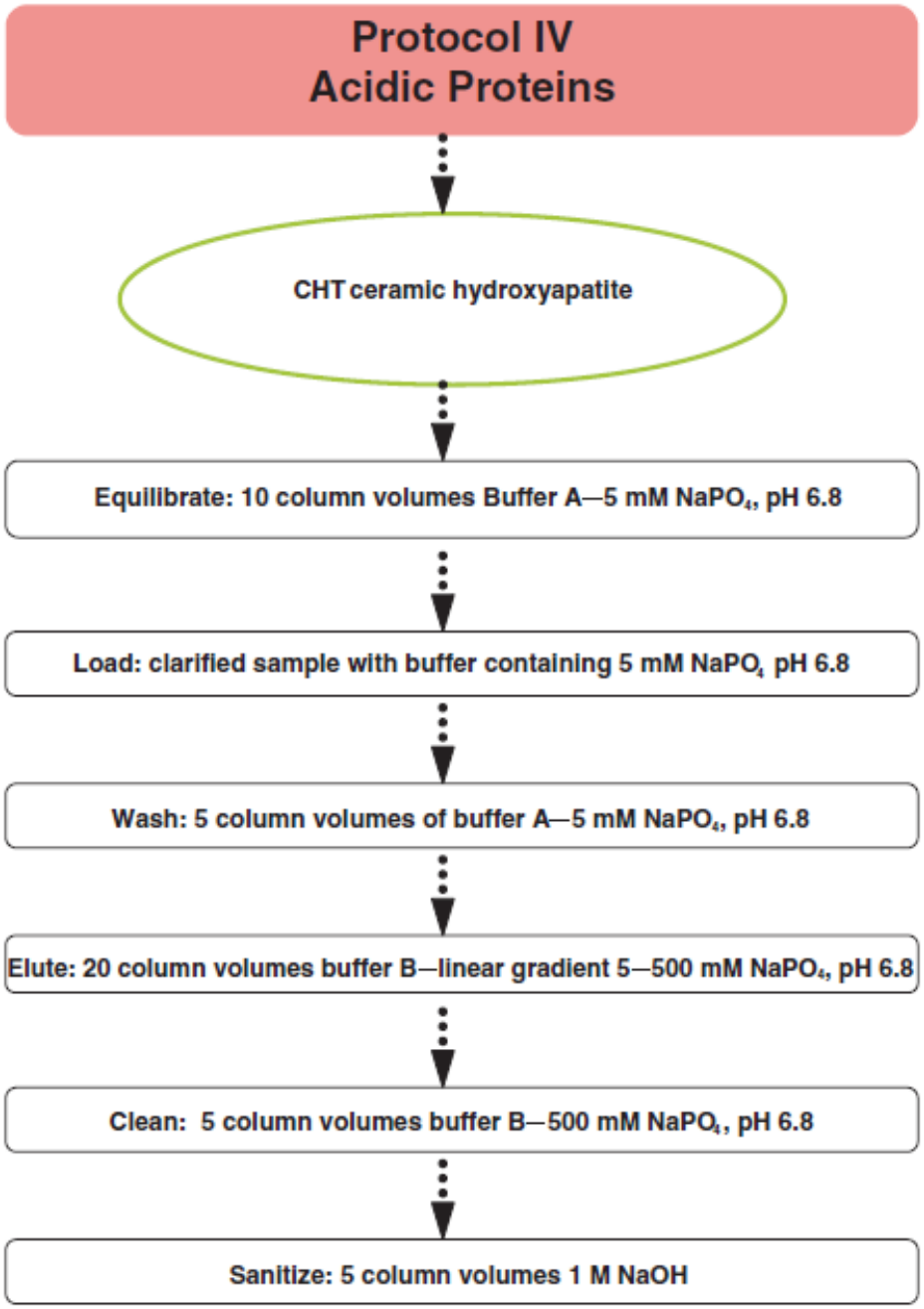
Calcium Interaction

BIO-RAD



Metal affinity and phosphoryl interaction

BIO-RAD



Metal (Ca^{++}) Interaction

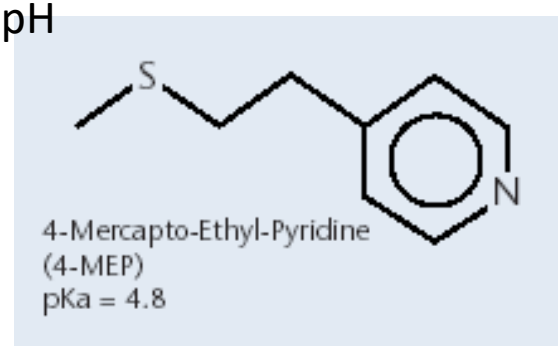
BIO-RAD

Hydrophobic Charge-Induction Chromatography (HCIC) - BIOSEPRA

Adsorption is based on mild hydrophobic interaction without the need of high salt concentration

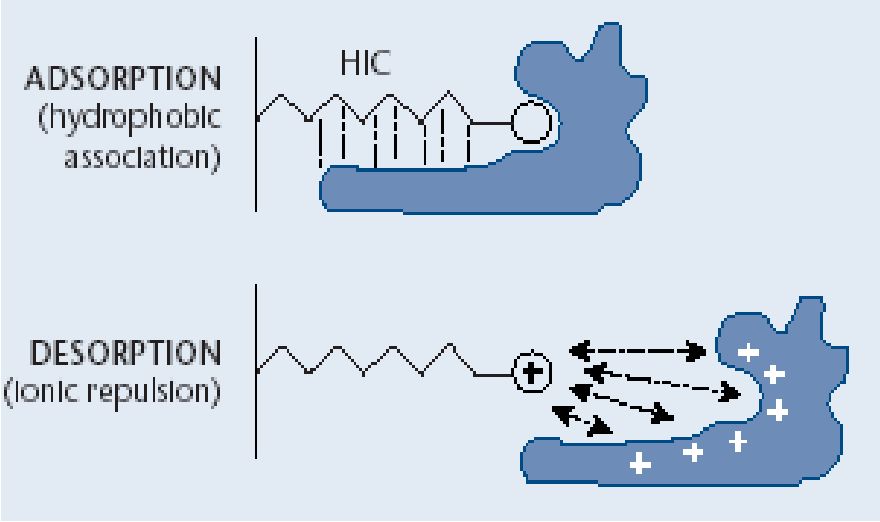
In contrast to traditional HIC, HCIC from is controlled on the basis of pH

Desorption is based on charge repulsion performed by reducing the pH



At physiological pH, the aromatic pyridine ring is uncharged and hydrophobic. Antibody binding is further enhanced by interaction with the thioether group.

By lowering the pH to ~4.0, both the ligand and the antibody take on a distinct positive charge that induced an electrostatic repulsion that desorbed the antibody.



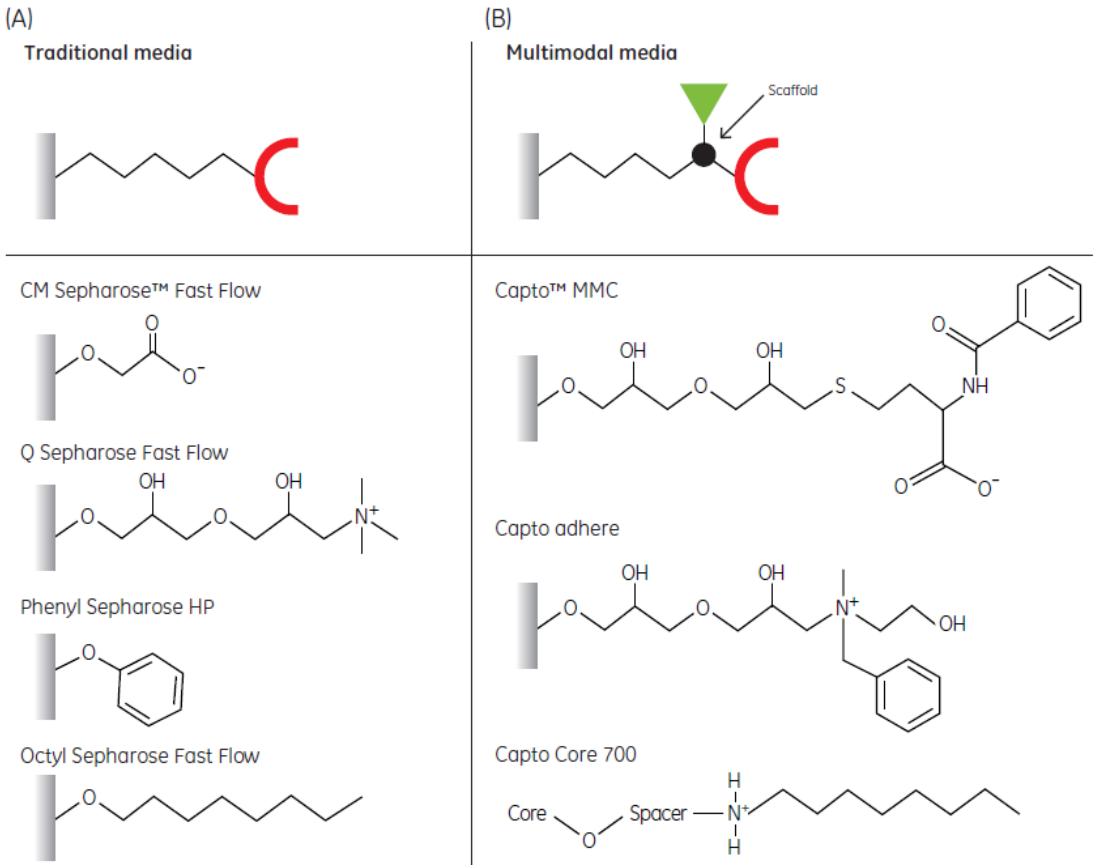
PALL

Pall Corporation

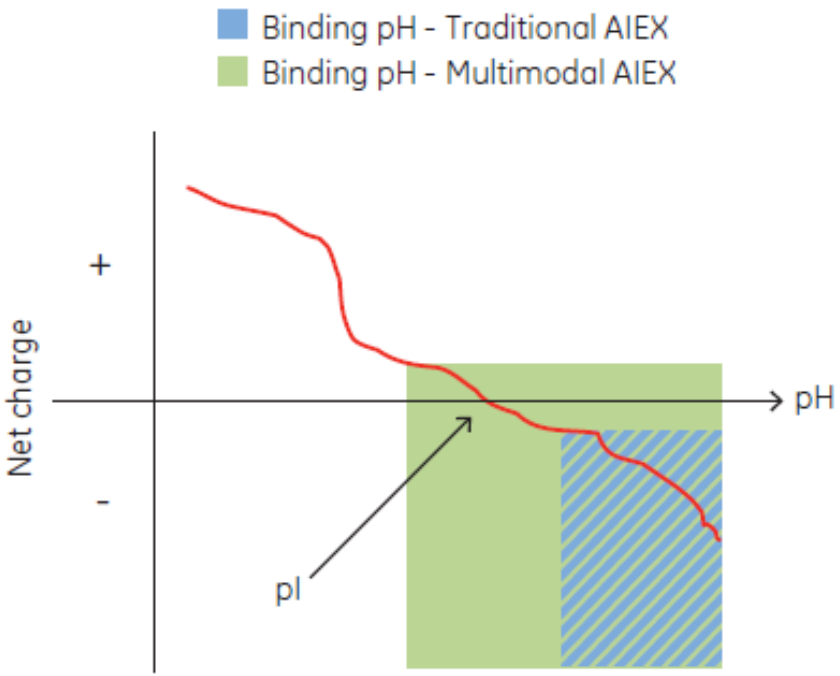
Comparison between traditional and Multimodal Media

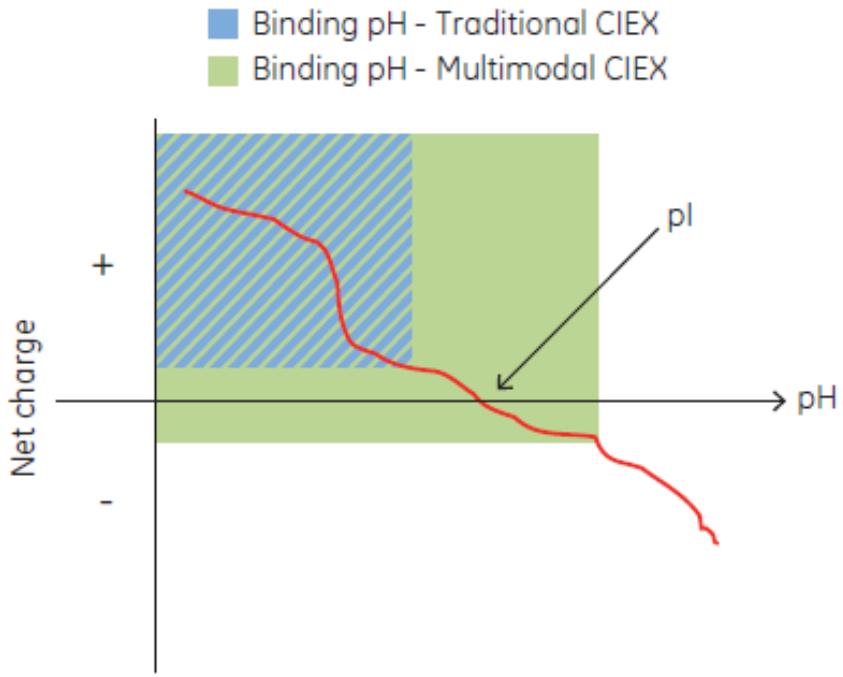
✓Multiple types of interactions are occurring in MMC, and the strength of these individual interactions often depends on the process conditions

- ✓ So, binding/elution parameters are more complex than IEX
- ✓ Moreover, MMC of different suppliers are different.
- ✓ Some MMC resins have weak instead of strong IEX ligands
- ✓ So, a wide spectrum of interactions is open



pH range for binding is extended for Multimodal Media compared with traditional IEX media





This gives the multimodal media unique selectivities and generally a wider operational window

pI is not a good indicator for choosing the correct pH for binding and elution

GE

Selectivity of multimodal media

pH range for binding is extended for multimodal media compared with traditional IEX

Salt types and additives can modulate the interactions of target molecule with MMC:

salt strength

Anion:

$SO_4^{2-} > HPO_4^{2-} >$ acetate $> Cl^- > NO_3^- > Br^- > ClO_3^- > I^- > ClO_4^- > SCN^-$

Cation:

$NH_4^+ > K^+ > Na^+ > Li^+ > Mg^{2+} > Ca^{2+} >$ guanidinium

GE Healthcare

Organic solvents, for example, ethanol and isopropyl alcohol, decrease the strength of hydrophobic interactions

Detergents and antifoaming agents such as Tween™ 80 and Triton™ X-100 can have a similar effect

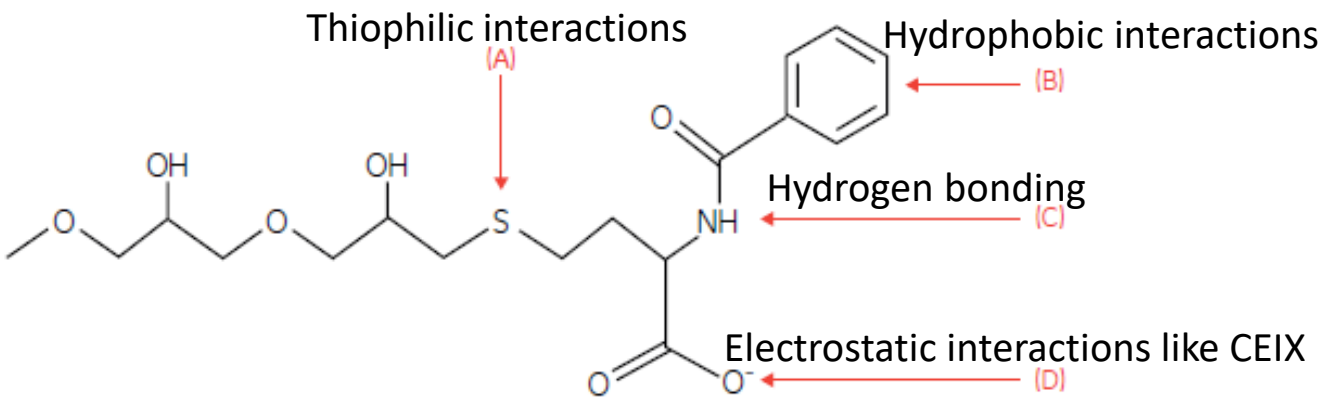
Hydrogen bond disruptors such as urea and guanidine hydrochloride also have the potential to impact the strength of MMC

Capto™ MMC a multimodal cation exchanger

According to: GE Healthcare (Amersham-Biosciences – Pharmacia)

✓Ligand design enables **binding at high conductivity** at 1-2 pH units below the protein pI. Requires an increase in both, pH (above the pI) and salt concentration, for effective elution of bound protein.

- ✓ Elution is affected by buffer strength and type of salt (NH_4Cl instead of NaCl)
- ✓ **Capto MMC**: Capture and intermediate for purification from large feed
- ✓ Purification can be performed at the conductivity of the feed material.
- ✓ High dynamic binding capacity (DBC) at high conductivity
- ✓ Different selectivity compared with traditional IEX
- ✓ **Capto MMC ImpRes**:
- ✓ higher resolution,
- ✓ Use in bind/elute mode



GE Healthcare

Capto adhere a multimodal anion exchanger

According to: GE Healthcare (Amersham-Biosciences – Pharmacia)

✓Electrostatic interaction, hydrogen bonding, and hydrophobic interaction

✓Gives a different selectivity compared to traditional ion exchangers

✓**Capto adhere** is designed for intermediate purification and polishing of MAbs.

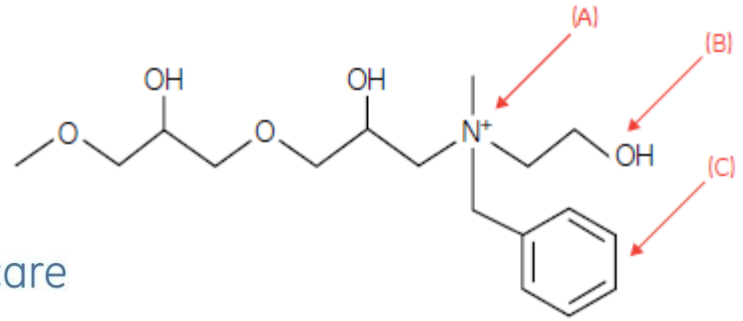
Removal of protein A, aggregates, host cell proteins, nucleic acids, and viruses

✓Recommended to be operated in flow through mode

✓Capto adhere is based on a rigid agarose matrix that allows high fluid velocities

✓**Capto ImpRes** particle are best utilized in bind/elute mode.

✓Selectivity affected by salt type and additives like isopropyl alcohol or chaotropic salts



electrostatic interactions like AEIX

hydrogen bonding

hydrophobic interactions.

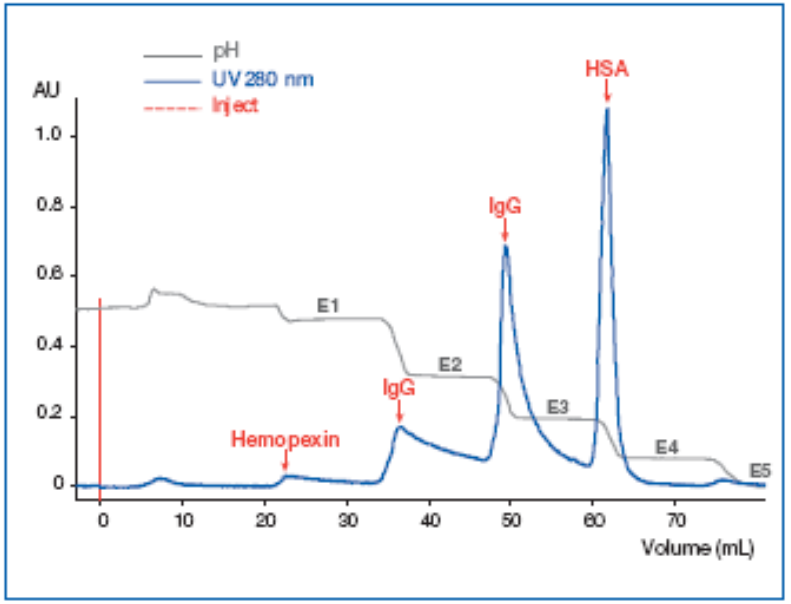
GE Healthcare

HEA and PPA HyperCel™ Mixed-mode Chromatography

Pall Corporation

- A combination of electrostatic and hydrophobic properties of the protein and ligands.
- Direct hydrophobic capture of proteins at low ionic strength
- **Protein binding** is achieved at neutral pH (i.e., PBS, pH 7.4), principally by hydrophobic interaction. Binding of very basic proteins may require increased pH (pH 9.0)
- Unlike traditional HIC, binding occurs at low ionic strength, in "physiological-like" conditions
- Elution is triggered by reducing the pH (from 5 to 3), usually in a step-elution mode

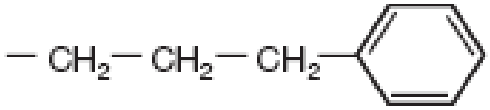
Example 2. Chromatography on HEA HyperCel sorbent: Separation of partially-purified polyclonal IgG from major plasma impurities.



Column: 0.66 cm I.D. x 7 cm height; Sorbent volume: 2.4 mL. Run at 100 cm/h; Equilibration in PBS, pH 7.4; Loading 5 mL of a partially-purified human polyclonal IgG (60% purity) at 3.8 mg/mL, pH 8.4, and 8.3 mS/cm; Wash with 5 CV in PBS; Elution in 0.2 M sodium phosphate / 100 mM citric acid, pH 7.0 (Elution 1), pH 5.4 (Elution 2), pH 4.4 (Elution 3), pH 3.4 (Elution 4), pH 2.6 (Elution 5); Regeneration in 1 M NaOH.

- **HEA HyperCel** → **n-hexyl** substituent

- **PPA HyperCel** → **phenylpropyl** substituent

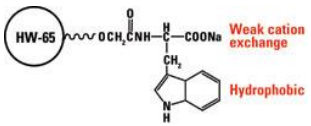


Selected commercially available mix-mode media

Preparative Purification of Recombinant Proteins: Current Status and Future Trends
Mayank Saraswat et al. Hindawi Publishing Corporation - BioMed Research International Volume 2013, Article ID 312709, http://dx.doi.org/10.1155/2013/312709

Media	Supplier	Type	Ligand	pH stability
CHT ceramic hydroxyapatite	Bio-Rad laboratories	Ion exchange, metal chelation	$[Ca_5[PO_4]_3OH]_2$	Operating pH: 5.5–14 Can be cleaned with 1-2 M NaOH
CHT Fluorapatite	Bio-Rad laboratories	Ion exchange, metal chelation	$[Ca_{10}[PO_4]_6F]_2$	Operating pH: 5–14 Can be cleaned with 1-2 M NaOH
MEP	Pall life sciences	Hydrophobic binding near neutral pH, elution by pH reduction	4-Mercapto ethyl pyridine	Working pH: 2–12 Cleaning pH: 2–14
HEA	Pall life sciences	Hydrophobic binding near neutral pH, elution by pH reduction	Hexylamino	Working pH: 2–12 Cleaning pH: 1–14
PPA	Pall life sciences	Hydrophobic binding near neutral pH, elution by pH reduction	Phenylpropylamino	Working pH: 2–12 Cleaning pH: 1–14
MBI	Pall life sciences	Hydrophobic binding at acidic pH, elution by raising the pH	2-Mercapto-5-benzimidazole sulfonic acid	—
Capto MMC	GE Healthcare	Multimodal cation exchange	2-Benzamido-4-mercaptobutanoic acid	Long term: 2–12 Short term: 2–14
Capto adhere	GE Healthcare	Multimodal strong anion exchange	N-benzyl-N-methyl ethanolamine	Long term: 3–12 Short term: 2–14

TOYOPEARL MX-Trp-650M	TOSOH	Multimodal cation exchange
TOYOPEARL®NH2-750F	TOSOH	**Salt Tolerant AEIX**
TOYOPEARL®SULFATE-650F	TOSOH	**Salt Tolerant CEIX**



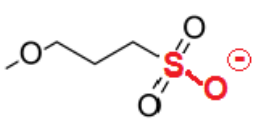
Salt Tolerant vs MMC resins

TOYOPEARL® SULFATE-650F SALT TOLERANT CATION EXCHANGE RESIN

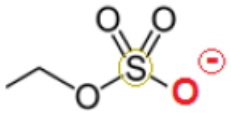
TOYOPEARL® NH2-750F SALT TOLERANT ANION EXCHANGE RESIN

- ✓ MMC resins: Both, charge and hydrophobic interactions are important
- ✓ Salt Tolerant resins: Binding to in ST-IEX depends on both, charge and hydrophobic interactions, but these hydrophobic interactions are less important here, and elution depends mainly of charge interactions
- ✓ Both resins allow protein binding at higher salt concentration than IEX, but only MMC allows broader pH range (lower pH for CEIX-HIC & higher pH for AEIX-HIC)
- ✓ So, charge and hydrophobic variables can be applied for MMC, while only charge variables can be applied for Salt Tolerant resins

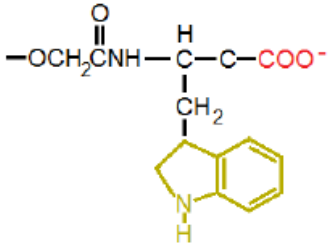
pure IEX



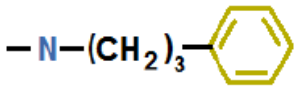
SP-650



Sulfate-650F

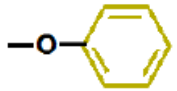


MX-Trp-650M



Phenylalkylamin

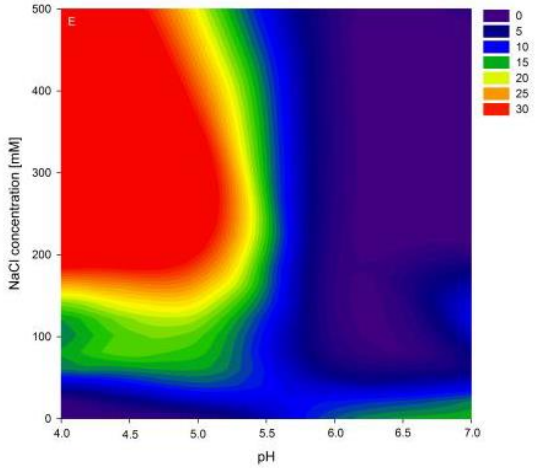
pure HIC



Phenyl-600

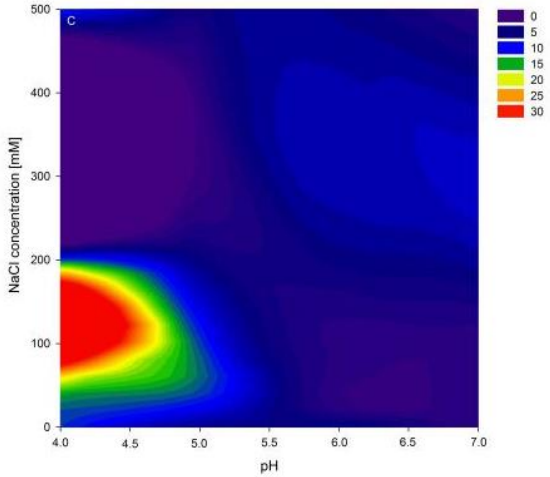
Comparing Dinamic Binding Capacity of different resins

DBC Sulfate-650F
Salt-tolerant sulfate strong CEX

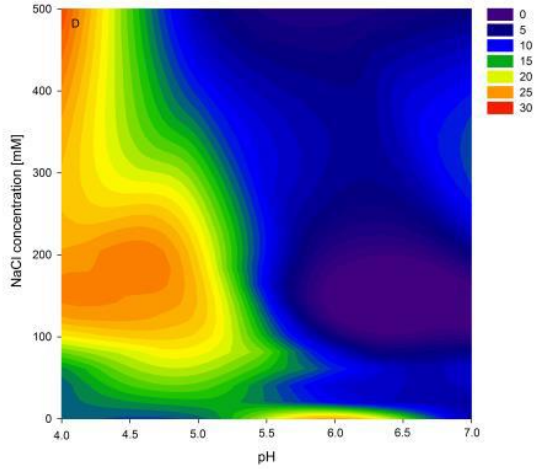


TOSOH

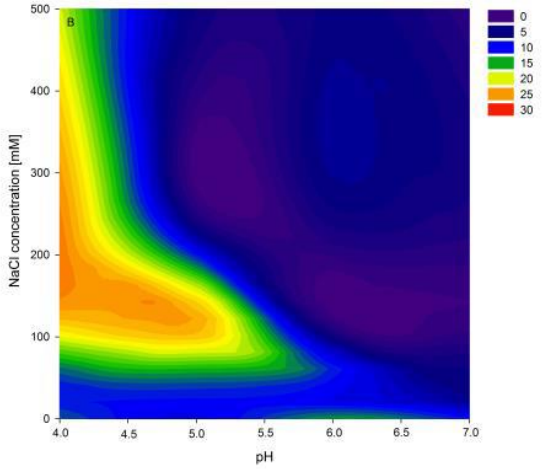
DBC Gica Cap S-650M
Sulfonic acid strong CEX



DBC MX Trp-650M
Mixed Mode



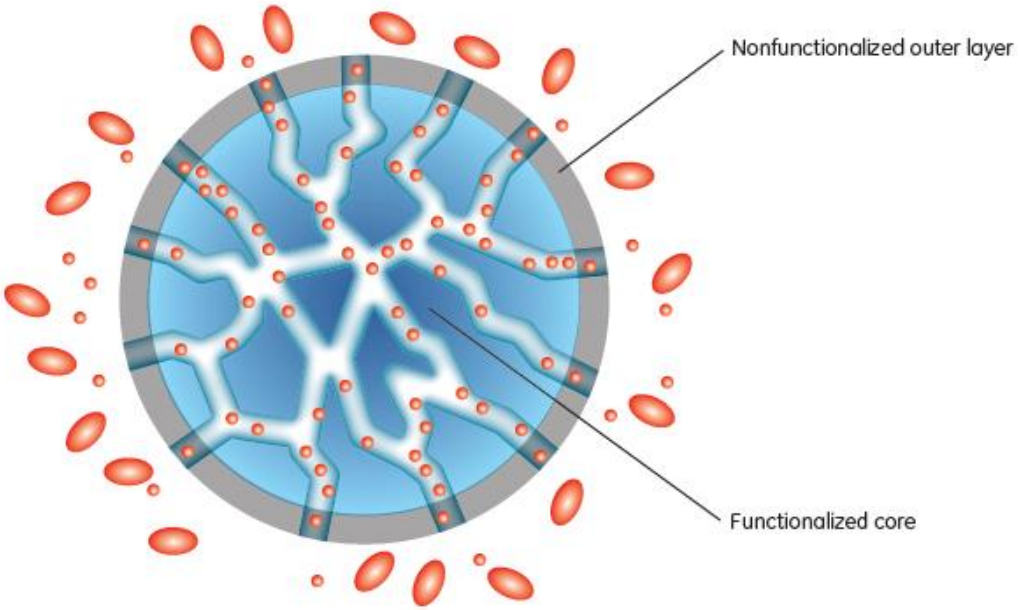
DBC Gica Cap CM-650M
Carboxy methyl weak CEX



Dual functionality: size exclusion, and binding chromatography - Capto Core 700 - GE

http://www.youtube.com/watch?v=YQRE9jdDmC8

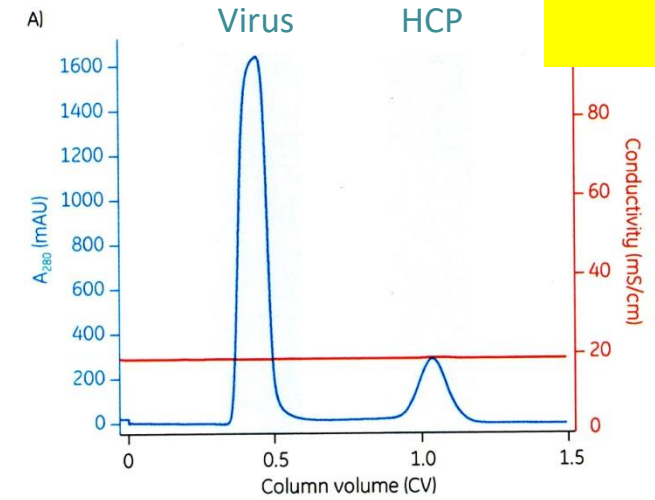
- For intermediate purification and polishing of viruses and other large biomolecules (M_r > 700 000) in flow-through mode.
- Efficient capture of contaminants (HCP, DNA)
- Target molecules are collected in the FT
- Significantly improved productivity and higher flow rates compared with GF

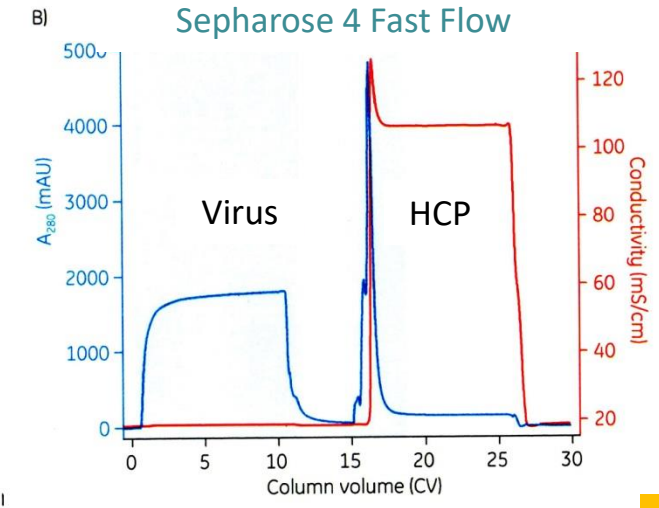


- Octylamine ligands inside the core of beads: both hydrophobic and positively charged, resulting in a highly efficient multimodal binding of various contaminants small enough to enter the core.
- The multimodal ligand ensure strong binding with most impurities over a wide range of pH and salt concentrations

GE Healthcare

Dual functionality: size exclusion, and binding chromatography
Capto Core 700





Capto Core 700

Sample: *Influenza H1N1 cultivated in MDCK cells, concentrated, and* diafiltrated on an Mr 500 000 hollow-fiber filter to 20 mM Tris, 150 mM NaCl, pH 7.5

Columns: *Tricorn 10/600 packed with Sepharose 4 Fast Flow, CV 47 ml*

Sample loads: *Sepharose 4 Fast Flow, 0.1 CV (4.7 ml)*

Flow velocities*: Sepharose 4 Fast Flow, 30 cm/h*

Columns: Tricorn 5/50 packed with Capto Core 700, CV 1 ml

Sample loads: Capto Core 700, 10 CV (10 ml)

Flow velocities*: Capto Core 700, 100 cm/h*

Buffer: *20 mM Tris, 150 mM NaCl, pH 7.5*

Cleaning-in-place (CIP)/elution: *Capto Core 700, 30% isopropanol in 1 M NaOH*

The sample load for Sepharose 4 Fast Flow was 0.1 CV

The equivalent load for the Capto Core 700 was 10 CV

Covalent or Thiophilic Chromatography

- The protein becomes covalently bound to the matrix through the thiol group of a Cysteine, while the other proteins are washed
- Elution under mild conditions using reducing agents like 2-mercaptoethanol, DTT or glutathione
- Can be used for the purification of antibodies at neutral pH

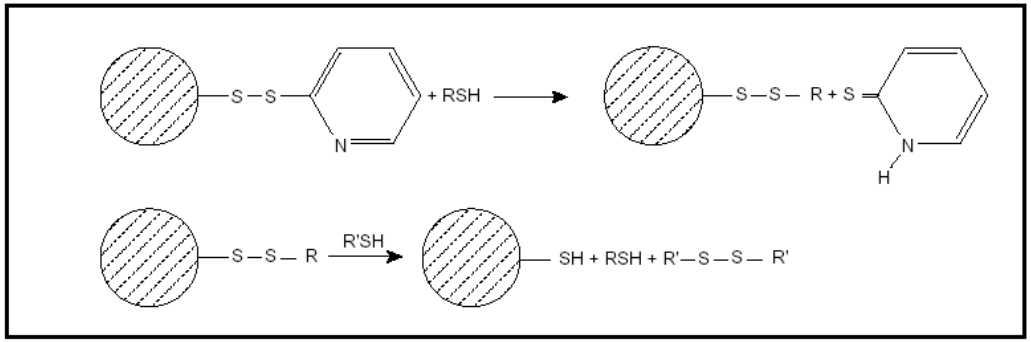
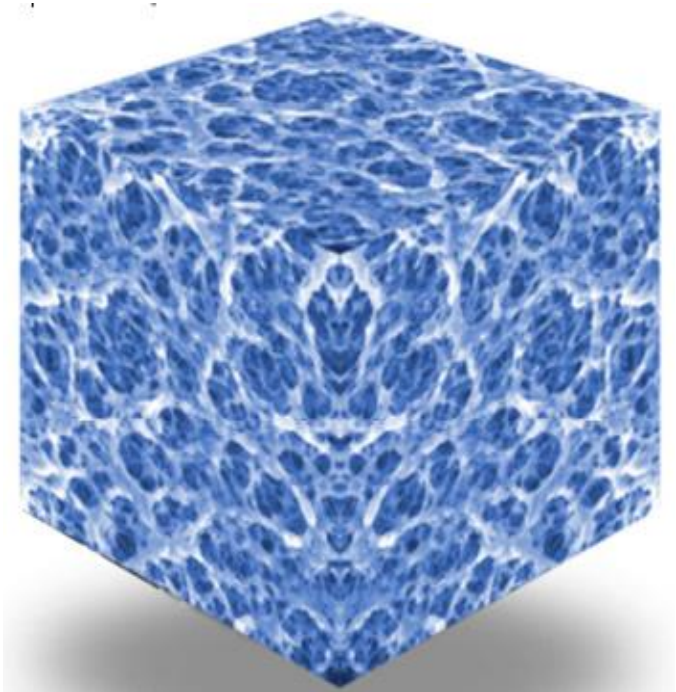


Fig. 2. Reaction scheme for covalent chromatography of a thiolated substance (RSH) on Thiopropyl Sepharose 6B. R'SH represents a low molecular weight thiol such as dithiothreitol.

GE Healthcare

Natrix Technology: HD membranes

- Advanced material with 3D macro porous hydrogel structure
- Contains high density of functional groups
- Rapid mass transfer
- High binding capacity like resins at high flow rates as membranes
- Identical functional binding group as resins



Free-flow electrophoresis (FFE)

https://www.youtube.com/watch?v=umQpk-ryqEk

FFE Service GmbH

GmbH

FFE is an analogous technique to capillary electrophoresis

Fast separation of preparative amounts of samples with a very high resolution

Samples dissolved in a liquid solvent and separated without the need of a matrix, polyacrylamide

Modes of separation

➢ **Isoelectric focusing (IEF)**

The separation buffers contain either commercial ampholytes or Prolyte reagents to form a pH gradient within the separation chamber. Mainly use for the separation of **proteins** and **peptides**

➢ **Zone electrophoresis (ZE)**

Continuous technique for separating different molecules by their net charge

Classical approach for separating **cells** and **organelles**

➢ **Interval Zone electrophoresis (iZE)**

High resolution separation technique for separating different molecules by their net charge.

Suitable for the high resolution separation of **organelles** and **particles** as well as for separating **membrane proteins**, **protein complexes**, **proteins** and **protein isoforms**

Reversed phase chromatography (RPC) Introduction

According to Reversed Phase Chromatography - Principles and Methods – GE

- Separations in reversed phase chromatography depend on the reversible adsorption/desorption of solute molecules with varying degrees of hydrophobicity to a hydrophobic stationary phase.
- Due to the nature of the RPC, the binding under aqueous conditions is usually very strong and requires the use of organic solvents and other additives (ion pairing agents) for elution. Elution is usually performed by increases in organic solvent concentration, most commonly acetonitrile.
- Excellent resolving power
- RPC is often used in the final polishing of oligonucleotides and peptides and is ideal for analytical separations, such as peptide mapping.
- Because of the use of organic solvents, is not recommended for protein purification (needs refolding after purification)
- Mainly use for protein analysis: QC-impurities, disulfide bridges, trypsin cleavage, etc

Reversed phase chromatography (RPC)

Choice of hydrophobic ligand

According to Reversed Phase Chromatography - Principles and Methods – GE

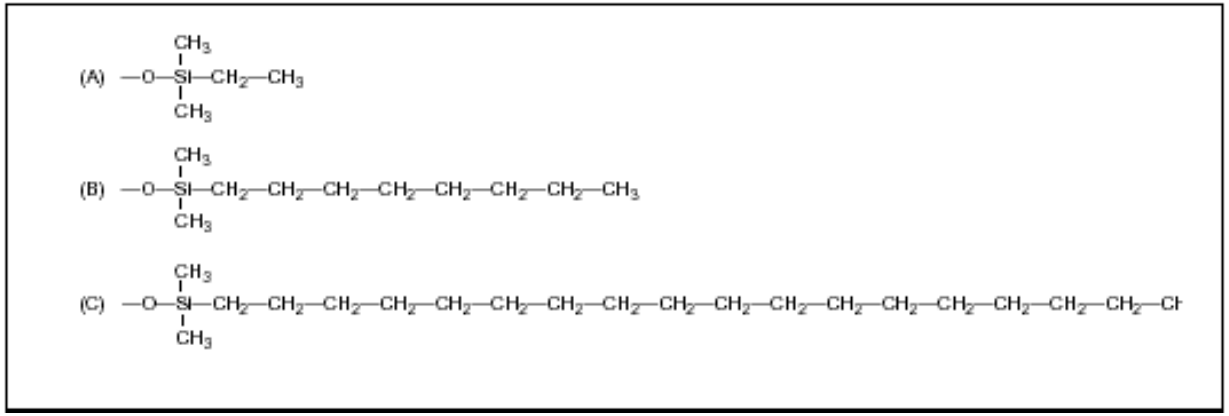
- Linear hydrocarbon chains (n-alkyl groups) are the most popular ligands used in RPC

- Although it is not possible to predict theoretically which ligand will be best for a particular application, a good rule of thumb is: the more hydrophobic the molecule to be purified, the less hydrophobic the ligand needs to be.

- Typically, chemically synthesised peptides and oligonucleotides are efficiently purified on the more hydrophobic C18 ligands

- Proteins and recombinant peptides, because of their size, behave as hydrophobic molecules and most often bind very strongly to C18 ligands. They are usually better separated on C8 ligands.

Fig. 6. Typical n-alkyl hydrocarbon ligands. (A) Two-carbon capping group, (B) Octyl ligand, (C) Octadecyl ligand.



Reversed phase chromatography (RPC)

Critical Parameters

According to Reversed Phase Chromatography - Principles and Methods – GE

- Chemical composition of the base matrix
- Particle size of the bead
- Type of immobilized ligand
- Ligand density on the surface of the bead
- The pore size of the bead.
- Column length
- Flow rate
- Temperature
- Mobile phase
- Organic solvent
- Gradient elution
- Ion pairing agents

The mobile phase used in reversed phase chromatography is generally prepared with strong acids such as trifluoroacetic acid (TFA) or ortho-phosphoric acid.

These acids maintain a low pH environment and suppress the ionisation of the acidic groups in the solute molecules.

Varying the concentration of strong acid components in the mobile phase can change the ionisation of the solutes and, therefore, their retention behaviour.

Reversed phase chromatography (RPC)

Method Development and Recommended Buffer

According to Reversed Phase Chromatography - Principles and Methods – GE

Method Development

1. Select medium from screening results.
2. Select gradient to give acceptable resolution. For unknown samples begin 0-100%B.
3. Select the highest flow rate which maintains resolution and minimises separation time.
4. For large scale purification transfer to a step elution.
5. Samples which adsorb strongly to a gel are more easily eluted from a less hydrophobic medium.

Recommended Buffer

Try these conditions first when sample characteristics are unknown:

Gradient: 2-80% elution buffer B in 20 column volumes

Start buffer A: 0.065% TFA (trifluoroacetic acid) in water

Elution buffer B: 0.05% TFA in acetonitrile