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: \( \text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2 \)

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
Most large proteins bind by a combination of mechanisms.
Calcium affinity occurs via interactions with carboxyl clusters and/or phosphoryl groups on proteins or nucleic acids.

These groups are repelled by the negative PO₄ 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: PO₄ gradient

Acidic proteins, such as albumin use this mechanism, and the presence of high NaCl almost not affect the binding.
Hydroxyapatite Chromatography
Cation exchange Interaction

- **Cation exchange**: protein amino groups interact with the negatively charged $\text{PO}_4$
- **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
Protocol I
IgG

CHT™ ceramic hydroxyapatite

- Equilibrate: 10 column volumes buffer A—10 mM NaPO₄, pH 6.5
- Load: clarified sample with buffer containing 5 mM NaPO₄, pH 6.5
- Wash: 5 column volumes of buffer A—10 mM NaPO₄, pH 6.5
- Elute: 20 column volume buffer B—linear gradient 10 mM NaPO₄, 0–2 M NaCl, pH 6.5
- Clean: 5 column volumes buffer C—500 mM NaPO₄, pH 6.5
- Sanitize: 5 column volumes NaOH

Cation exchange Interaction
Protocol II
Globular Proteins

CHT ceramic hydroxyapatite

Equilibrate: 10 column volumes Buffer A–5 mM NaPO₄, 150 mM NaCl, pH 6.8

Load: clarified sample with buffer containing 5 mM NaPO₄, pH 6.8

Wash: 5 column volumes of buffer A–5 mM NaPO₄, 150 mM NaCl, pH 6.8

Elute: buffer B–linear gradient 500 mM NaPO₄, 0–150 mM NaCl, pH 6.8

Clean: 5 column volumes buffer C–0.5 mM NaPO₄, pH 6.8

Sanitize: 5 column volumes NaOH
Metal affinity and phosphoryl interaction

Protocol III

Plasmids

CHT ceramic hydroxyapatite

Equilibrate: 10 column volumes buffer A–10 mM NaPO₄, 1 mM EDTA, pH 7.0

Load: clarified sample with buffer containing 0.5 M NaCl

Wash: 5 column volumes of buffer A–10 mM NaPO₄, 1 mM EDTA, pH 7.0

Elute: 20 column volumes of buffer B–linear gradient 0–0.4 mM NaPO₄, 1 mM EDTA, pH 7.0

Clean: 5 column volumes buffer A–10 mM NaPO₄, 1 mM EDTA, pH 7.0

Sanitize: 5 column volumes 1 M NaOH
Protocol IV
Acidic Proteins

- CHT ceramic hydroxyapatite

1. Equilibrate: 10 column volumes Buffer A—5 mM NaPO₄, pH 6.8
2. Load: clarified sample with buffer containing 5 mM NaPO₄, pH 6.8
3. Wash: 5 column volumes of buffer A—5 mM NaPO₄, pH 6.8
4. Elute: 20 column volumes buffer B—linear gradient 5—500 mM NaPO₄, pH 6.8
5. Clean: 5 column volumes buffer B—500 mM NaPO₄, pH 6.8
6. Sanitize: 5 column volumes 1 M NaOH

Metal (Ca⁺⁺) Interaction
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.

4-Mercapto-Ethyl-Pyridine (4-MEP)

Palla 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

pl is not a good indicator for choosing the correct pH for binding and elution
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:*
\[ SO_4^{2-} > HPO_4^{2-} > \text{acetate} > Cl^- > NO_3^- > Br^- > ClO_3^- > I^- > ClO_4^- > SCN^- \]

*Cation:*
\[ NH_4^+ > K^+ > Na^+ > Li^+ > Mg^{2+} > Ca^{2+} > \text{guanidinium} \]

**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 pl.
- Requires an increase in both, pH (above the pl) and salt concentration, for effective elution of bound protein.
- Elution is affected by buffer strength and type of salt (NH₄Cl 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

Electrostatic interactions like CEIX
Hydrogen bonding
Hydrophobic interactions
Thiophilic interactions

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

![Chemical structure](image)

electrostatic interactions like AEIX
hydrogen bonding
hydrophobic interactions.
HEA and PPA HyperCel™ Mixed-mode Chromatography

- 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
### Selected commercially available mix-mode media

**Preparative Purification of Recombinant Proteins: Current Status and Future Trends**  

<table>
<thead>
<tr>
<th>Media</th>
<th>Supplier</th>
<th>Type</th>
<th>Ligand</th>
<th>pH stability</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHT ceramic hydroxypatite</td>
<td>Bio-Rad laboratories</td>
<td>Ion exchange, metal chelation</td>
<td>$[Ca_5[PO_4]_3OH]_2$</td>
<td>Operating pH: 5.5–14. Can be cleaned with 1-2 M NaOH</td>
</tr>
<tr>
<td>CHT Fluorapatite</td>
<td>Bio-Rad laboratories</td>
<td>Ion exchange, metal chelation</td>
<td>$[Ca_{10}[PO_4]_6F]_2$</td>
<td>Operating pH: 5–14. Can be cleaned with 1-2 M NaOH</td>
</tr>
<tr>
<td>MEP</td>
<td>Pall life sciences</td>
<td>Hydrophobic binding near neutral pH, elution by pH reduction</td>
<td>4-Mercapto ethyl pyridine</td>
<td>Working pH: 2–12. Cleaning pH: 2–14</td>
</tr>
<tr>
<td>MBI</td>
<td>Pall life sciences</td>
<td>Hydrophobic binding at acidic pH, elution by raising the pH</td>
<td>2-Mercapto-5-benzimidazole sulfonylic acid</td>
<td>—</td>
</tr>
<tr>
<td>Capto MMC</td>
<td>GE Healthcare</td>
<td>Multimodal cation exchange</td>
<td>2-Benzamido-4-mercaptobutanoic acid</td>
<td>Long term: 2–12. Short term: 2–14</td>
</tr>
</tbody>
</table>

- **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

![Chemical structures](image)
Comparing Dynamic Binding Capacity of different resins

DBC Sulfate-650F
Salt-tolerant sulfate strong CEX

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 ($M_r > 700 \text{,}000$) 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
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.
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 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 used 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.
**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