Chromatography
(More)

Voet & Voet:
Pages 133-144
Chromatography

Most powerful separation technique in Biochemistry

- Mixture is dissolved in 'mobile phase' and percolated through a 'stationary phase'
- Continuous process that subjects sample to repeated, identical separations

Chromatographic methods are classified according to:

1) type of mobile and stationary phase
   eg. liquid-liquid, gas-liquid, ... 
2) type of retarding force
   eg. ion exchange, size exclusion, hydrophobic interaction, ...
Ion Exchange Chromatography

Stationary phase is an insoluble, inert bead uniformly coated with charged functional groups

- **Anion exchange**: beads have positive charges and bind anions
- **Cation exchange**: beads have negative charges and bind cations

Ions (including proteins) in the mobile phase reversible bind stationary phase

Ions compete for available binding sites based upon their concentration and strength of binding

Typically, the mobile phase is chosen to maximize the binding of the sample of interest

Note: The net charge (and strength of binding) of a protein is highly dependent on the pH
Stepwise vs. Gradient Elution

For IEC, bound proteins are removed or eluted by

1) changing the concentration of a competing ion (ie. High salt concentration)
2) changing the pH of the mobile phase (ie. Changing the net charge of the protein)

The pH or salt concentration change can be abrupt or continuous

**Stepwise elution** – abruptly change mobile phase pH or salt concentration

**Gradient elution** – continuously change mobile phase pH or salt concentration using a gradient maker

---

**Diagram:**

- **Stepwise:**
  - Absorbance (blue)
  - Conductance (red)

- **Gradient:**
  - Absorbance (blue)
  - Conductance (red)

**Legend:**
- **High Salt**
- **Low Salt**
- **Simple Gradient Maker**
- **To column**
- **Stir bar**
Matrix and Detection

Common ion exchange stationary phases

- **Strongly Basic**: eg. Trimethylammonium (Q column)
- **Strongly Acidic**: eg. Sulfonates (S column)
- **Weakly Basic**: eg. Ethyleneamine (DEAE column)
- **Weakly Acidic**: eg. Carboxylates (CM column)

Wide range of available detection methods:

- **UV absorbance**: proteins have intrinsic UV absorbance
- **Fluorescence**: requires labeling of sample prior to separation
- **Activity**: requires assay (eg. Enzyme activity, ligand binding, ...)
- **Radioactivity**: requires labeling of sample prior to separation
- ...
- ...
Size Exclusion Chromatography

Separation based upon molecular size and shape

Stationary phase contains small pores that preferentially retard small samples

Behaviour of molecules can be quantitatively characterized (ie. $M_w$)

\[ V_t = V_x + V_o \], where $V_t$ is the total column volume, $V_x$ is volume occupied by the stationary phase and $V_o$ is void (remaining) volume

The ratio of the elution and void volumes ($V_e/V_o$) varies approximately linearly with log $M_w$ over a wide range
# SEC Matrices

<table>
<thead>
<tr>
<th>Name</th>
<th>Type</th>
<th>Fractionation Range (kD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sephadex G-10</td>
<td>Dextran</td>
<td>0.05 – 0.7</td>
</tr>
<tr>
<td>Sephadex G-25</td>
<td>Dextran</td>
<td>1 – 5</td>
</tr>
<tr>
<td>Sephadex G-50</td>
<td>Dextran</td>
<td>1 – 30</td>
</tr>
<tr>
<td>Sephadex G-100</td>
<td>Dextran</td>
<td>4 – 150</td>
</tr>
<tr>
<td>Sephadex G-200</td>
<td>Dextran</td>
<td>5 – 600</td>
</tr>
<tr>
<td>Sepharose 6B</td>
<td>Agarose</td>
<td>10 – 4000</td>
</tr>
<tr>
<td>Sepharose 4B</td>
<td>Agarose</td>
<td>60 – 20000</td>
</tr>
<tr>
<td>Sepharose 2B</td>
<td>Agarose</td>
<td>70 – 40000</td>
</tr>
<tr>
<td>Bio-Gel P-2</td>
<td>Polyacrylamide</td>
<td>0.1 – 1.8</td>
</tr>
<tr>
<td>Bio-Gel P-6</td>
<td>Polyacrylamide</td>
<td>1 – 6</td>
</tr>
<tr>
<td>Bio-Gel P-10</td>
<td>Polyacrylamide</td>
<td>1.5 – 20</td>
</tr>
<tr>
<td>Bio-Gel P-30</td>
<td>Polyacrylamide</td>
<td>2.4 – 40</td>
</tr>
<tr>
<td>Bio-Gel P-100</td>
<td>Polyacrylamide</td>
<td>5 – 100</td>
</tr>
<tr>
<td>Bio-Gel P-300</td>
<td>Polyacrylamide</td>
<td>60 – 400</td>
</tr>
</tbody>
</table>
Dialysis is a commonly used, non-chromatographic filtration method

Preferred method for changing non-protein solutes (buffers, salts, ligands, etc.)

Dialysis membranes contain small pores that allow molecules smaller than the pore size to freely diffuse (eg. Non-protein solutes)

Molecules larger than the pore size are retained (eg. Proteins)
Assume you have 10.0 mL of a protein solution in a solution containing 1.00 M NaCl. If you dialyze the protein sample (until equilibrated) against a 0.500 L solution containing 0.100 M NaCl what is the concentration of NaCl in the protein solution?

\[
(C_{NaCl, \text{protein}}) (V_{\text{protein}}) + (C_{NaCl, \text{dialysate}}) (V_{\text{dialysate}}) = (C_{NaCl, \text{total}}) (V_{\text{total}})
\]

\[
(1.00 \text{ M}) (0.0100 \text{ L}) + (0.100 \text{ M}) (0.500 \text{ L}) = (C_{NaCl, \text{total}}) (0.510 \text{ L})
\]

\[
\frac{(0.0100 \text{ moles}) + (0.0500 \text{ moles})}{0.510 \text{ L}} = (C_{NaCl, \text{total}})
\]

\[
0.118 \text{ M} = (C_{NaCl, \text{total}})
\]

Process can be repeated by replacing the dialysate until desired solute concentrations are obtained.
Affinity Chromatography

Exploits characteristic or engineered ligand binding properties of proteins

Recombinant DNA technology can be used to add a specific tag (binding activity) to a recombinant protein (fusion protein)

Elution is typically accomplished by adding excess ligand

Ligand is attached to inert, porous matrix by one of several chemical methods

Note: A portion of the ligand is sterically blocked by the attachment to the matrix
Paper Chromatography

Historically important technique used to separate amino acids, nucleotides and metabolites

Stationary phase is cellulose-based paper

Mobile phase can be more polar or less polar than the stationary phase
- water, acetic acid, formic acid are more polar than stationary phase
- organic solvents (hexane, ketones, ethers) are less polar

 Separates small, relatively polar samples due to polar stationary phase (typically non-recoverable)

Resolving power can be readily improved by adding a second dimension using a second solvent system
Thin Layer Chromatography

**Improvement over paper chromatography**

Stationary phase can be any of a variety of materials (ie. Silica gel – polar, C18 – non polar) attached to a glass or plastic plate

- allows for many less polar stationary phases when compared to 'paper chromatography'

Mobile phase can be **more polar or less polar** than the stationary phase

- water, acetic acid, formic acid are very polar
- organic solvents (hexane, ketones, ethers) are very non polar

Often used to separates non polar organic compounds (eg. Lipids)

Has largely replaced paper chromatography due to the variety of stationary phases and their mechanical stability

---

Often requires comparison to standard samples
Hydrophobic Interaction
Chromatography

Stationary phase is lightly substituted hydrophobic matrix

- Separation is based upon the hydrophobic surface of a native protein
- Difficult to predict how a protein will behave

Mobile phase is highly polar (high salt, aqueous solution)

- Elute by decreasing ionic strength (lowering salt concentration)

Particularly useful for separating:

- A) membrane associated proteins
- B) protein subunits
Reverse Phase Chromatography

Conceptually similar to Hydrophobic Interaction Chromatography (HIC)

Stationary phase is far more hydrophobic in Reverse Phase

Essentially an immobilized organic solvent layer

Mobile phase is the more polar solvent (hence reverse phase)

Unlike HIC, proteins are denatured when bound or partitioned into stationary phase

Elute samples with organic solvents

Wide variety of stationary phases allow separation of virtually any non-polar or weakly polar compound

Note: Require powerful pumps due to exceptionally large back pressures (up to 5000 psi)