# Chromatography Column Mass Balance with Negligible Dispersion

# Examples: $\alpha$ and elution volume

- **Example1**

**: large sample volume**

  - Sample emerging after 1/(1-$\alpha$) column volume
  - Sample volume = 5 x column volume (Vc) and washing with 5 x column volume
    - $\alpha$ of a protein to remain in the column
    - 1/(1-$\alpha$) x Vc > 10 Vc
    - $\alpha$ > 0.9

- **Example 2**

**: Protein of interest has $\alpha$ of near 0**

  - Purification of protein in the flow-through fraction

# Example : Linear equilibrium

- **Example3 : Chromatographic separation of two solutes**
  - Conditions
    - Linear equilibrium constant $K_{eq,1}$ = 7.5, $K_{eq,2}$ = 7.8
    - Flow rate : 1.5 liter/min
    - Column diameter: 63 cm
    - Void fraction : 0.33
  - (Q) What column length (L) is required to separate the two solute by 5 min ?
  - (S)
    - $u_1$ =
    - $v$=
    - Elution time for solute 1 =

$$u_i = \frac{v}{\varepsilon + (1-\varepsilon)q_i'(c_i)}$$

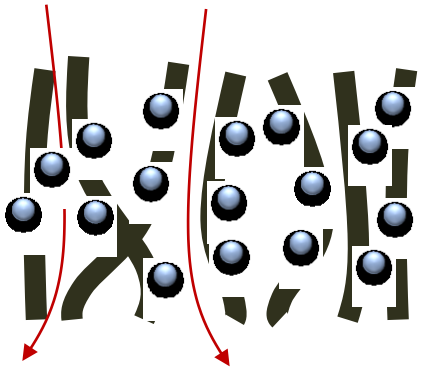
# Example : Shock Wave Velocity

- **Effective solute velocity at the front**
  - The concentration change is not continuous but is a step change
  - $u_{i,sh} = v/ [\varepsilon + (1- \varepsilon)(\Delta q_1/ \Delta c_1)]$
- **Example4 : Shock wave velocity for a nonlinear isotherm**
  - Conditions
    - Solute 1 with Langmuir isotherm characteristics, $K_{eq}$ = 7.5 ml/mg
    - $S_{tot}$ = 120μg/ml
    - Same column conditions as Example 3
  - (Q) Calculate shock wave velocity for an injection of 1 mg/ml
  - (S)
    - [CS] = $\{K_{eq}S_t [C]\}/ \{1+ K_{eq}[C]\}$ =
    - $\Delta q_1/ \Delta c_1$ =
    - $u_{i,sh}$ =

# Membrane Adsorbents: Radial Flow Columns

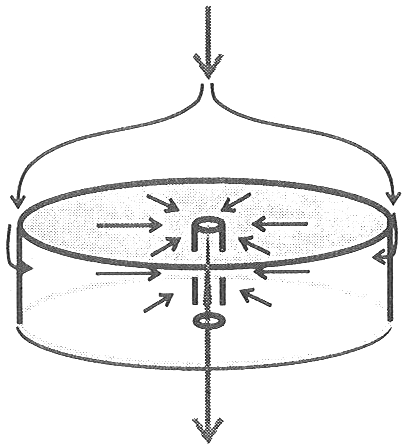
# Membrane adsorbent

- **A stack of many membranes**
- **Advantage of membrane**
  - Large surface area per unit volume
  - No diffusion of solute away from the buffer flow
  - Fast flow rate
    - Resistance against pressure is determined mainly by pore size
    - The increase in required pressure is proportional to the stack thickness
    - Can apply fast flow rate (100-500 cm/h)
      - No significant time delay for binding
- **Capacity**
  - 0.1 ~ 0.3 mg/cm$^2$, 10 mg/cm$^3$ (50 membrane ~ 1 cm)
  - 5 to 10 times less than conventional column beads (ion exchange)



# Radial Flow Column

- **Increase flow rate**
  - Flow from outside of the column cylinder to the inside
  - Collection tube receives the buffer flow
  - High flow rate
    - Because of large cross-sectional area of initial contact



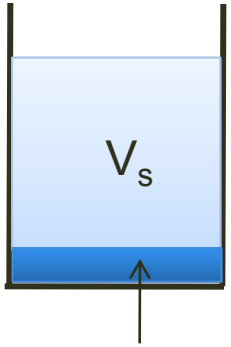
# Batch Adsorption

# General Principles

- **Methods**
  - Mix adsorbent with proteins
  - Recovery and washing by flocculation, filtration, or centrifugation
  - Can be used to remove unnecessary proteins
- **Requirement**
  - $\alpha$ should be very close to 1
  - Concentration of free protein is the same throughout the volume
    - The amount of protein adsorbed : $qV_a$
      - q : conc. of adsorbed protein
    - The amount of protein not adsorbed : $p(V_a + V_s)$
      - p : conc. of free protein
    - Partition coefficient

      $\alpha = q/(p + q)$, $p = q(1- \alpha)/\alpha$
    - Fraction protein adsorbed

      $f = qV_a/[p(V_a + V_s) + qV_a] = V_a \alpha /[V_a + (1- \alpha)V_s]$



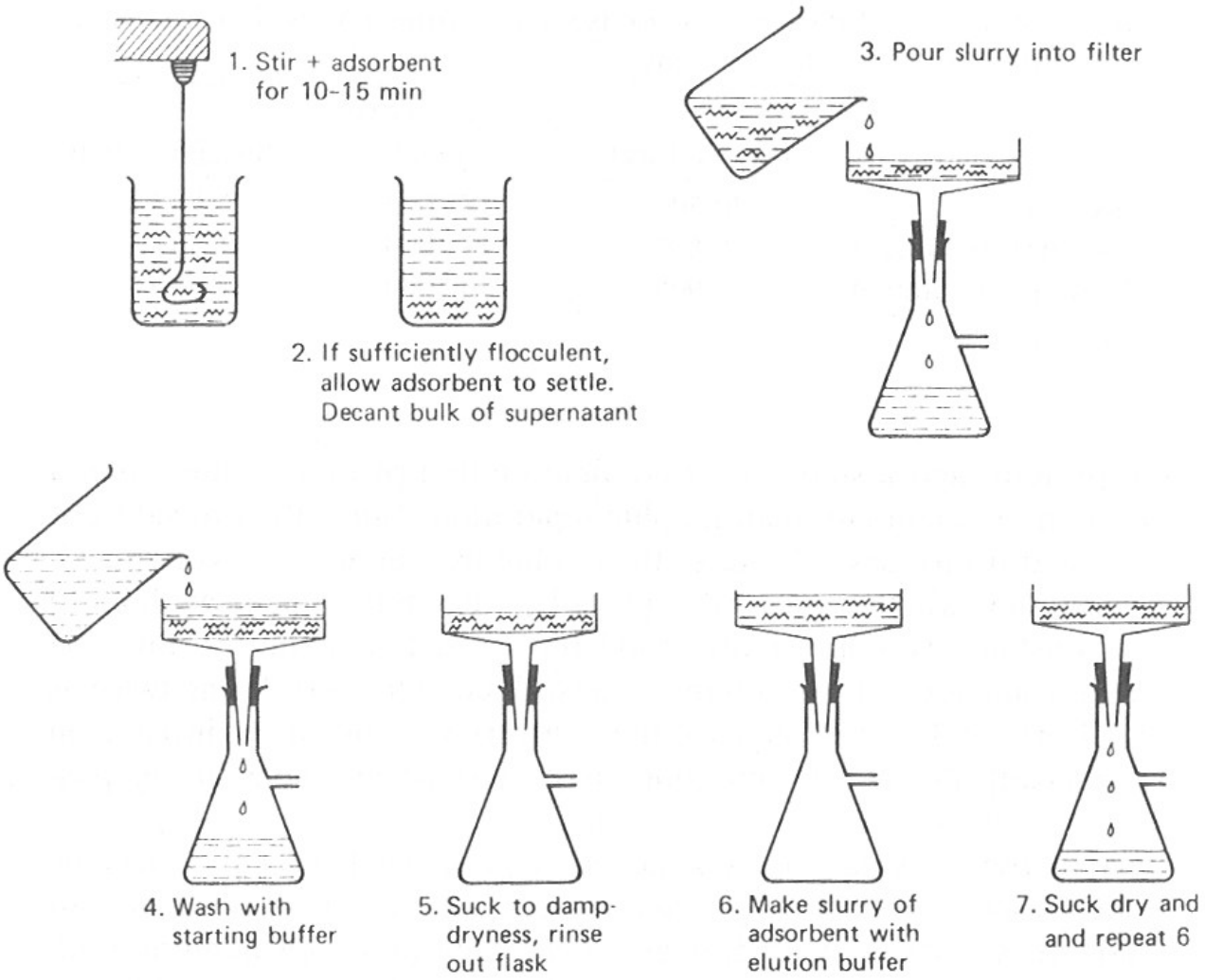

$V_a$ : Volume of adsorbent when settled

# Application of Batch Adsorption

- **Requirement for high α**
  - α ≈ 1 for 80-90% yield
    - Need to optimize α by changing binding buffer
  - $K_p < 10^{-6}$ M for α > 0.98
- **Suitable for highly specific adsorbents**
  - Immunoadsorbents
  - Dye ligand adsorbents
- **Batch vs. column**
  - Batch
    - Fast
    - Can treat large volume
    - Loss of protein if α <0.98
  - Column
    - 100 % adsorption even at α =0.95
    - Slower
    - Possible clogging with crude extracts

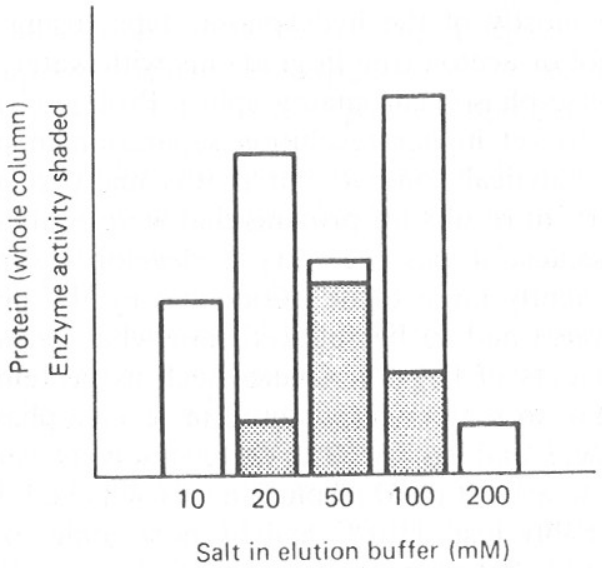
| $V_a/V_s$ | α | f |
|---|---|---|
| 0.05 | 0.9 | 0.30 |
| 0.1 | 0.9 | 0.45 |
| 0.2 | 0.9 | 0.60 |
| 0.5 | 0.9 | 0.75 |
| 0.1 | 0.95 | 0.63 |
| 0.2 | 0.95 | 0.76 |
| 0.5 | 0.95 | 0.86 |
| 0.05 | 0.98 | 0.70 |
| 0.1 | 0.98 | 0.82 |
| 0.1 | 0.98 | 0.89 |
| 0.1 | 0.99 | 0.99 |
| 0.02 | 0.998 | 0.91 |
| 0.05 | 0.998 | 0.96 |

# Practical Approach



# Practical Approach

- **Step-wise elution**
  - Wash with 20 mM salt
  - Elution with 50-60 mM salt
- **Typical batch adsorbents**
  - Hydroxyapatite
  - Ion exchangers
  - Affinity adsorbents
  - Dye ligand adsorbents
  - Hydrophobic adsorbents
  - Immunoadsorbents



# High-Performance Liquid Chromatography

# HPLC

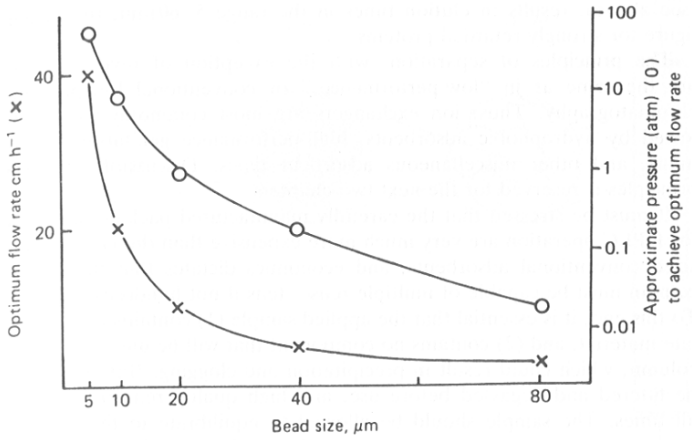
- **High pressure liquid chromatography**
  - Developed for separation of low-molecular weight compound
    - Fast flow rate to prevent diffusion
    - Smaller beads for faster flow rates
    - Higher pressure for smaller beads
  - Reverse-phase chromatography
    - Using organic solvents methanol, acetonitrile in gradients with water or acidic solutions
- **High performance liquid chromatography**
  - High pressure is not as important as high quality of the adsorbent material

# Bead Size, Flow Rate, Pressure, and Optimum Performance

- **Ways to increase plate number**
  - Long column → increase in operation time
  - Small and spherical beads → decrease in flow rate → need to develop pressure-resistant column
- **Relationship between parameters**
  - Rate of attainment of equilibrium
    - $\propto$ $1/D$ (diffusion coefficient of solute)
    - $\propto$ $1/d_p$ ( bead diameter)
  - Flow rate
    - $\propto$ $d_p^2$ (assuming ideal packing)
  - Resolution
    - $\propto$ $\sqrt{L}$ (column length)
  - Optimum performance
    - At the smallest value of plate height which depending on flow rate

# Reduced Velocity

- **Determination of an ideal flow rate**
  - $V_{red} = v\, d_p/D$
    - $v$= linear flow rate
    - $d_p$ = bead diameter
    - $D$= diffusion coefficient of solute
  - Optimum $V_{red}$ : 3~10
    - 50-200 kDa protein, $d_p$ 100 μm (conventional low-pressure chromatography) → $v$ < 2cm/h : normal operation : 20 cm/h
    - $d_p$ 10 μm (HPLC) → $v$ < 20cm/h, normal operation: 10 times faster
    - For $d_p$ 20-40 μm
      - Optimum flow rate ∝ 1/ $d_p$
      - Pressure to obtain the same flow rate ∝ 1/ $d_p^2$



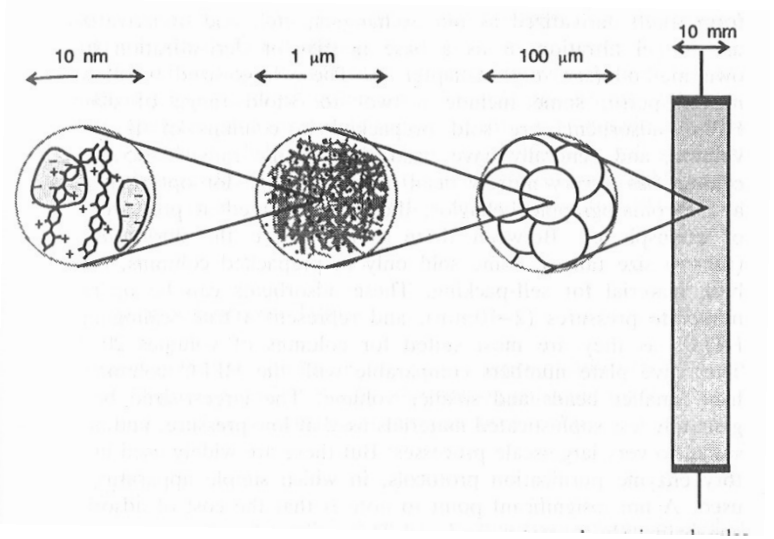
# HPLC

- **Bead size and Resolution**
  - Optimum resolution in the presence of $5 \times 10^8$ beads
    - 0.5 ml column for 10 μm beads
    - 200 ml for 80 μm beads
- **HPLC columns for protein works**
  - Adsorbents
    - Silica particles
    - Synthetic organic polymers with hydrophilic groups
  - 1-20 ml total volume
  - Theoretical plate height > 0.05 mm
  - 0.5-2 ml/min → protein elution after 5-60 min
  - Cautions
    - Do not use particulate material nor unstable compounds in the sample to prevent precipitation of clogging
    - Filtration and degassing of the buffer before use
    - Use high quality reagents
    - Equilibrate the sample to the operation conditions and filtration through a 0.2 μm filter

# Types of Adsorbent Used in Protein Chromatography

# Adsorbents

- **Essential features of adsorbents**
  - The nature of the matrix
  - The types of functional group that carries out the adsorption
- **Nature of bead matrix**
  - Matrix
    - Insoluble polymer which is cast into bead form
    - Requirements
      - Porosity : allow protein penetration
      - Rigidity
  - Matrix materials
    - Carbohydrate polymer
      - Agarose, cellulose, dextran
    - Synthetic organic polymers
    - Inorganic matrices
      - Silica
    - Composite structures



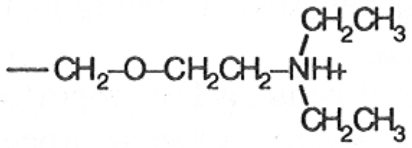
# Adsorbents

- **Bead size**
  - For operation at low pressure (up to 2 atm)
    - 50-150 μm, not monodisperse
  - For operation at moderate pressure (2-10 atm)
    - 30-40 μm
    - Most suited for columns of 20-500 ml volume
  - HPLC adsorbents (10s atm)
    - 5-15 μm
    - Narrow bead diameter range for optimum packing
    - Packed in 1-20 ml volume
- **Attachment of ligand to the bead matrix**
  - By simple chemical reaction
    - Ion exchangers, hydrophobics
  - Introduction of spacer arms for affinity ligands

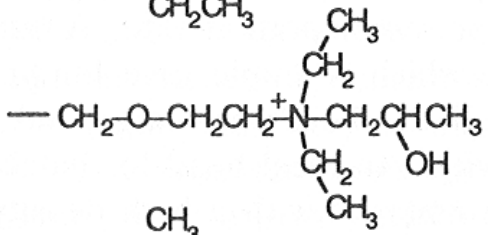
# Adsorbent Types 1: Ion Exchangers

- **Principles**
  - Interaction of proteins by electrostatic attraction
- **Types**
  - Anion exchangers
    - Positively charged matrix
    - Substituents attached to –OH on the matrix
      - Diethylaminoethyl(DEAE)
        - » $NH^+$ : complete deprotonation above pH 9.5
        - » Partly charged at pH7-8 : buffering effect for protein separations
      - Quaternary amino ethyl (QAE) : attached
        - » $N^+$

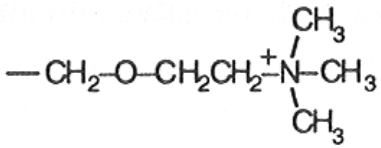
DEAE



Hydroxypropyl diethyl aminoethyl, Q-



Trimetyl aminoethyl, TMAE-, Q-



# Adsorbent Types 1: Ion Exchangers

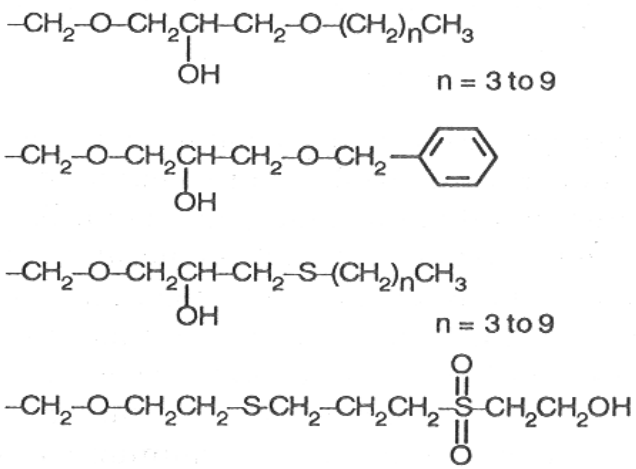
- Cation exchangers
  - Negatively charged matrix
  - Substituents
    - Weak carboxymethyl susbtituents (CM)
      - » Fully charged above pH 4.5
    - Strong sulfonate groups (S- and SP-)
      - » < pH 4
    - Phosphates : certain affinity actions
      - » Interaction with enzymes that bind multiply-phosphorylated substrates (e.g. nucleic acids), sugar phosphates

Carboxymethyl (CM) —$CH_2$–O–$CH_2COO^-$

Sulfopropyl (SP- or S-) —$CH_2$–O–$CH_2CH_2CH_2SO_3^-$

# Hydrophobic and Reverse Phase Adsorbents

- **Principles**
  - Binding to proteins by hydrophobic interactions
  - Strength of interaction
    - Higher interaction: high salt, high temperature
    - Weak interaction: presence of detergents or miscible organic solvents
  - Poorer resolution than ion exchange chromatography
- **Substituents to -OH**
  - Short aliphatic chains ($C_4$ to $C_{10}$)
  - Benzyl group
- **Reverse phase adsorbents**
  - Short aliphatic chains (C8 to C18)
  - For HPLC, hydrophobic matrix, hydrophilic solvent
  - Useful for small, stable proteins up to 30 kDa
    - Binding of denatured protein to the adsorbent
    - Bioactivity depends on the reversibility of the denaturation
  - High resolving power



# Other Adsorbents

- **Inorganic adsorbents**
  - Inorganic oxides and phosphates : batch mode
  - Hydroxyapatite : column
- **Affinity adsorbents**
  - Mimicking protein-ligand interaction
  - Biomimetic
    - Dyes, ralatively nonspecific multifunctional adsorbents, metal chelaters binding to target proteins
- **Immunoadsorbents**
  - Antibody adsorbents for protein purification
  - Protein A/G for antibody purification

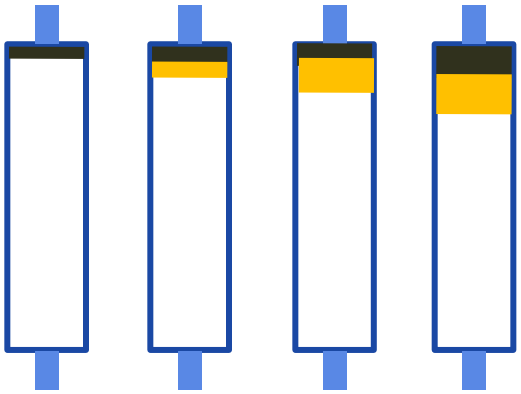
# Operating Conditions for Column Chromatography

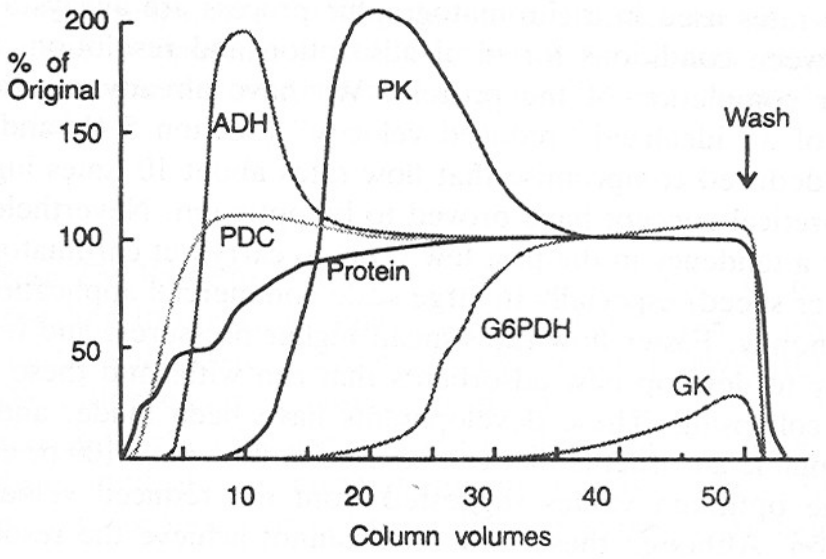
# Sample Application

- **No particulate matter in the sample**
  - Filtration
  - Prevent using conditions (pH, temperature, salt concentration) which can induce protein aggregation
- **Adjust protein concentration of the sample**
  - Ultrafiltration or dilution
  - <10 mg/ml in general
    - At higher concentration
      - Proteins may affect the adsorption behavior
      - Generate osmotic pressure
  - For protein with low $\alpha$ value
    - High protein concentration and low volume
- **Determining the size of column**
  - Consider capacity of adsorbents
    - Ion exchangers : 1-10 mg/ml of adsorbent
      - Smaller loading → high loss, unnecessary cost, reduction of speed
  - If resolution is important : limited loading volume
  - If resolution is good or not very important : high loading is possible (e.g. affinity chromatography)

# Overload and Displacement Chromatography

- **Principle**
  - Displacement of weakly binding proteins by strongly binding proteins
  - Use insufficient adsorbent to bind all proteins
  - Suitable for purification of proteins with high binding affinity





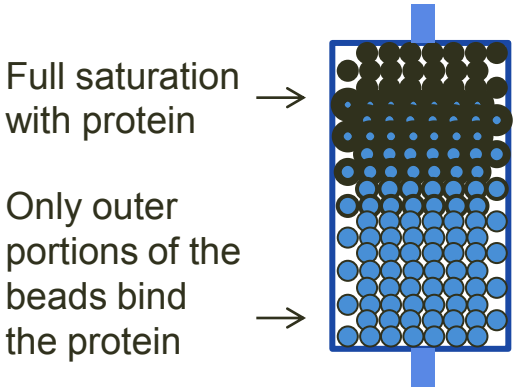

Purification of glucokinase (GK) using dye adsorbent

# Flow Rates

- **Optimal flow rate**
  - 10 times faster flow rate than that suggested from reduced velocity ($V_{red}$)
  - For large beads (80-150 μm) : 20-30 cm / h at 20 °C, half of the rate in the cold room
  - For 30-40 μm beads : 50-100 cm / h at 20 °C
  - HPLC column with 10 μm beads : 2-4 cm / min
- **Fast flow rate**
  - 100~1,000 times faster flow rate than that suggested from $V_{red}$
  - Need adsorbents resistant to high pressure
  - Loss of resolution
  - Gain of operational and economical advantage
- **Dynamic capacity**
  - Reduction of capacity at high flow rate
  - Depending on the time needed for proteins to penetrate the beads and find the binding sites

| Flow rate, cm/h | Mg IgG bound/ml of adsorbent |
|---|---|
| 0 | 23 |
| 30 | 18 |
| 100 | 12.3 |
| 200 | 7.1 |
| 300 | 4.7 |
| 500 | 2.3 |
| 1000 | 0.83 |

- ProteinG Sepharose™ Fast Flow
- $V_{red}$: 1-3 cm/h




Effect of fast flow rate during column loading