

Biological Chemistry Laboratory  
Biology 3515/Chemistry 3515  
Spring 2017

Lecture 24:

Gel Filtration Chromatography

11 April 2017

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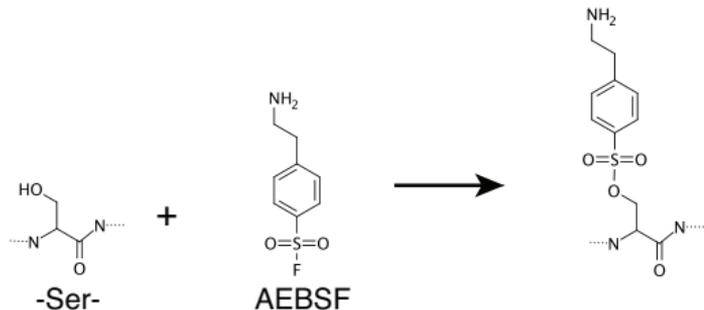
# Public Service Announcement

## American Chemical Society Student Chapter TA of the Semester Award

<https://chem.utah.edu/undergraduate/ta-nomination-form.php>

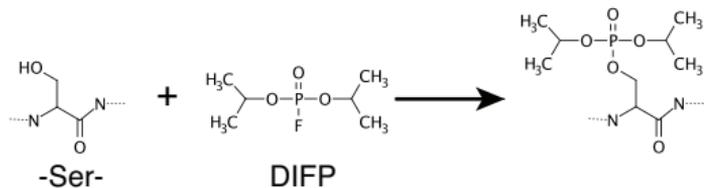
- Winner gets fame and glory, a \$25 gift certificate **and** is eligible for TA of the year award (\$1,000!)
- Anyone in Biol 3515 or Chem 3515 can nominate their TA.
- “Please describe how this TA has enriched your experience in chemistry. Please be as specific as possible. The winner will be chosen based on the nomination statements, not a popular vote.”
- Last day for nominations: Thursday, 13 April

# An Irreversible Enzyme Inhibitor in the News



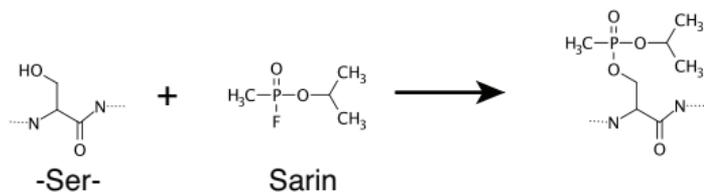
## 4-(2-aminoethyl)-benzenesulfonyl fluoride(AEBSF)

- Relatively specific for proteases, versus acetylcholine esterase.
- LD<sub>50</sub> for mice ≈ 2,800 mg/kg.



## Diisopropyl fluorophosphate (DIFP)

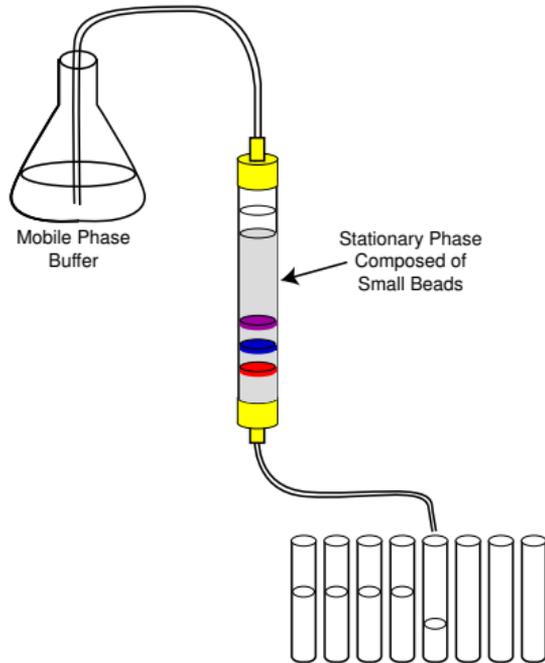
- Highly reactive with serine proteases and acetylcholine esterase.
- LD<sub>50</sub> for mice ≈ 8 mg/kg.



## Isopropyl methylfluorophosphate (sarin)

- Sarin
- LD<sub>50</sub> for mice ≈ 0.2 mg/kg.

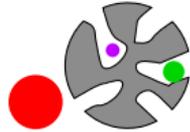
# Column Chromatography: The Most Commonly Used Format for Biochemistry



- Sample is placed at top of column.
- Buffer flows through and around beads in stationary phase and carries sample molecules.
- Molecules that interact the least with the beads elute from the column first.
- Eluent is fractionated into tubes as it leaves the column. (automatic fraction collector)
- Eluent can be analyzed as it leaves the column (e.g., spectrophotometer with flow cell) or from fractions.

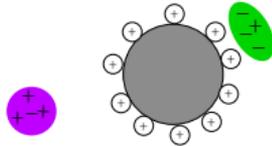
# Chromatography Methods Commonly Used for Biomolecules

- Gel filtration chromatography - based on molecular size

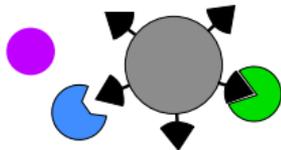


Form that we will use in lab.

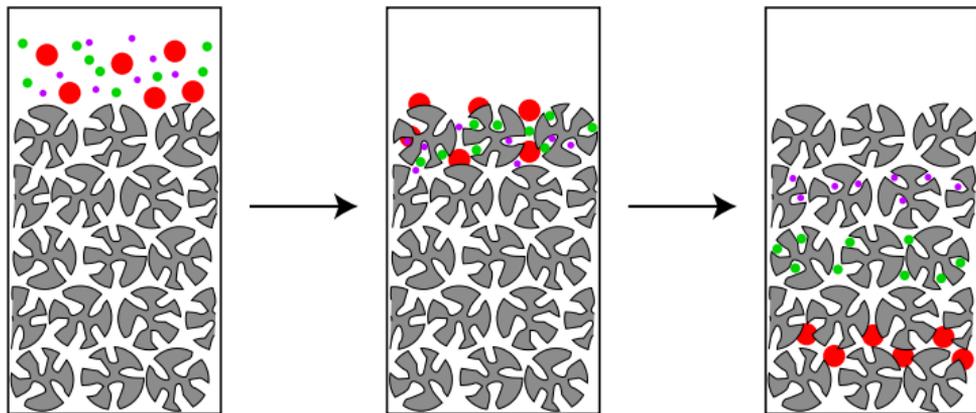
- Ion exchange chromatography - based on electric charge



- Affinity chromatography - based on specific biochemical interactions

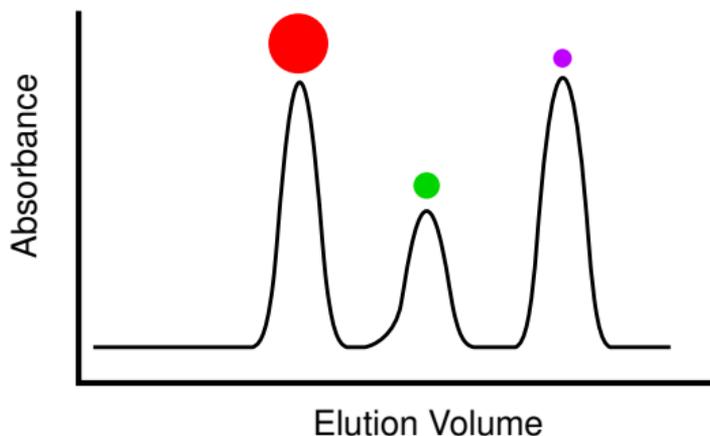


# Gel Filtration Chromatography



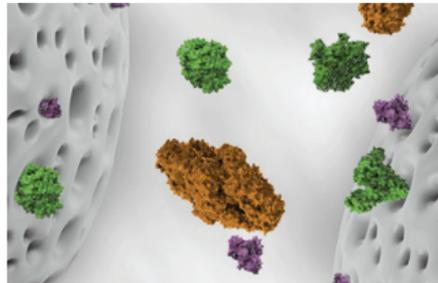
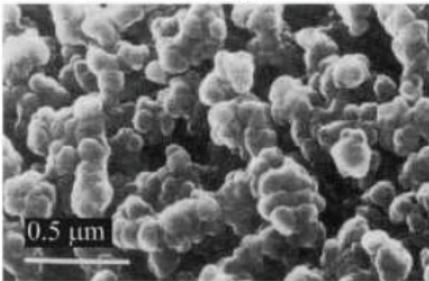
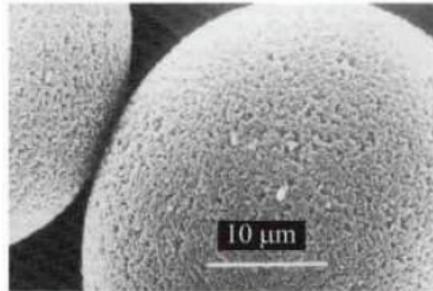
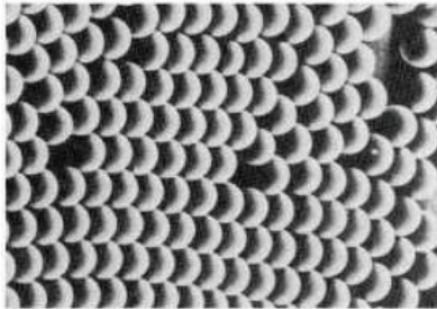
- Also called “gel permeation”, “size exclusion”, “sizing” or “molecular sieve” chromatography.
- Beads are made of a porous gel. (similar to gels used for electrophoresis)
- Separates molecules on basis of size.
- Larger proteins elute first. (opposite of gel electrophoresis!)
- Beads have a distribution of pore sizes.

## Elution Profile for a Gel Filtration Column



- Elution volume is the volume of buffer that flows through the column between when the sample is applied to the top and when a particular protein leaves the column.
- Can be calibrated with proteins of known size in order to estimate molecular weights of other proteins.
- Unless a denaturant is present, elution volume usually reflects molecular weight of native protein, with quaternary and tertiary structure intact, unlike SDS gel electrophoresis.
- Media with different distributions of pore sizes are used to separate molecules of different size ranges.

# Scanning Electron Micrographs (and a cartoon) of Gel Filtration Media



Micrographs by A. Medin, Department of Biochemistry, Uppsala University.

Found at: <http://macromol.sbcs.qmul.ac.uk/oldsite/expertise/Beads2a.jpg>

Cartoon by GE HealthCare:

<http://www.gelifesciences.com/webapp/wcs/stores/servlet/catalog/en/GELifeSciences-us/brands/superdex/>

## Clicker Question #1

- Suppose that we have a protein with a molecular weight of 85 kDa, and we want to separate this protein from both smaller and larger molecules?
- What range of pore diameters should the gel filtration media have?
  - 1 0.4–2 nm
  - 2 2–10 nm
  - 3 10–50 nm
  - 4 50–250 nm

All answers count for now!

# How Do We Estimate the Diameter of a Protein?

## ■ What do we know?

- The mass of the molecule:  
 $85,000 \text{ g/mol} \div 6.02 \times 10^{23} \text{ molecule/mol}$   
 $= 1.41 \times 10^{-19} \text{ g/molecule}$

## ■ What else do we need to know (or assume)?

- The density of the molecule (mass/vol)
- Is the density of a protein greater or less than 1 g/mL?
- Proteins sediment in the ultracentrifuge!  
Density must be greater than that of water.
- Because of protein interaction with water, defining density is a bit tricky.  
But, we will assume  $\approx 1 \text{ g/mL}$ .

## ■ Anything else?

- What is the shape of the molecule?
- Determines relationship between volume and linear dimensions.
- We will assume a sphere.

# Calculating the Diameter of a Protein

## ■ Volume

$$1.41 \times 10^{-19} \text{ g} \div 1 \text{ g/mL} \\ = 1.41 \times 10^{-19} \text{ mL} = 1.41 \times 10^{-19} \text{ cm}^3$$

$$1.41 \times 10^{-19} \text{ cm}^3 \times \left( \frac{1 \text{ m}}{100 \text{ cm}} \right)^3 \\ = 1.41 \times 10^{-25} \text{ m}^3$$

## ■ Radius

$$V = \frac{4}{3} \pi r^3$$

$$r = \sqrt[3]{\frac{3}{4\pi} V} = \sqrt[3]{\frac{3}{4\pi} 1.41 \times 10^{-25} \text{ m}^3} = 3.2 \times 10^{-9} \text{ m} = 3.2 \text{ nm}$$

## ■ Diameter

$$d = 2r = 6.5 \text{ nm}$$

## Clicker Question #1 Revisited (Without Clickers)

- Suppose that we have a protein with a molecular weight of 85 kDa, and we want to separate this protein from both smaller and larger molecules?
- What range of pore diameters should the gel filtration media have?

1 0.4–2 nm

2 2–10 nm

3 10–50 nm

4 50–250 nm

## Clicker Question #2

- Suppose that we have a protein with a molecular weight of 170 kDa, twice that of the previous example.
- What is the diameter of this protein, if it is also spherical?

1  $\approx 3$  nm

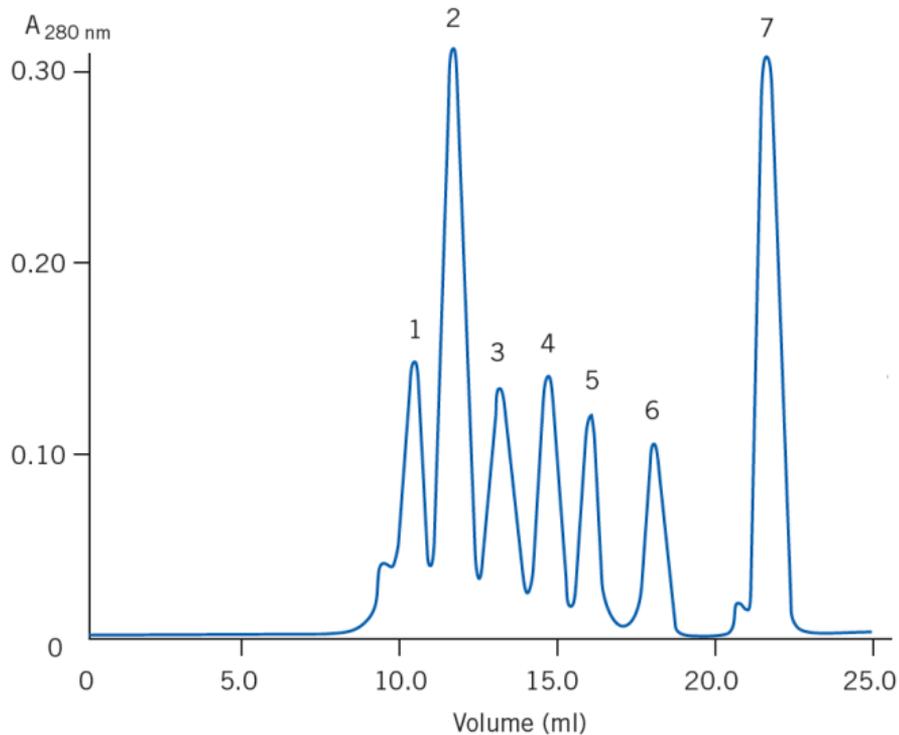
2  $\approx 6$  nm

3  $\approx 8$  nm

4  $\approx 12$  nm

- Doubling the mass of a spherical particle only increases its diameter by about 25%.

# Example Gel Filtration Separation of Proteins



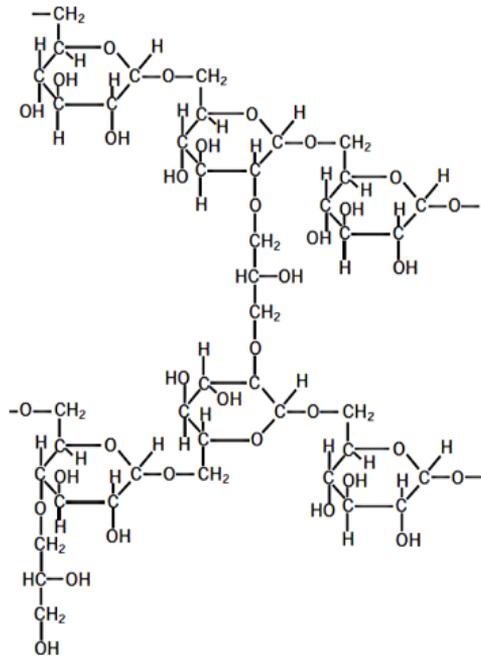
## ■ Proteins:

1. Thyroglobulin (669,000 Da)
2. Ferritin (440,000 Da)
3. Human IgG (150,000 Da)
4. Ovalbumin (43,000 Da)
5. Myoglobin (17,000 Da)
6. Vitamin B<sub>12</sub> (1,335 Da)

## ■ Media: Superdex 200

- Good separation by gel filtration requires a difference in MW of about 2-fold, or greater.

# Sephadex: The First Practical Gel Filtration Media for Proteins



- Cross-linked dextran
  - Dextran: Glucose-based polysaccharide linked by  $\alpha$ -1,6 glycosidic bonds.  
Slime made by bacteria (dental plaque)
  - Crosslinked with epichlorohydrin
- Produced with different pore sizes: G-10 - G100.

	Exclusion limit (Da)
G-10	8,000
G-25	30,000
G-75	60,000
G-100	100,000

- Larger pore sizes previously available, but fragile and replaced by more modern materials.
- Made by GE HealthCare, previously Amersham Biosciences, previously Pharmacia Fine Chemicals.

Originally described by: Porath, J. & Flodin, P. (1959). Gel filtration: A method for desalting and group separation. *Nature*, pp. 1657–1659. <http://dx.doi.org/10.1038/1831657a0>

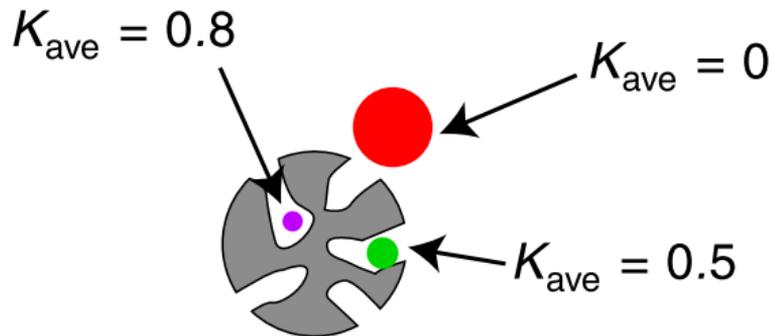
Illustration from: "Gel Filtration: Principles and Methods", Amersham Biosciences (2002 ed.)

## Other Gel Filtration Media

- Cross-linked agarose
  - GE Superose (earlier version called Sepharose)
  - Bio-Rad Bio-Gel A
  - Suitable for very large molecules and complexes (up to  $1 \times 10^6$  Da).
- polyacrylamide
  - Bio-Rad Bio-Gel P
- GE Superdex
  - Sephadex supported by cross-linked agarose matrix.
  - Modern replacement of Sephadex.
  - Large range of pore sizes.
- GE Sephacryl
  - Dextran cross-linked with bis-acrylamide
  - Suitable for very wide range of molecular sizes.

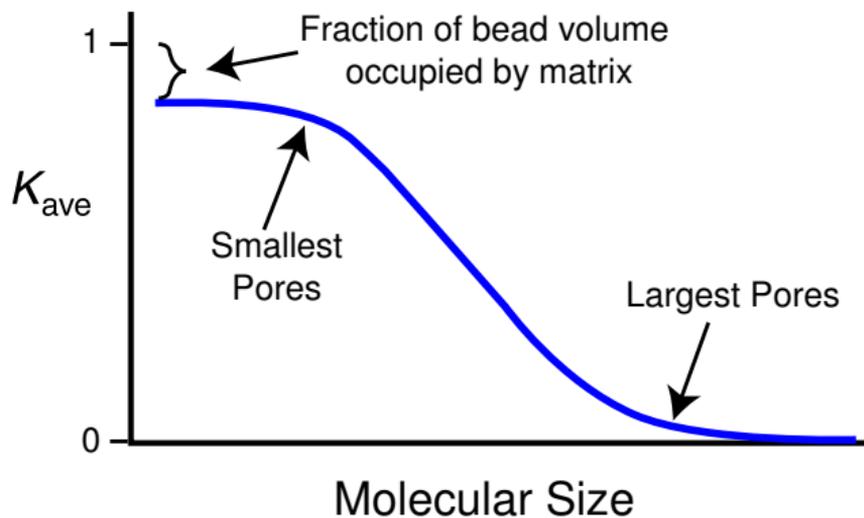
## Quantitative Description of Gel Filtration Chromatography

- Partition between mobile and stationary phases is determined by  $K_{ave}$ , the average fraction of the bead volume that is accessible to the molecule.



- $K_{ave}$  is a property of the particular molecule and the beads used.

## $K_{ave}$ Depends on Molecular Size and Distribution of Pore Sizes



- Beads should have a range of pore sizes in order to separate molecules of different sizes.

# Relationship Between Elution Volume and $K_{ave}$

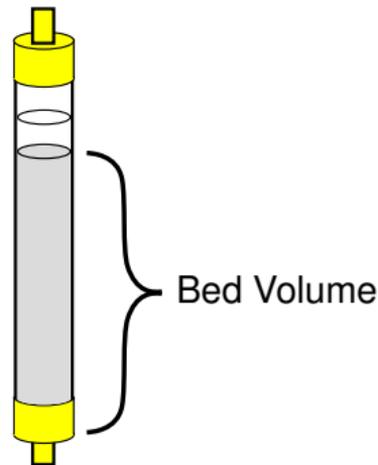
First, define some volumes:

$V_E$  = Elution volume, volume of buffer that passes through the column between when the sample is applied and when it leaves the column.

$V_T$  = Total bed volume

$V_S$  = Stationary phase volume  
= volume occupied by beads (including pores)

$V_0$  = Void volume (space between beads)  
=  $V_T - V_S$



## Relationship Between Elution Volume and $K_{ave}$

- if  $K_{ave} = 0$ , molecule is excluded from all pores,  $V_E = V_0$ .
- if  $K_{ave} > 0$ ,  $V_E = V_0 +$  volume of beads that molecule can enter.

$$\begin{aligned}V_E &= V_0 + K_{ave} \cdot \text{stationary phase volume} \\ &= V_0 + K_{ave}(V_T - V_0)\end{aligned}$$

- Rearranged to calculate  $K_{ave}$ :

$$K_{ave} = \frac{V_E - V_0}{V_T - V_0}$$

## Calculation of $K_{ave}$

$$K_{ave} = \frac{V_E - V_0}{V_T - V_0}$$

- $V_T$  determined from dimensions of column ( $V = \pi r^2 h$ )
- $V_0$  determined as elution volume of molecule much larger than pores. Blue dextran is often used.
- Elution volume depends on the column dimensions, but  $K_{ave}$  should be independent of column size, for a given molecule and type of bead.
- $K_{ave}$  should lie between 0 and  $\sim 1$ , but it might not!

# Outline of Chromatography Experiment

- 1 Prepare column (Day 1)
  - Pour hydrated beads into glass column.
  - Flow several column-volumes of buffer through column.
- 2 Calibrate column (Day 1)
  - Apply a mixture of a large and a small molecule, blue dextran (MW  $\approx$  2,000,000) and phenol red (MW 354).
  - Elute column and collect fractions.
  - Measure absorbance of fractions.
- 3 Separate trypsin and benzamidine (Day 2)
  - Mix trypsin and benzamidine - inhibit trypsin activity.
  - Apply mixture to column.
  - Elute with buffer.
  - Measure  $A_{280}$  of fractions.
  - Measure trypsin activity of peak fraction. Is activity restored?