Biological Chemistry Laboratory
Biology 3515/Chemistry 3515
Spring 2021

Lecture 25:

Quick Review of Quiz 2 and

Gel Filtration Chromatography, Continued

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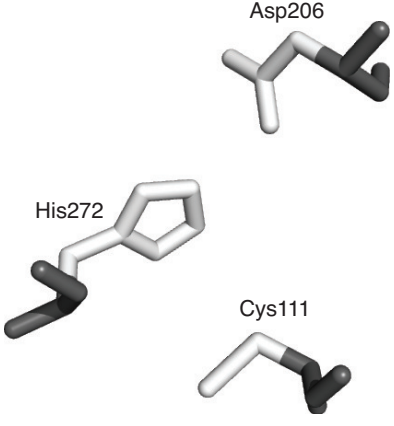
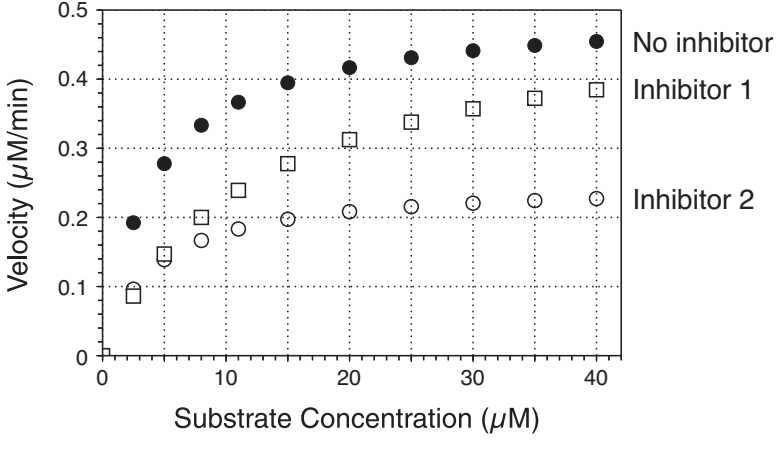
©David P. Goldenberg
University of Utah
goldenberg@biology.utah.edu

Quiz 2: Problem 1

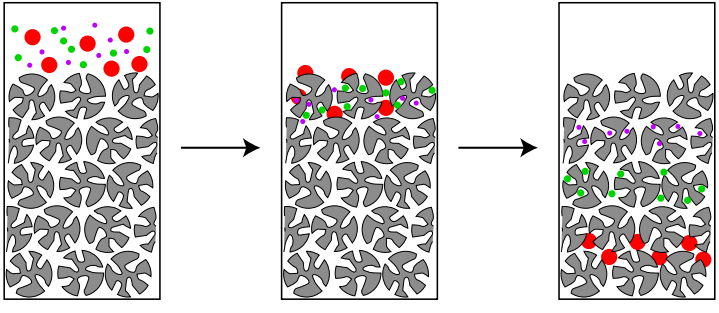
The catalytic triad of PLPro of SARS-CoV-2



Quiz 2: Problems 2 and 3

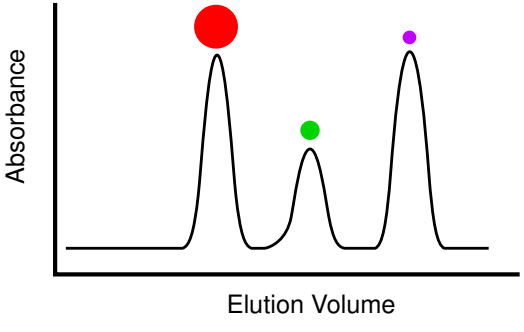


Gel Filtration Chromatography



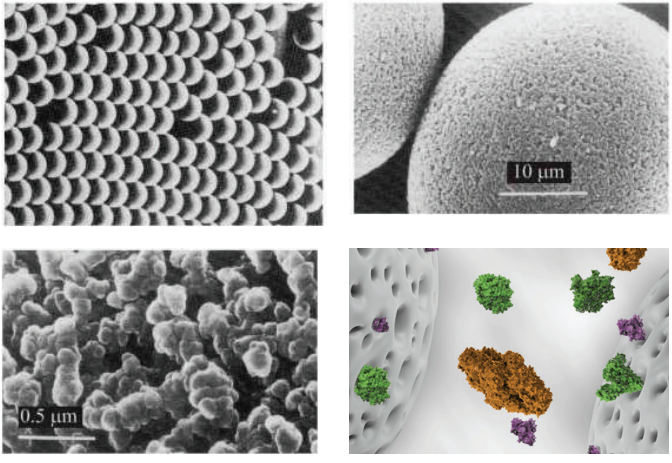
- Also called “gel permeation”, “size exclusion”, “sizing” or “molecular sieve” chromatography.
- Beads are made of a porous gel. (similar to gels used for electrophoresis)
- Separates molecules on basis of size.
- Larger proteins elute first. (opposite of gel electrophoresis!)
- Beads have a distribution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

- 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?

A) 0.4–2 nm

B) 2–10 nm

C) 10–50 nm

D) 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:

$$85,000\,\mathrm{g/mol} \div 6.02 \times 10^{23}\,\mathrm{molecule/mol}$$

$$= 1.41 \times 10^{-19}\,\mathrm{g/molecule}$$

- 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 $\approx 1\,\mathrm{g/mL}$.
- 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

$$
\begin{aligned}
& 1.41 \times 10^{-19}\,\mathrm{g} \div 1\,\mathrm{g/mL} \\
=& 1.41 \times 10^{-19}\,\mathrm{mL} = 1.41 \times 10^{-19}\,\mathrm{cm}^3 \\
& 1.41 \times 10^{-19}\,\mathrm{cm}^3 \times \left(\frac{1\,\mathrm{m}}{100\,\mathrm{cm}}\right)^3 \\
=& 1.41 \times 10^{-25}\,\mathrm{m}^3
\end{aligned}
$$

- Radius

$$
\begin{aligned}
& V = \frac{4}{3}\pi r^3 \\
& r = \sqrt[3]{\frac{3}{4\pi}V} = \sqrt[3]{\frac{3}{4\pi}1.41 \times 10^{-25}\,\mathrm{m}^3} = 3.2 \times 10^{-9}\,\mathrm{m} = 3.2\,\mathrm{nm}
\end{aligned}
$$

- Diameter

$$
d = 2r = 6.5\,\mathrm{nm}
$$

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?

A) 0.4–2 nm

B) 2–10 nm

C) 10–50 nm

D) 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?

A) $\approx 3\,\text{nm}$

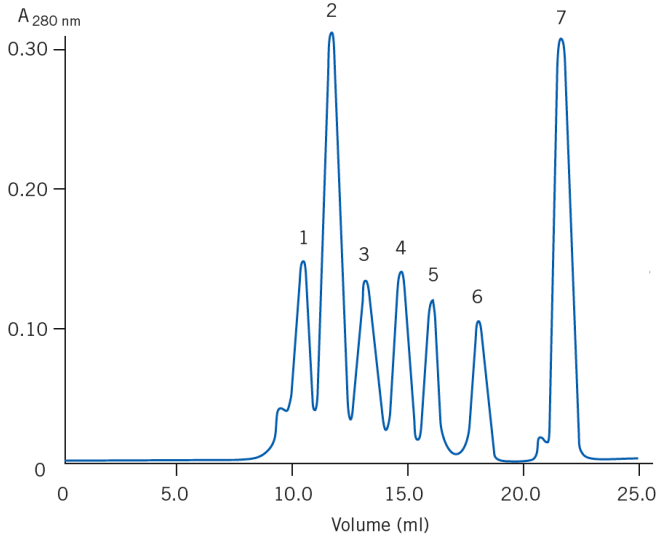
B) $\approx 6\,\text{nm}$

C) $\approx 8\,\text{nm}$

D) $\approx 12\,\text{nm}$

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

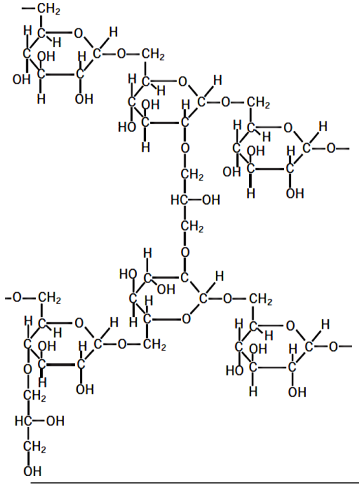
Example Gel Filtration Separation of Proteins



- 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_{12} (1,335 Da)
- Media: Superdex 200
- Good separation by gel filtration requires a difference in MW of about 2-fold, or greater.

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

Sephadex: The First Practical Gel Filtration Media for Proteins



- Cross-linked dextran
  - Dextran: Glucose-based polysaccaride linked by α-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.

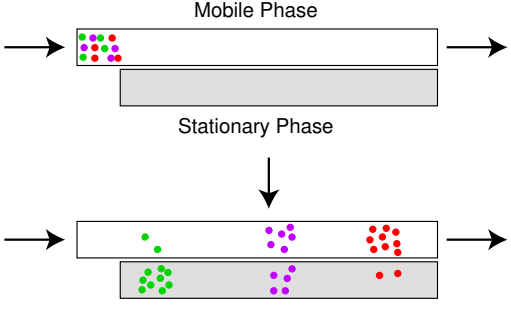
Originally described by: Porath, J. & Flodin, P. (1959). Gel filtration: A method for desalting and group separation. *Nature*, pp. 1657–1659. http://dx.doi.org/10.1038/1831657a0

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

Other Gel Filtration Media

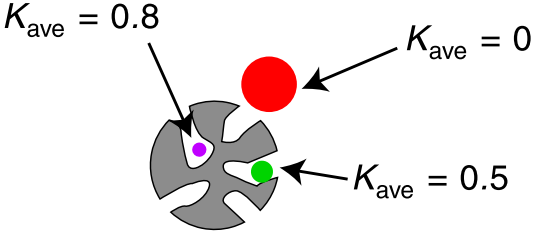
- Cross-linked agarose
  - GE Superose (earlier version called Sepharose)
  - Bio-Rad Bio-Gel A
  - Suitable for very large molecules and complexes (up to 1×10^6 Da).
- polyacrylamide
  - Bio-Rad Bio-Gel P
- GE Superdex
  - Sephadex supported by cross-linked agarose matrix.
  - Modern replacement of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

Chromatography as an Equilibrium Process



- Stationary and mobile phases have distinct chemical or physical properties.
- Molecules partition (thermodynamic equilibrium) between two phases.
- Molecules that partition preferentially into mobile phase move more rapidly than molecules that prefer the stationary phase.
- Exchange between the phases has to be rapid, or the molecules will smear out.

Quantitative Description of Gel Filtration Chromatography



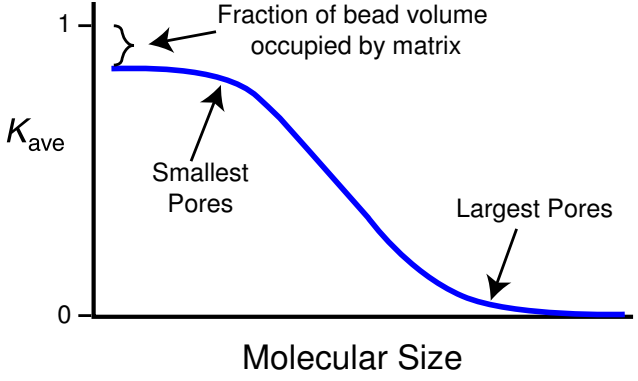
- Partition between mobile and stationary phases is determined by K_{ave}:

$$K_{ave} = \frac{\text{concentration in stationary phase}}{\text{concentration in mobile phase}}$$

- K_{ave} is the average fraction of the bead volume that is accessible to the molecule.
(assuming no interactions with the stationary phase)

- 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}

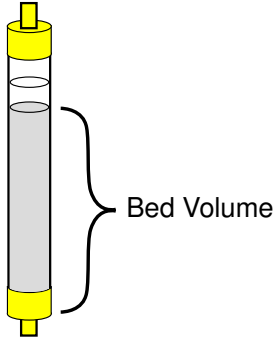
First, define some volumes:

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 $K_{\text{ave}} = 0$, molecule is excluded from all pores, $V_{\text{E}} = V_0$ (the space between the beads).

- if $K_{\text{ave}} > 0$, V_{E} = V_0 + volume of beads that molecule can enter.

$$V_{\text{E}} = V_0 + K_{\text{ave}} \cdot \text{stationary phase volume}$$

$$= V_0 + K_{\text{ave}}(V_{\text{T}} - V_0)$$

- Rearranged to calculate K_{ave}:

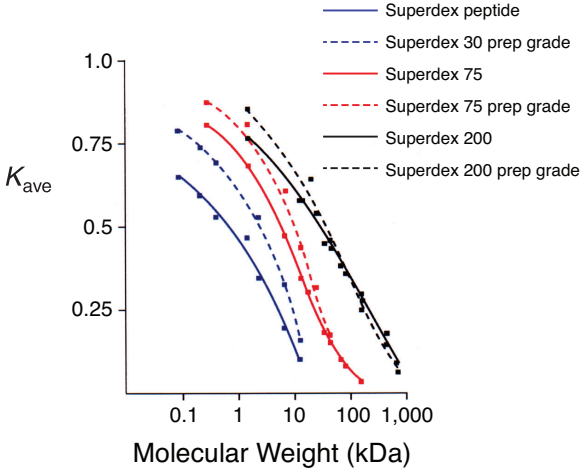
$$K_{\text{ave}} = \frac{V_{\text{E}} - V_0}{V_{\text{T}} - V_0}$$

Calculation of K_{ave}

$$K_{\text{ave}} = \frac{V_{\text{E}} - V_0}{V_{\text{T}} - V_0}$$

- 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 ~ 1, but it might not!

Effects of Media and Molecular Size on K_{ave}



- Media are defined by two properties:
  - Distribution of pore sizes (number)
  - Bead size (grade)
    Smaller beads give better resolution, but slower flow.
- For a given pore size distribution, proteins over a range of 10 to 100-fold can be separated.
- Columns can be calibrated with proteins of known molecular weight and then used to estimate MW of other proteins.

Illustration adapted from: “Gel Filtration: Principles and Methods”, free from GE Healthcare (formerly Amersham Biosciences, formerly Pharmacia)
Link to free download: https://goldenberg.biology.utah.edu/courses/biol3515/internet_3515.shtml

Outline of Chromatography Experiment

Day 1:

1. Prepare column
   - Pour hydrated beads into glass column.
   - Flow several column-volumes of buffer through column.
2. Calibrate column
   - 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.

Day 2:

1. Separate trypsin and benzamidine
   - 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?