SIZE EXCLUSION CHROMATOGRAPHY

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INTRODUCTION

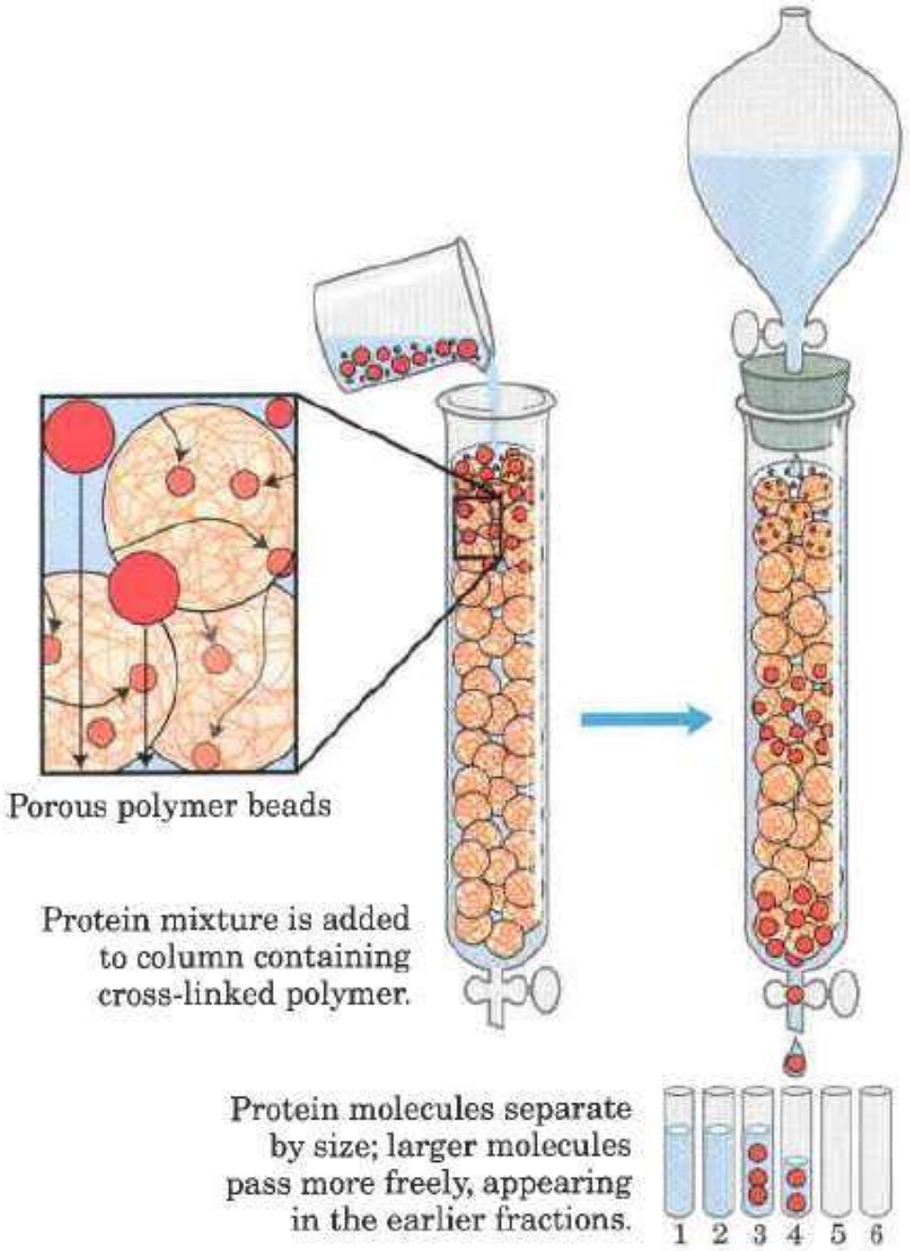
- Size-exculsion chromatography (SEC), also called gel-filtration or gel-permeation chromatography (GPC), uses porous particles to separate molecules of different sizes.
- It is generally used to separate biological molecules, and to determine molecular weights and molecular weight distributions of polymers
- It is usually applied to large molecules or macromolecular complexes such as proteins and industrial polymers.

- When an aqueous solution is used to transport the sample through the column, the technique is known as Gel-filtration chromatography .
- When an organic solvent is used as a mobile phase,the technique is known as Gel-permeation chromatography.
- The separation of molecules is called fractionation.
- Size of pores in beads determines the exclusion limit (what goes through the beads and what goes around the beads)

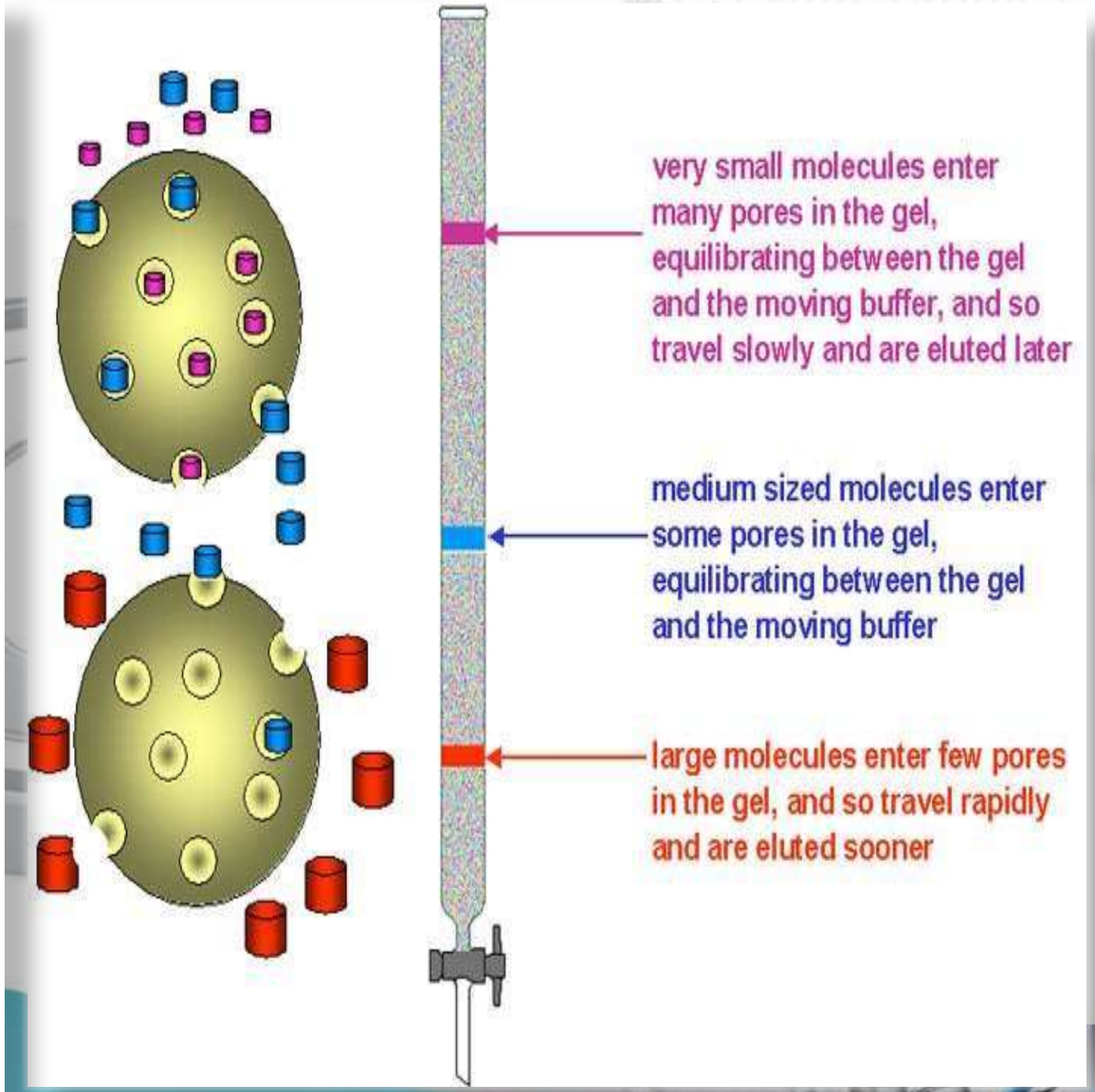
PRINCIPLE

- A mixture of molecules dissolved in liquid (the mobile phase) is applied to a chromatography column which contains a solid support in the form of microscopic spheres, or “beads” (the stationary phase).
- The mass of beads within the column is often referred to as the column bed.
- The beads act as “traps” or “sieves” and function to filter small molecules which become temporarily trapped within the pores.

- Larger molecules pass around or are “excluded” from the beads .
- Large sample molecules cannot or can only partially penetrate the pores, whereas smaller molecules can access most or all pores.
- Thus, large molecules elute first, smaller molecules elute later, while molecules that can access all the pores elute last from the column.
- Particles of different sizes will elute (filter) through a stationary phase at different rates.



(b) Size-exclusion chromatography



❖ Total column volume (V_t)

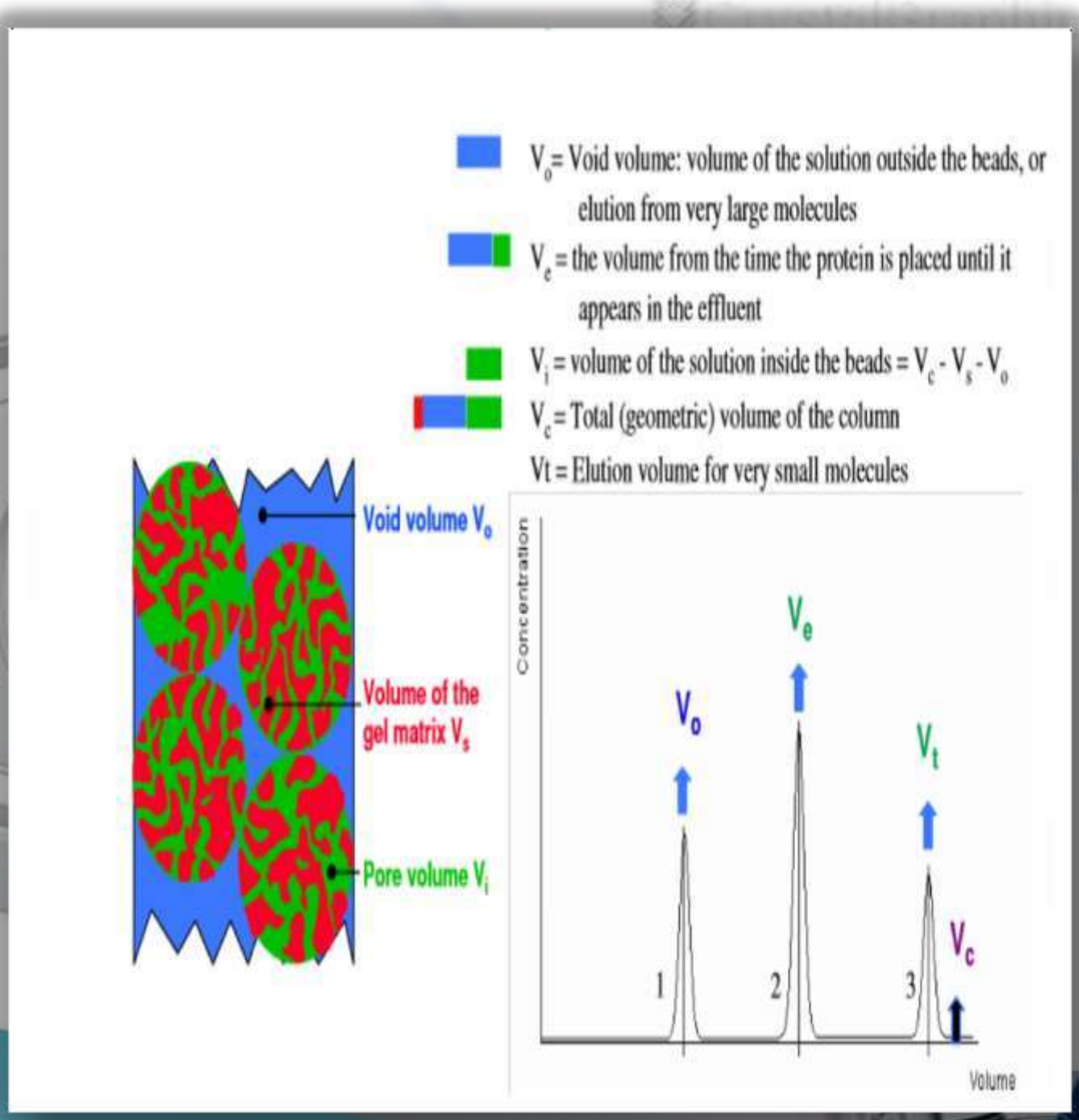
$$V_t = V_g + V_i + V_0$$

where

V_g --is the volume occupied by the packing

V_i --is the volume of solvent in the pores

V_0 --is the free solvent volume (similar to injection volume)



COMPONENTS OF A SEC

1. Stationary Phase
2. The Mobile Phase
3. The Columns
4. The Pump
5. Detectors

STATIONARY PHASE:

- Stationary Phase Semi-permeable, porous beads with well-defined range of pore sizes .
- Beads are crosslinked polymers
- Degree of crosslinking is controlled carefully to yield different pore sizes.
- Smaller pore sizes are used for rapid desalting of proteins or for protein purification.
- Intermediate pore sizes are used to separate relatively small proteins.

- Very large pore sizes are used for purification of biological complexes.
- Stationary phase used for gel exclusion chromatography include dextran (Sephadex™), polyacrylamide and dextran-polyacrylamide (Sephacryl™).
- Each is available with a variety of different ranges of pore size in the beads, permitting separation of macromolecules of different size

A good stationary phase should have following properties:

- It should be chemically inert.
- It should be inexpensive.
- It should not react with component to be separated.
- It should not react with eluent.
- It should be colorless, uniform in size and shape.
- It should be mechanically stable.

- **Soft gel** e.g.- dextran(Sephadex), Polyacrylamide gels

  Separation of proteins.

- **Semi-rigid** gel e.g.- bio beads

  Separation of non-polar polymers in non-polar solvents.

- **Highly rigid** gels and glasses

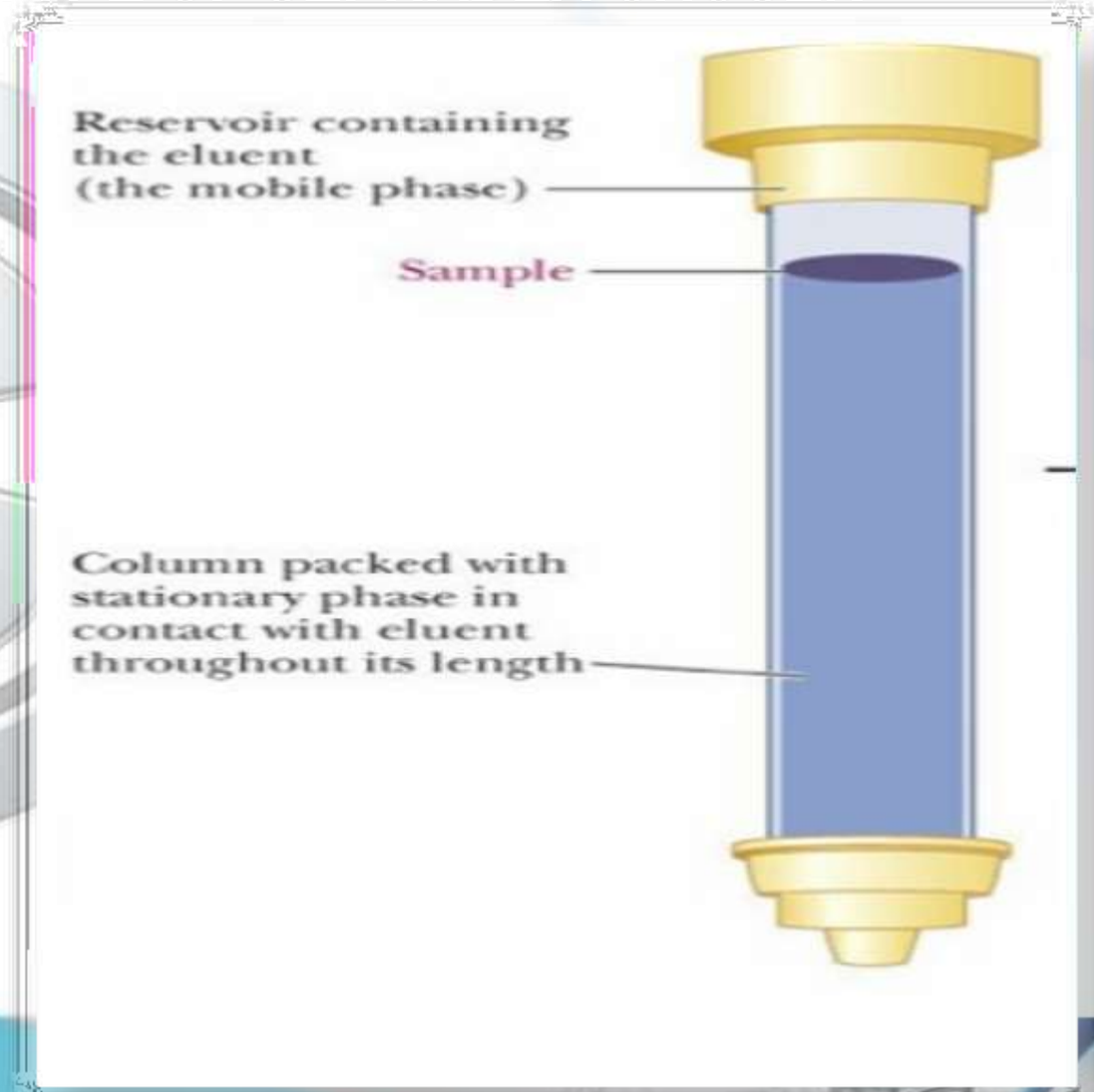
  Separation of polar systems.

Dextran

- A homopolysaccharide of glucose residues.
- It’s prepared with various degrees of cross-linking to control pore size.
- It’s bought as dry beads, the beads swell when water is added.
- The trade name is sephadex.
- It’s mainly used for separation of small peptides and globular proteins with small to average molecular mass.

TYPICAL SEPARATION RANGES THAT CAN BE ACHIEVED USING SEPHADEX ARE GIVEN BELOW. MOLECULES RANGING FROM 100 TO 600,000 DA CAN BE SEPARATED DEPENDING ON THE TYPE OF SEPHADEX CHOSEN

SEPHADEX	RANGE
G10	100-800 Da
G15	(500-1500 Da),
G25	(1000-5,000 Da),
G50	(1,500-30,000 Da)
G75	(3,000-80,000 Da)
G100	(4,000-150,000 Da)
G150	(5,000-300,000 Da)
G200	(5,000-600,000 Da).



Polyacrylamide

- these gels are prepared by cross linking acrylamide with N,N-methylene bis acrylamide.
- The pore size is determined by the degree of cross-linking.
- The separation properties of polyacrylamide gels are mainly the same as those of dextrans.
- They are sold as bio-gel P. They are available in wide range of pore sizes.

Agarose

- Linear polymers of D-galactose and 3,6 anhydro-1-galactose.
- It forms a gel that’s held together with H bonds. It’s dissolved in boiling water and forms a gel when it’s cold.
- The concentration of the material in the gel determines the pore size.
- The pores of agarose gel are much larger than those of sephadex or bio-gel p.
- It’s useful for analysis or separation of large globular proteins or long linear molecules such as DNA

Mobile phase

- The liquid used to dissolve the biomolecules to make the mobile phase is usually called a **buffer.**
- The mixture of biomolecules dissolved in the buffer is called the **sample.**
- The choice of mobile phase to be used in any separation will depend on the type of separation to be achieved and component to be separated.

❖ The most common eluents in for polymers that dissolve at room temperature.**e.g.-**Tetrahydrofuran,Chloroform, Dimethyl formamide.

MOBILE PHASE

material	solvent
Synthetic elastomers (polybutadiene , polyisoprene)	Toluene
PS, PVC, Styrene-Butadiene Rubber , Epoxy resins	Tetrahydrofuran
Polyolefins	Tri- chloro -benzene
Polyurethane	Di- methylformamide
Proteins, polysaccharides	Water / Buffers

SOLVENT SELECTION

The solvents used for mobile phase of SEC are limited to those follows following criteria:

- The solvent must dissolve the sample completely.
- The solvent has different properties with solute in the eluent: typically with solvent refractive index (RI) .
- solvent must not degrade the sample during use Otherwise, the viscosity of eluent will gradually increase over times.
- The solvent is not corrosive to any components of the equipment

Mobile Phase Preparation

- high purity of solvent is recommended.
- Filter mobile phase solvents using 0.5 micron filter to remove any particular impurities such as dusts, insoluble salts.
- Antioxidant is added to trichlorobenzene to keep solvent stable in high temperature.
- Other additives eliminate adsorption or interaction of solutes with column packing materials Size Exclusion Chromatography

Sample preparation

- The sample solutions are supposed to be prepared in dilute concentration (less than 2 mg/mL)
- A good solvent can dissolve a sample in any proportion in a range of temperatures.
- Samples with broad molecular weight distribution may require higher concentrations.
- It is recommended to filter the sample solutions before injecting into columns in order to get rid of clogging and excessively high pressure problems.

- Agitation and filtration Generally filtration is required to remove insoluble impurities.

- Do not agitate and filter samples that contain very high MW (>1 million).

COLUMNS

Commercially Available Columns

- analytical column- 7.5–8mm diameters.
- Preparative columns-22–25mm for.
- Usual column lengths-25, 30, 50, and 60 cm.
- Recently, narrow bore columns- 2–3mm diameter have been introduced, which save time and solve

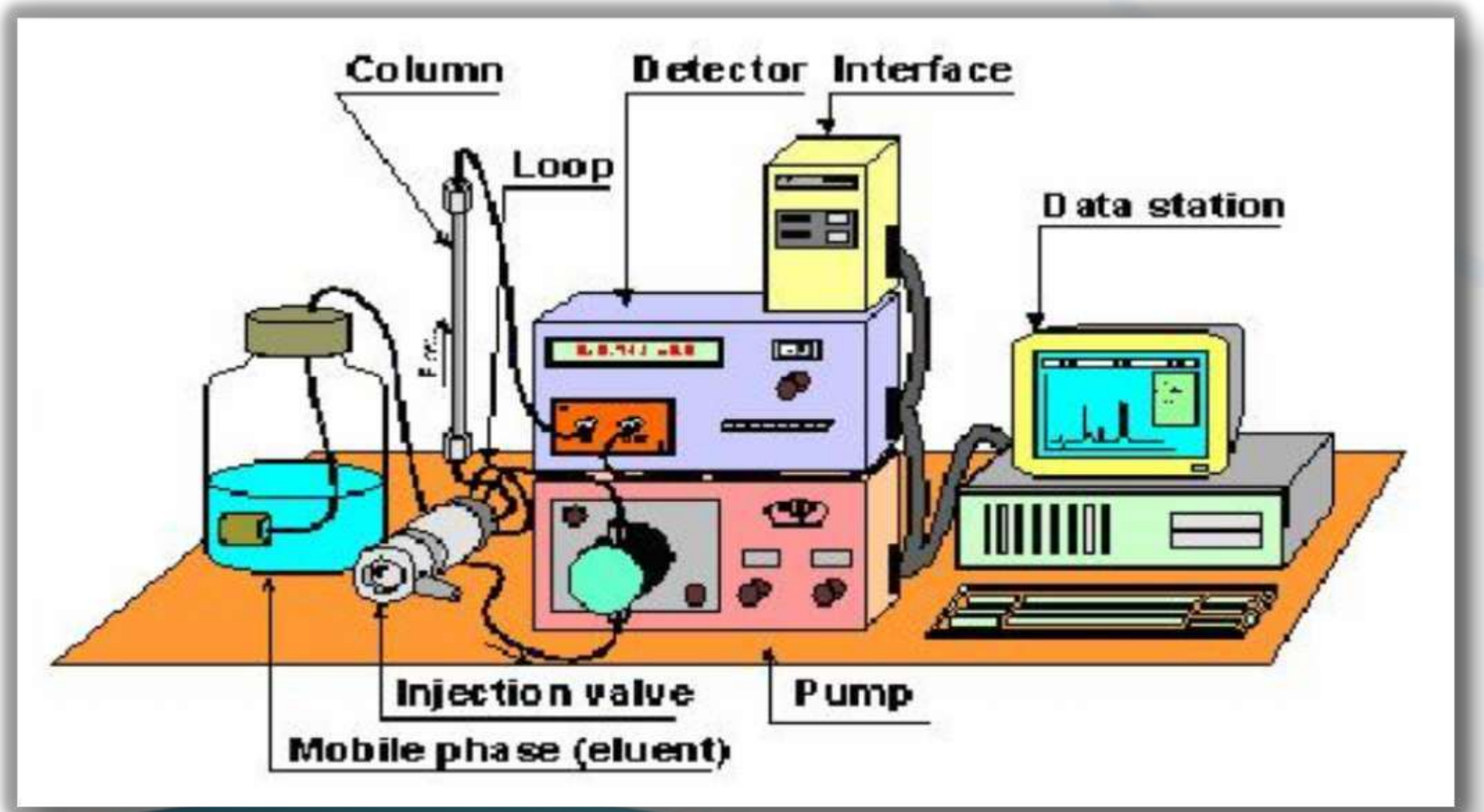
Selecting SEC column

- Shorter columns save time and solvent.
- Small particles (typically 5 mm) provide a better resolution.
- On the other hand, 5 mm (or even 3 mm) packings are more sensitive towards contamination by samples containing impurities.
- Particles as large as 20 mm have been recommended for very high-molecular-weight polymers.
- ion.

- Small particle size packings can sometimes result in shear degradation of large polymer molecules because the space between particles is very narrow.
- Columns with different porosity or mixed-bed columns, provide a better separation.

Handling SEC Columns

- A column set in SEC should be always run in the same mobile phase.(isocratic)
- SEC columns should never be operated in a backward direction.
- Care should also be taken in connecting columns or in sample injection.
- Replacing a clogged inlet frit is a dangerous operation which can reduce column performance.
- A damaged or dirty check valve of pump, can also reduce column life.


Column
Detector
Interface
Loop
Data station
Injection valve
Pump
Mobile phase (eluent)

PUMP

- A highly constant flow rate has to be maintained during the entire chromatogram. This is very important in SEC.
- A change of the flow rate of only 0.1% can cause an error in molar mass of up to 10%.
- Most pumps can only reproduce the flow rate to 0.2–0.3%.
- In-line filters in the solvent reservoir may prevent particles from coming into the pump heads, which might damage the check valves or the pump seals.

➢ Types- ,**Syringe pumps, Reciprocating pumps**

DETECTOR

❖ Concentration sensitive detectors

- Bulk Property Detectors- Refractive Index (RI) Detector
- Solute Property Detectors- Ultraviolet (UV) Absorption Detector
- Evaporative Detectors- Evaporative Light Scattering Detector (ELSD)

❖ Molar mass sensitive detectors

➢ Light Scattering Detectors

- Low Angle Light Scattering (LALS) Detectors
- Multiangle Light Scattering (MALS) detectors

- Viscosity Detectors- Differential Vscometers

Other :- Flame Ionization Detector (FID), A Mass Spectrometer or A Fourier Transform Infrared (FTIR) Spectromet

ADVANTAGES

- Short analysis time.
- Well defined separation.
- Narrow bands and good sensitivity.
- There is no sample loss.
- Small amount of mobile phase required.
- The flow rate can be set.

DISADVANTAGES

- Limited number of peaks that can be resolved within the short time scale of the GPC run.
- Filtrations must be performed before using the instrument to prevent dust and other particulates from ruining the columns and interfering with the detectors.
- The molecular masses of most of the chains will be too close for the GPC separation to show anything more than broad peaks
- .

APPLICATION

- Proteins fractionation
- Purification
- Molecular weight determination.
- Separation of sugar, proteins, peptides, rubbers and others on the basis of their size.
- This technique can be determine the quaternary structure of purified proteins.

- SEC is a widely used technique for the purification and analysis of synthetic and biological polymers, such as protein, polysaccharides and nucleic acid.
- Various species of RNA and viruses have been purified using agarose gels.
- For Desalting
- For copolymerisation studieS