Size exclusion chromatography (Gel chromatography)

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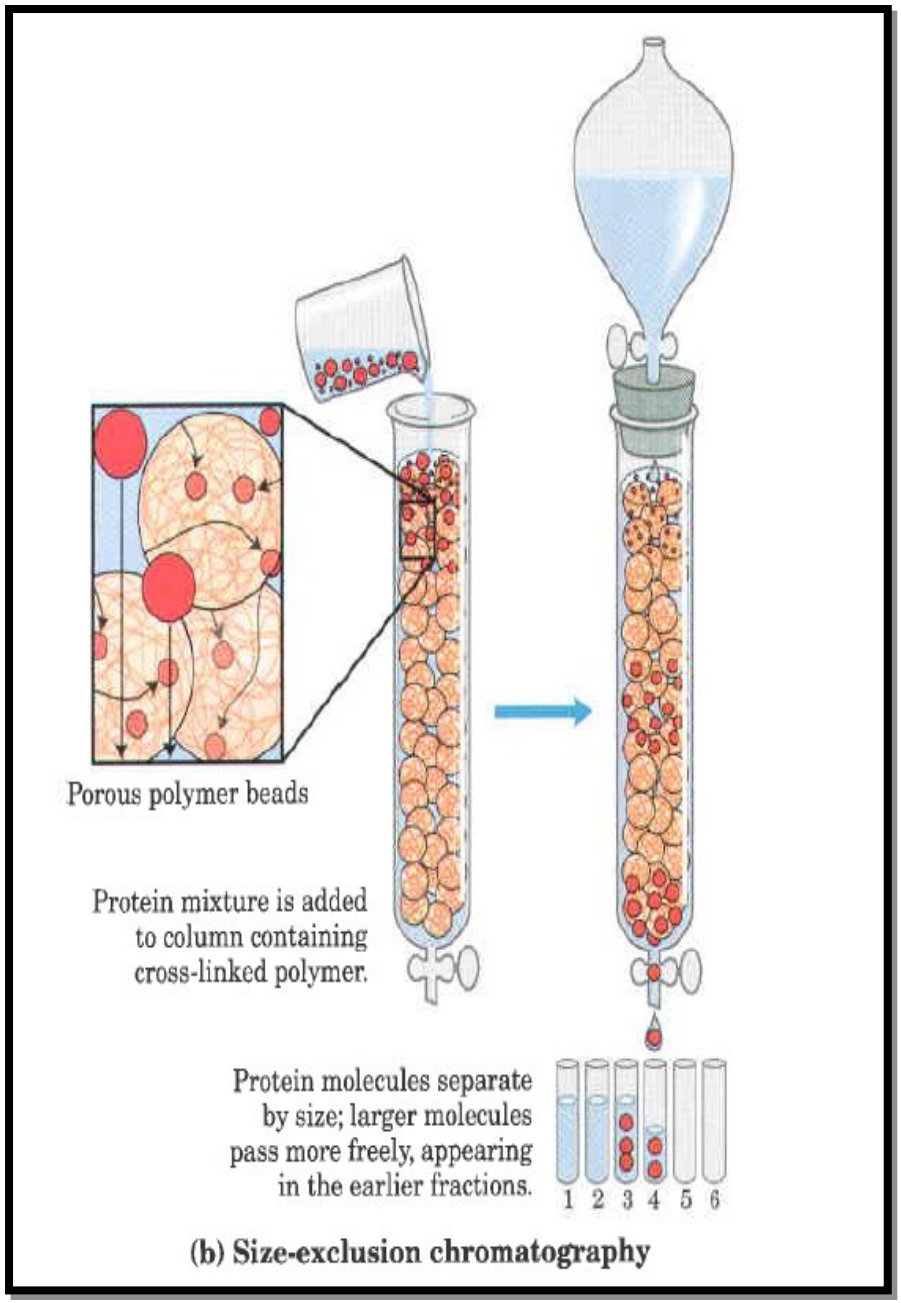
INTRODUCTION

- The technique is also known as **gel-filtration** or **gel-permeation chromatography (GPC)**, uses porous particles to separate molecules of different sizes.
- Used to separate biological molecules, and to determine molecular weights and molecular weight distributions of polymers
- Applied to large molecules or macromolecular complexes such as proteins and industrial polymers.
- Fractionation: separation of molecules

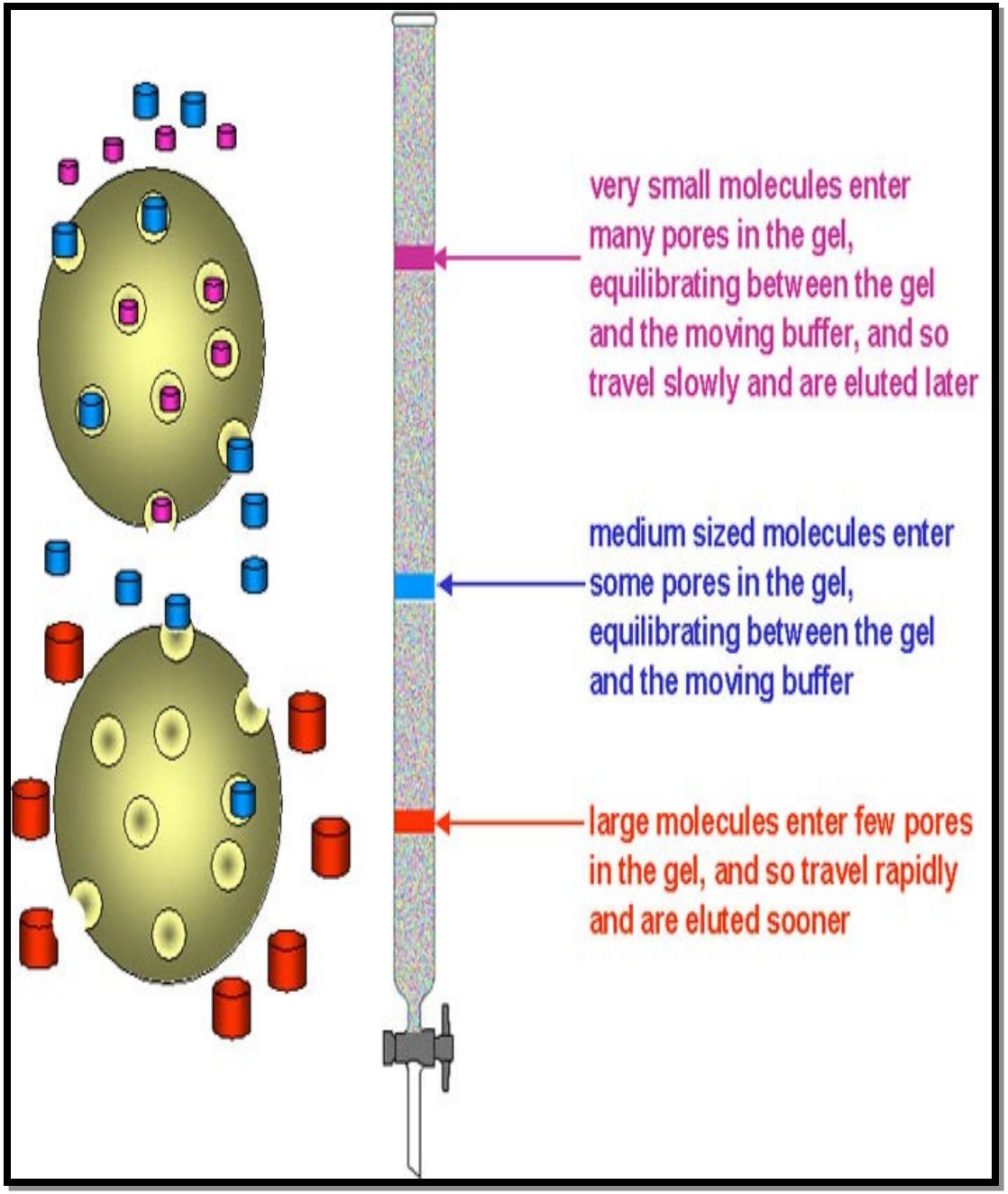
PRINCIPLE

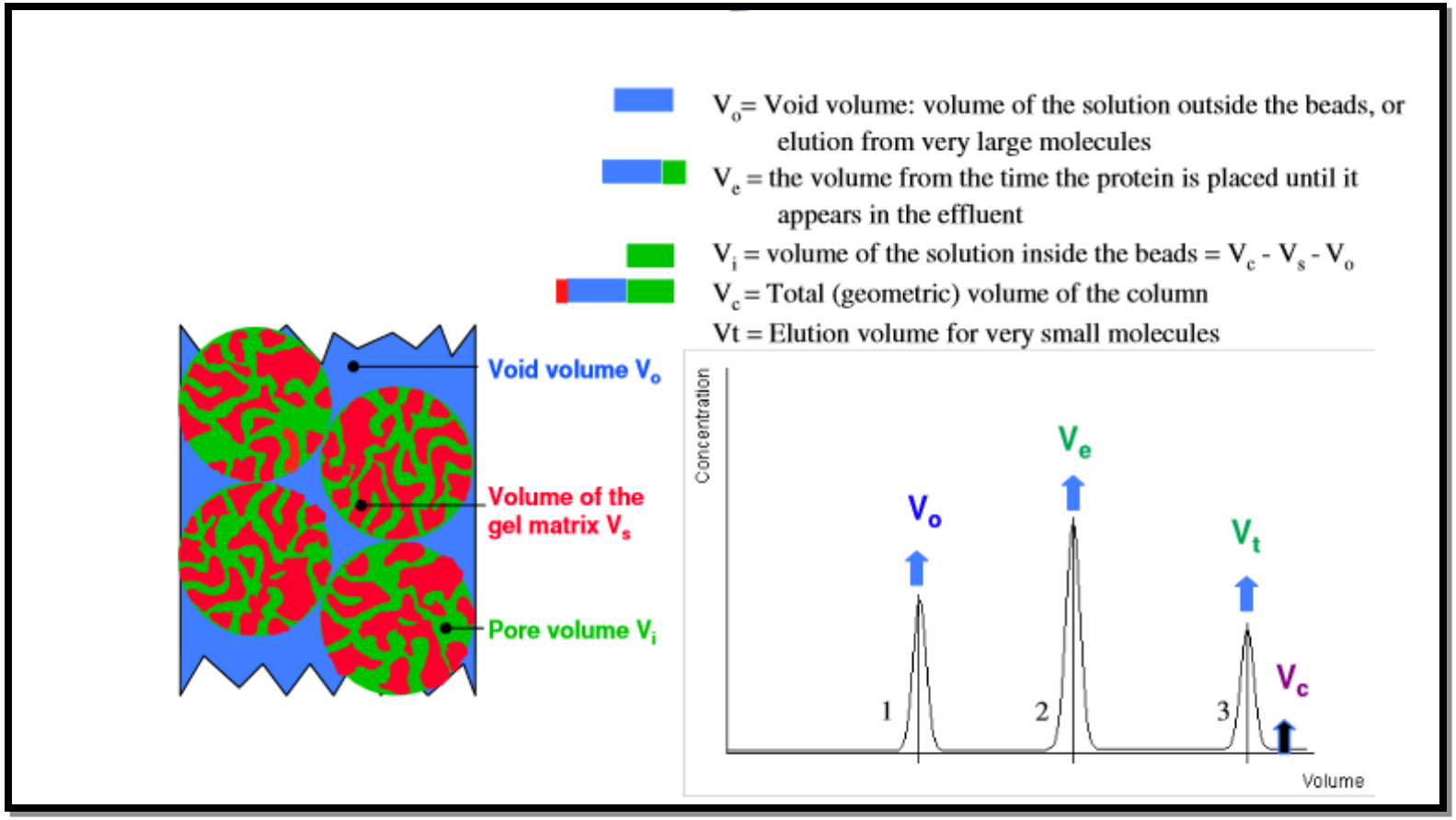
- A mixture of molecules dissolved in liquid mobile phase is applied to a chromatography column which contains a solid support in the form of microscopic spheres, or '**beads**' (i.e. Stationary Phase).
- Beads act as traps/ sieves and function to filter small molecules which become temporarily trapped within the pores.
- The mass of beads within the column is also known as **column bed.**
- As sample moves through a bed of porous beads, molecules of different size diffuse into the beads to greater or lesser extent.

- 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 through a stationary phase at different rates.



(b) Size-exclusion chromatography


very small molecules enter many pores in the gel, equilibrating between the gel and the moving buffer, and so travel slowly and are eluted later
medium sized molecules enter some pores in the gel, equilibrating between the gel and the moving buffer
large molecules enter few pores in the gel, and so travel rapidly and are eluted sooner



Total column volume (V_t)

$$V_t = V_g + V_i + V_0$$

V_i -volume of solvent in the pores

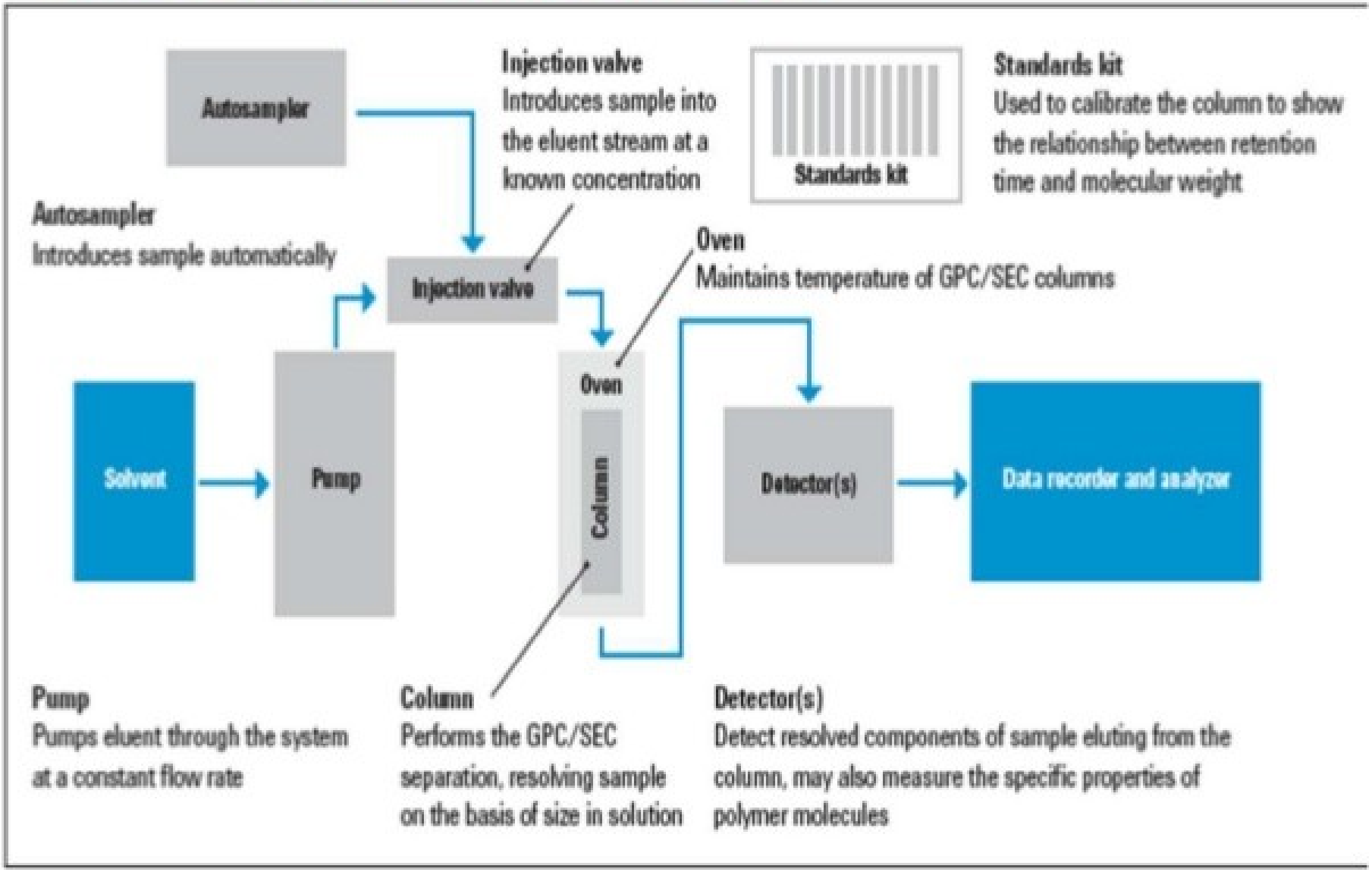
V_g -volume occupied by the packing

V_0 -free solvent volume

COMPONENTS OF SEC

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

INSTRUMENTATION



STATIONARY PHASE:

- Semi-permeable, porous beads with well-defined range of pore sizes . Beads are cross-linked polymers.
- Degree of cross-linking is controlled carefully to yield different pore sizes.
- **Examples**- 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:

- Chemically inert.
- Inexpensive.
- Not react with component to be separated.
- Not react with eluent.
- Colorless, uniform in size and shape.
- Mechanically stable.

- **Soft gel –**

  Examples- Dextran (Sephadex), Polyacrylamide gels

  Use- Separation of proteins.

- **Semi-rigid** gel –

  Examples- - bio beads

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

- **Highly rigid** gels and glasses-

  Use- Separation of polar systems.

Dextran

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

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-l-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 bio-molecules to make the mobile phase is usually called a **buffer.**
- The mixture of bio-molecules dissolved in the buffer is called the **sample.**
- The most common eluents in for polymers that dissolve at room temperature. **e.g.-**Tetrahydrofuran, Chloroform, Dimethyl formamide, toluene, water

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
- Usual column lengths-25, 30, 50, and 60 cm.
- Recently, narrow bore columns- 2–3mm diameter

have been introduced, which save time

Column packing techniques

- **Swelling the gel:**

- Add appropriate amount of filtered doubled distilled water to the gel matrix depending on the swelling capacity of the beads as per manufacturer's recommendations.
- Once the gel has swollen and settled in the bottom of the beaker, decant the doubled distilled water on the top.
- Then, add appropriate buffer to the gel, mix gently (do not use magnetic stirrer for mixing) and wait for it to settle down.
- Decant and resuspend the gel in equal volume of the buffer and degas the gel in order to remove trapped air.
- Rapid swelling of the gel can be achieved by heating the slurry at 90°C for 5 hours. Swelling at room temperature is considerably slower, and takes approximately 72 hrs.

Column packing techniques

- **Pouring the gel into the column:**
- Mount the glass column vertically on a metal stand and adjust the bottom adapter.
- Add some buffer to the empty column and allow it to pass through outlet tube, so that any trapped air bubble in the path can be removed.
- Now, block the outlet tubing and pour the gel slurry to fill the column up to the required height with the help of a glass rod. The glass rod should be used in such a way that it touches inner wall of the column.
- This avoids introduction of any air bubble. A gel reservoir should be used to smooth and continuous pouring of the gel.
- Allow it to settle down, decant the excessive buffer and the gel and put the top adapter.

Column packing techniques

- **Equilibration of the column:**
- Attach the top tubing with peristaltic pump and buffer reservoir. Keep the bottom tubing to the waste.
- The operating pressure should be according to the manufacturer's recommendations for the gel matrix. It's always better to keep flow rate slower.
- Choose the buffer as per your purification needs and equilibrate the column with 2-3 bed volumes of this buffer.

- 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%.
- Types- **Syringe pumps, Reciprocating pumps**

❖ **Concentration sensitive detectors**

- Bulk Property Detectors-

- Refractive Index (RI) Detector

- Solute Property Detectors-

- Ultraviolet (UV) Absorption Detector

- Evaporative Detectors-

- Evaporative Light Scattering Detector (ELSD)

DETECTORS

- **Molar mass sensitive detectors**
- Light Scattering Detectors
  - Low Angle Light Scattering (LALS) Detectors
  - Multiangle Light Scattering (MALS) detectors

- Viscosity Detectors- Differential Viscometers

Others:- Flame Ionization Detector (FID),
Mass Spectrometer,
Fourier Transform Infrared (FTIR) detector

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 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

APPLICATIONS

- 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.

APPLICATIONS

- 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 co-polymerisation studies

Thank you