Protein Extraction and Purification Methods-3

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Reference: Protein Purification techniques Second edition
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Detergents

- The attraction of chemical methods of cell disruption is that the cell will be left substantially intact after release of its contents. This facilitates separation of the cell debris from the supernatant. However, the chemicals used must be compatible with further downstream processes. It will, probably, be necessary to remove the DNA from the lysate, since it is likely to be released in a high molecular weight form, and therefore, greatly increases the viscosity of the lysate.

- (a) Chelating agents such as EDTA sequest divalent cations. Loss of Mg^{2+} and Ca^{2+} from Gram negative cell walls results in the loss of their permeability barrier. In fact, the inner membrane of E. colt remains intact so only periplasmic enzymes are released (35). Chelating agents are not usually used on their own but in combination with another lysis method. Thus, they could be viewed as pre-treatments.

- (b) Chaotropic agents such as guanidine, ethanol, and urea weaken interaction between hydrophobic molecules. They generally have to be used at high concentrations, which does not make them suitable for large scale extraction processes. Some details are shown in Table 5.

Triton X-100 has often been used on its own or in combination with other disruption methods. The 0.2 M guanidine releases periplasmic proteins, treatment with 0.2 M guanidine and 0,5% Triton X-100 releases cytoplasmic protein as well.

A method for using Triton X-100 and details of other systems are given in Table 5.

Anionic detergents (e.g. SDS at low concentrations, 0.05%) released 24% of intracellular protein (and similar amounts of nucleic acids).

Examples for SDS lysis of bacterial cells and animal cells are given in Protocols 11 and 12.

Table 5 Examples of chemical and enzymic extraction of cells

Cell type (and lysis method)	Details	Ref
Bacillus subtilis with lysozyme and surface active agent	Cells suspended in buffer with sucrose, dithiothreitol, lysozyme (300 μg/ml, EDTA, and Brij 58). Incubated for 1 h on ice. Supernatant obtained after 30 min at 40 000 *g*.	36
β-Galactosidase release from the yeast: *Kluyveromyces lactis*	2% chloroform, with 10% ethanol. 5–37 °C, toluene also tested.	37
Protein and β-lactamase release from *E. coli*	Cell suspension mixed with lysis buffer to give 0.4 M guanidine and 0.5% Triton X-100. Treatment time about 4 h. Clarification by centrifugation.	38

Protocol 11

SDS lysis of E. coli cells[a]

Equipment and reagents

- Suspension of *E. coli* cells in 0.5 M glycerol, 1 mM sodium phosphate at 4 °C, made up to an OD (660 nm) of between 10–15
- Ice bath
- Lysis buffer: 2% SDS (v/v) in 0.25 M NaOH with 0.2 M Na_2EDTA, stored at 4 °C

Method

1. Add 1 ml of lysis buffer to 4.0 ml cell suspension.
2. Mix by inversion once and store on ice.
3. Lysis should occur in about 5 min. The lysate will be very viscous due to the release of DNA, which should remain intact since minimum agitation has been used.

[a] Stephenson *et al.* (46).

Protocol 12

Lysis of animal cells (from cell lines) with SDS[a]

Reagents

- Chloroform
- Cell suspension
- 0.1% SDS

Method

1. Add 15 µl of chloroform and 15 µl of 0.1% SDS to 1 ml of cell suspension.
2. Agitate the mixture for 10 sec.
3. Centrifuge at 12 000 *g* for 15 min at 4 °C.
4. Assay of protein can be carried out but there is interference from SDS. SDS of the same concentration needs to be included in blanks and standards.

[a] Shin *et al.* (25).

Concentration of the extract

Concentration is achieved by removal of water and other small molecules:

(a) By addition of a dry matrix polymer with pores that are too small to allow entry of the large protein molecules (Section 2).

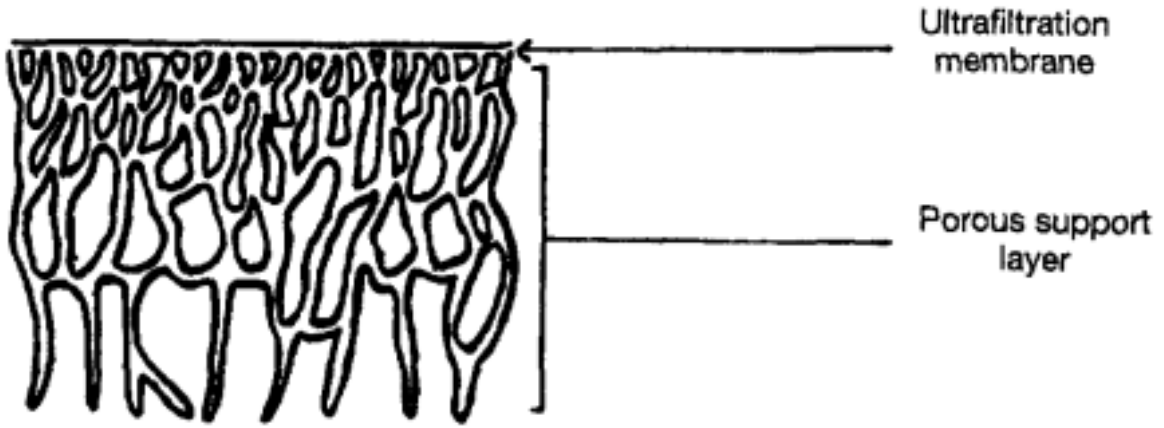
(b) By removal of the small molecules through a semi-permeable membrane which will not allow the large molecules through (i.e. ultrafiltration, Section 3).

(c) By removal of water in vacua

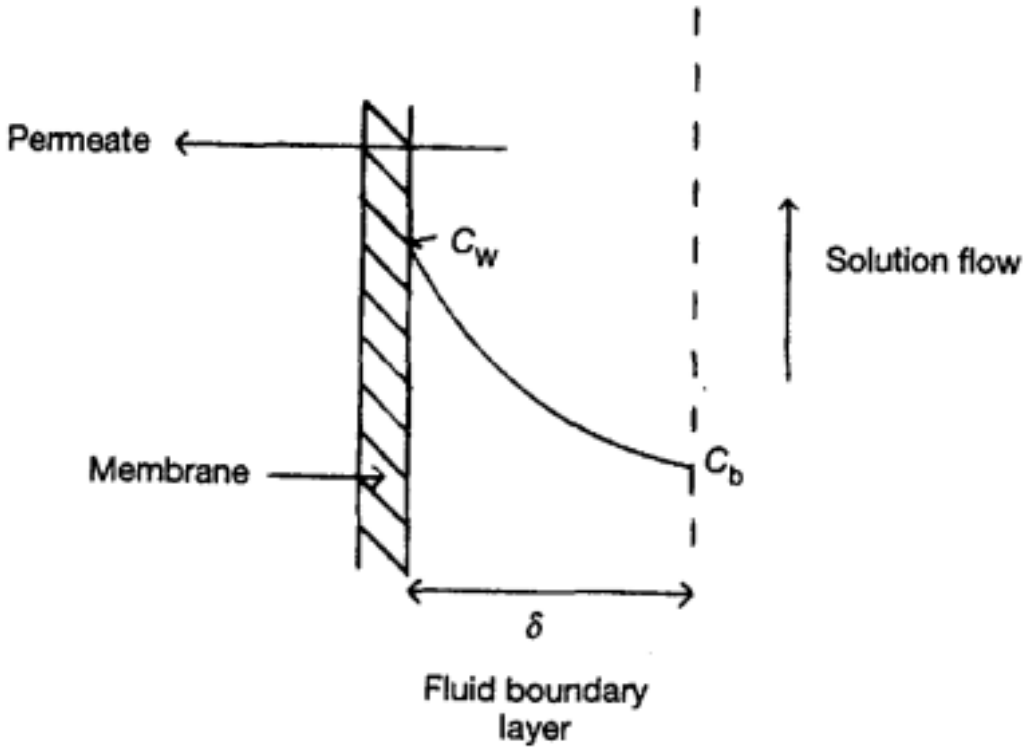
Precipitation can also be used to concentrate proteins if the pellet is redissolved in a smaller volume, and in addition often results in some degree of purification of the protein of interest. However, as mentioned above precipitation is more effective if the total protein concentration is above 100 ug/ml

Ultrafiltration

- In ultrafiltration, water and other small molecules are driven through a semipermeable membrane by a transmembrane force such as centrifugation or high pressure.
- For ultrafiltration, the membrane pores range in diameter from 1-20 nm; the diameter is chosen such that the protein of interest is too large to pass through. Pore sizes of microfiltration membranes range from 0.1-10 µm diameter and allow proteins and other macromolecules to pass through, whilst retaining larger particles such as cells.


Ultrafiltration membrane
Porous support layer

(a)


Permeate
C_w
Solution flow
Membrane
C_b
δ
Fluid boundary layer

(b)

Rather than quote the pore size of the ultrafiltration membranes it is more common to quote a nominal molecular weight cut-off (NMWC) for the membrane. The NMWC is defined as the minimum molecular weight globular molecule which will not pass through the membrane. Concentration by ultrafiltration offers several advantages over alternative methods.

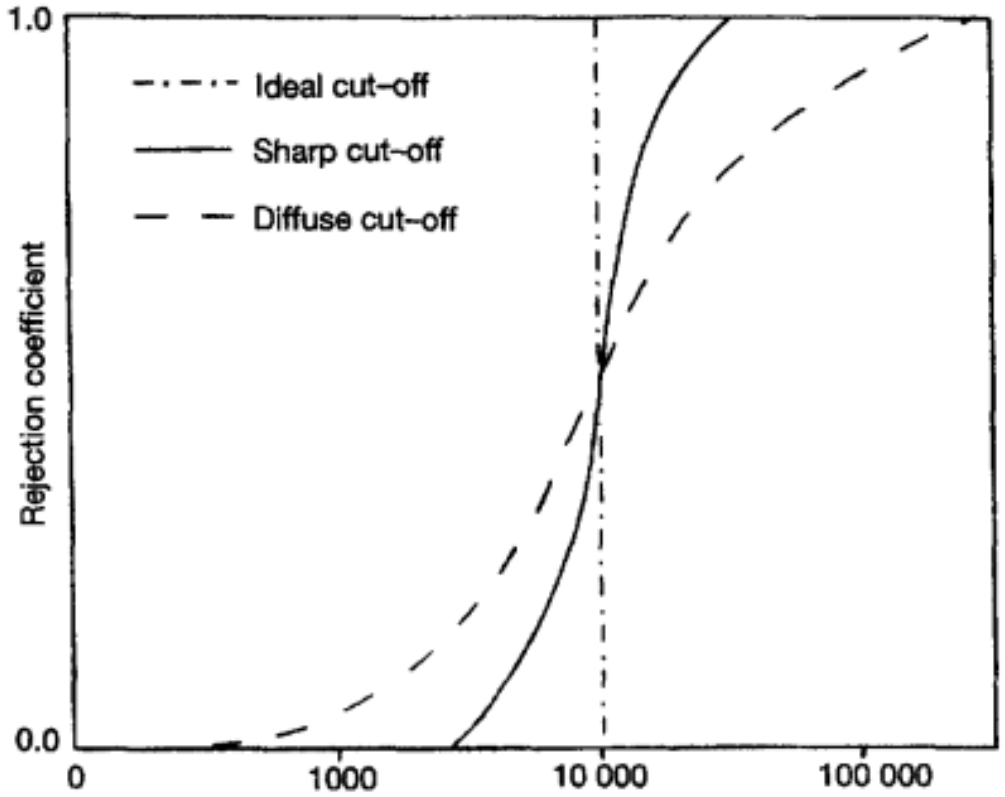
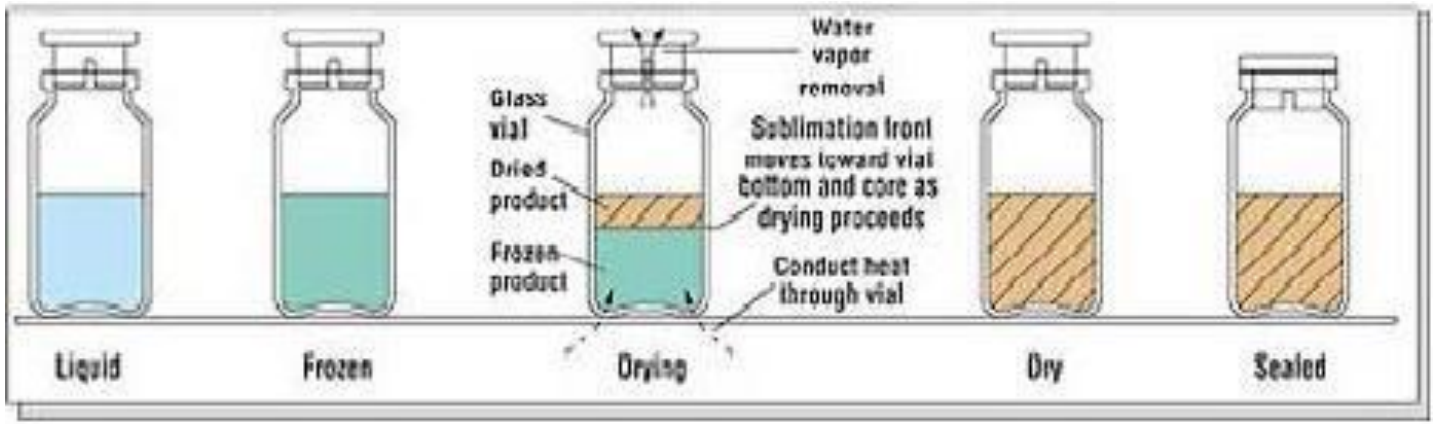


Figure 1 Rejection characteristics of a membrane with a NMWC of 10 000. Ideally all molecules of ≥ 10 000 molecular weight will not pass through the membrane whilst those of < 10 000 will. However, due to the distribution of pore sizes in membranes there is also a distribution in molecular weight of the molecules able to pass through the membrane. Thus, for a particular membrane the filtrate will contain a certain percentage of molecules with molecular weight less than the NMWC and a similar percentage with a higher molecular weight.

Freeze-drying or lyophilization

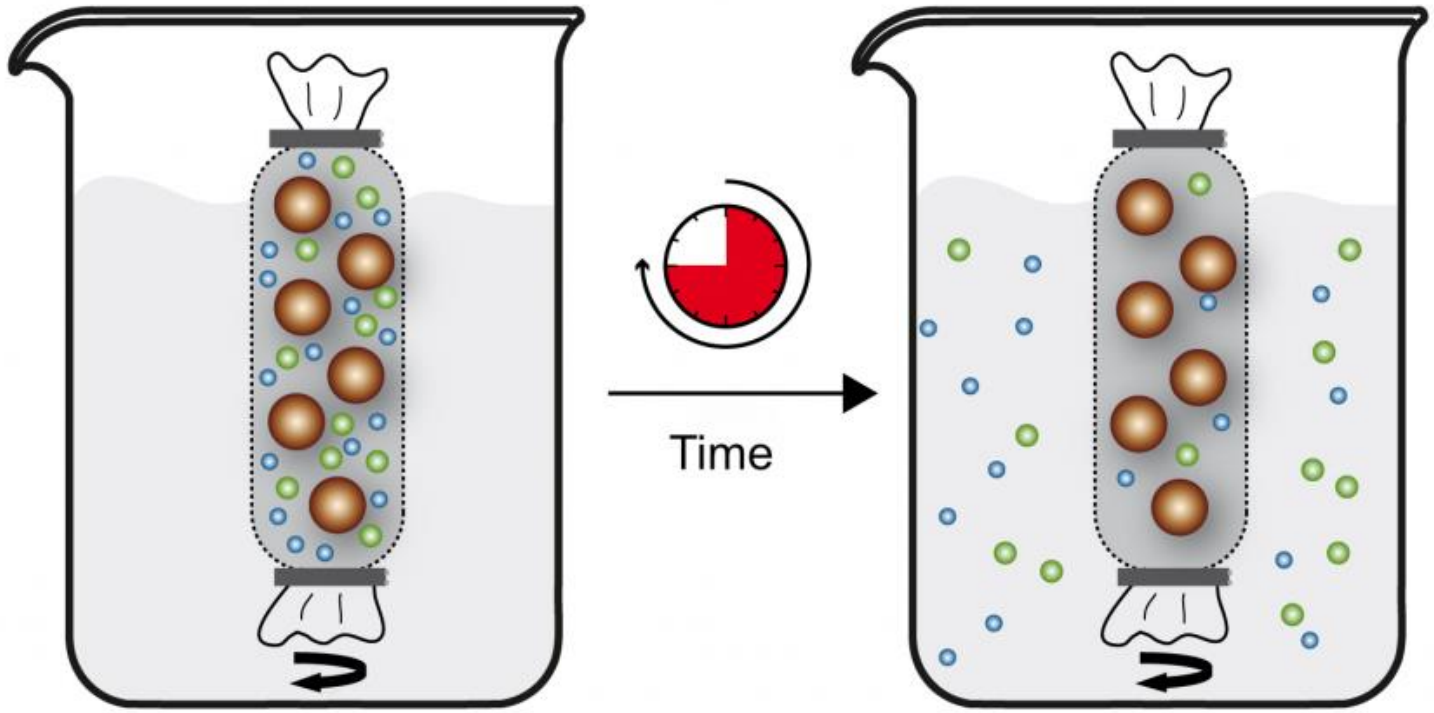
In contrast to ultrafiltration, lyophilization also results in concentration of any salts present in the initial solution; in addition lyophilization may cause greater losses in enzyme activity. Lyophilization is, however, an invaluable method both for concentrating small molecular weight peptides which are not retained by ultrafiltration membranes, and for obtaining a dry powder of protein. Once obtained, a dry powder of enzyme is more stable than an aqueous preparation of enzyme, since many degradation processes require the presence of water. Hence many commercially available proteins are obtained as freeze-dried powders.


Water vapor removal
Glass vial
Sublimation front moves toward vial bottom and core as drying proceeds
Dried product
Frozen product
Conduct heat through vial
Liquid
Frozen
Drying
Dry
Sealed

Freeze-drying flasks come in a variety of shapes, pear-shaped flasks are more convenient for small volumes, whilst flat-bottomed flasks are best used for larger volumes. The freeze-drying flask is then rapidly attached to a mechanical vacuum pump (e.g. Edwards), ensuring that all the solution remains frozen prior to applying the vacuum. Any thawed liquid will rapidly degas and 'bump' with possible loss of solution out of the flask.

Removal of salts and exchange of buffer 1. Dialysis

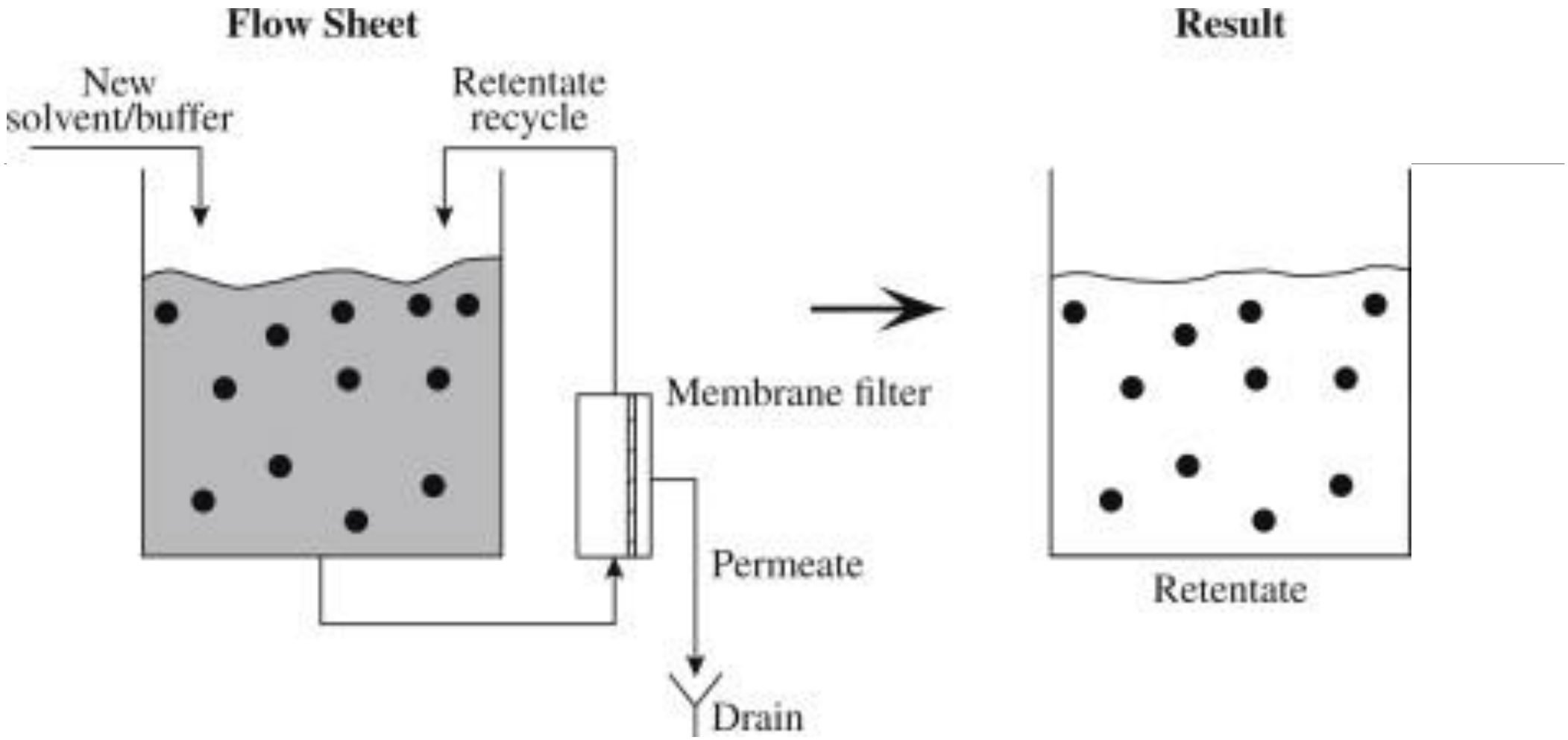
Frequently it is necessary to remove salts or change the buffer after one step in the purification for the next step to work efficiently (e.g. for ion Exchange chromatography, the pH and/or the ionic strength may need to be changed to ensure that the protein will bind to the matrix). This is often achieved by dialysis; the protein solution is placed in a bag of semi-permeable membrane and placed in the required buffer, small molecules can pass freely across the membrane whilst large molecules are retained. The semi-permeable dialysis tubing is usually made of cellulose acetate, with pores of between 1-20 nm in diameter. The size of these pores determine the minimum molecular weight of molecules which will be retained by the membrane (NMWC).


Time
Start of dialysis
(high concentration gradient)
End of dialysis
(Equilibrium)
Globular Protein
Ionic contamination
Semipermeable
membrane


SPECTRAPOR
membrane
tubing
1
2
3
4

2. Diafiltration

A quicker, alternative method for desalting or buffer exchange is diafiltration. This method is also more applicable to larger scale applications (i.e. > 100 ml). Ultrafiltration equipment is used for diafiltration. With some types of equipment, water or buffer is added to the protein solution, which is then concentrated by ultrafiltration; this process is repeated until the ionic strength of the filtrate reaches that of the added buffer or water. With other types or equipment (e.g. Millipore's hollow fibre systems) the plumbing is altered to allow uptake of water or buffer at a rate equal to the flux through the membrane; this method has the advantage that it can be left unattended whilst equilibrium is achieved.


Flow Sheet
Result
New solvent/buffer
Retentate recycle
Membrane filter
Permeate
Drain
Retentate

3. Gel filtration

Another quicker alternative to dialysis is gel filtration. This method is only applicable to small volumes. The maximum sample volume should not exceed 25-30% of the volume of the column to ensure adequate resolution between the protein and salt. A gel filtration matrix with a small pore size (e.g. Sephadex G-25 Amersham Pharmacia) is poured into a column to give a bed volume of approximately five times the volume of sample to be desalted.