Protein Extraction and Purification Methods Chromatography

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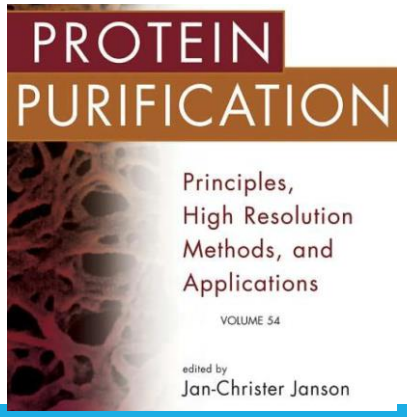


- The term chromatography refers to a group of separation techniques characterized by a distribution of the molecules to be separated between two phases, one stationary and the other mobile.

- The most common physical configuration is column chromatography, in which the stationary phase is packed or molded into a tube, a column, through which the mobile phase, the eluent, is pumped.
- The sample to be separated is introduced into one end of the column.
- The various sample components travel with different velocities through the column and are subsequently detected and collected at the other end.
- Other configurations, such as thin-layer chromato-graphy and paper chromatography are also used, but are less commonly applied for protein separation.

TABLE 2.1 Versions of Protein Liquid Chromatography

Separation Principle	Type of Chromatography
Size and shape	Gel filtration/size exclusion chromatography
Net charge	Ion exchange chromatography
Isoelectric point	Chromatofocusing
Hydrophobicity	Hydrophobic interaction chromatography
	Reversed phase chromatography
Biological function	Affinity chromatography
Antigenicity	Immunoadsorption
Carbohydrate content	Lectin affinity chromatography
Content of free –SH	Chemisorption ("Covalent chromatography")
Metal binding	Immobilized metal ion affinity chromatography
Miscellaneous	Hydroxyapatite chromatography
	Dye affinity chromatography

- The stationary phase in a chromatographic experiment is composed of a porous matrix and imbibed immobile solvent.

- Typically, the solvent constitutes most of the stationary phase, often more than 90%, and such materials are generally referred to as gels. In protein chromatography the solvents are normally aqueous buffers, and the gel-forming materials are usually composed of hydrophilic polymers.

- In addition to being hydrophilic, an ideal general matrix for protein chromatography should not contain groups that spontaneously bind protein molecules.
- However, it should contain functional groups that allow the controlled synthesis of a wide variety of protein adsorbents.
- A wide variety of materials have been used for the design of protein chromatography matrices. These can be classified as either inorganic materials, synthetic organic polymers, or polysaccharides.

Examples include the following:

† inorganic materials

– porous silica

– controlled pore glass

– hydroxyapatite

† synthetic organic polymers

– polyacrylamide

– polymethacrylate

– polystyrene

† polysaccharides

– cellulose

– dextran

– agarose

Among the polysaccharides, cellulose (Figs. 2.1 and 2.2) is still used for the synthesis of protein ion exchangers, more than 50 years after its introduction by Peterson and Sober in 1956, and is marketed under the trade names WhatmanTM

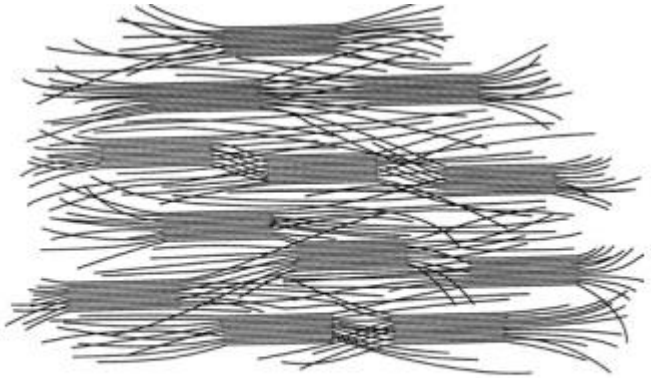


Figure 2.2 Schematic representation of part of a cellulose fiber composed of ordered (crystalline) and disordered (amorphous) regions.

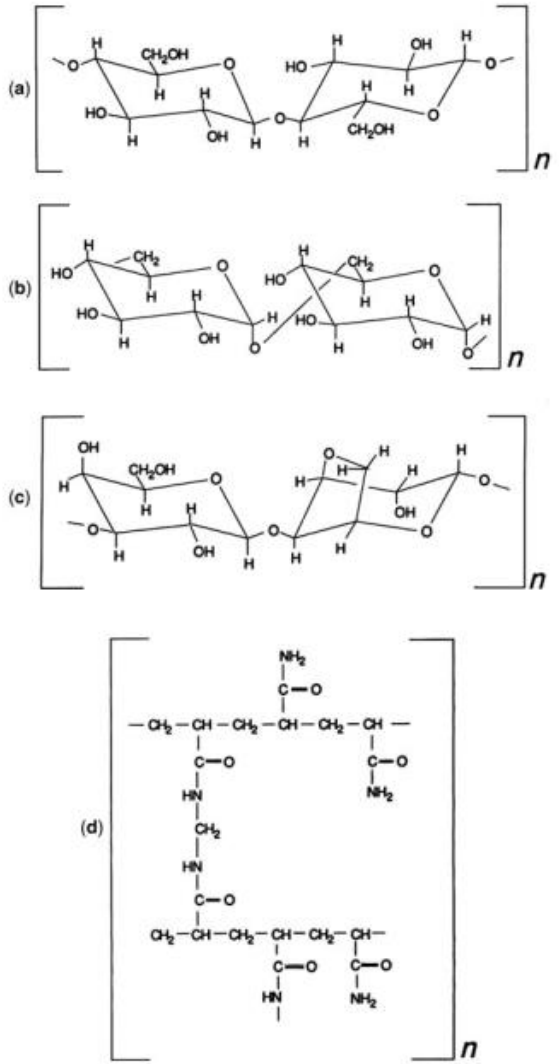


Figure 2.1 Partial structures of common gel-forming polymers. (**a**) Cellulose (ß-1,4-linked D-glucose). (**b**) Dextran (α-1,6-linked D-glucose; the glycosidic bond is stretched out for layout reasons). (**c**) Agarose (alternating 1,3-linked ß-D-galactose and 1,4-linked, 3,6-anhydro-α-L-galactose). (**d**) Polyacrylamide crosslinked with *N,N*-methylenebisacrylamide.

Ligand–Protein Interactions

- The binding of a dissolved protein to an immobilized ligand arises because of one or several of the following interactions:
- † ion–ion or ion–dipole bonds
- † hydrogen bonds
- † dispersion or van der Waals forces
- † aromatic or p–p interactions
- † hydrophobic effect or hydrophobic interaction.

- The surface of globular proteins typically has ≐45% hydrophobic residues and 55% charged or hydrophilic uncharged residues accessible to surrounding water. In the latter groups, the positive charges of lysine, arginine, and histidine side chains, as well as the a-amino group, change according to pH. The negative charge of glutamic acid and aspartic acid side chains and the a-carboxyl group are also pH-dependent.

- Of these, the histidine side chain and the a-amino groups titrate within the pH region that is commonly used in chromatography. The cysteine thiol side chain is not typically available in extracellular proteins, but might be in intracellular proteins. It does not play a role as an anion, although it has a pK of ≐8.5. Other hydrophilic groups such as asparagine, glutamine, serine, and threonine, as well as peptide bonds, are mostly involved in hydrogen-bonding interactions.

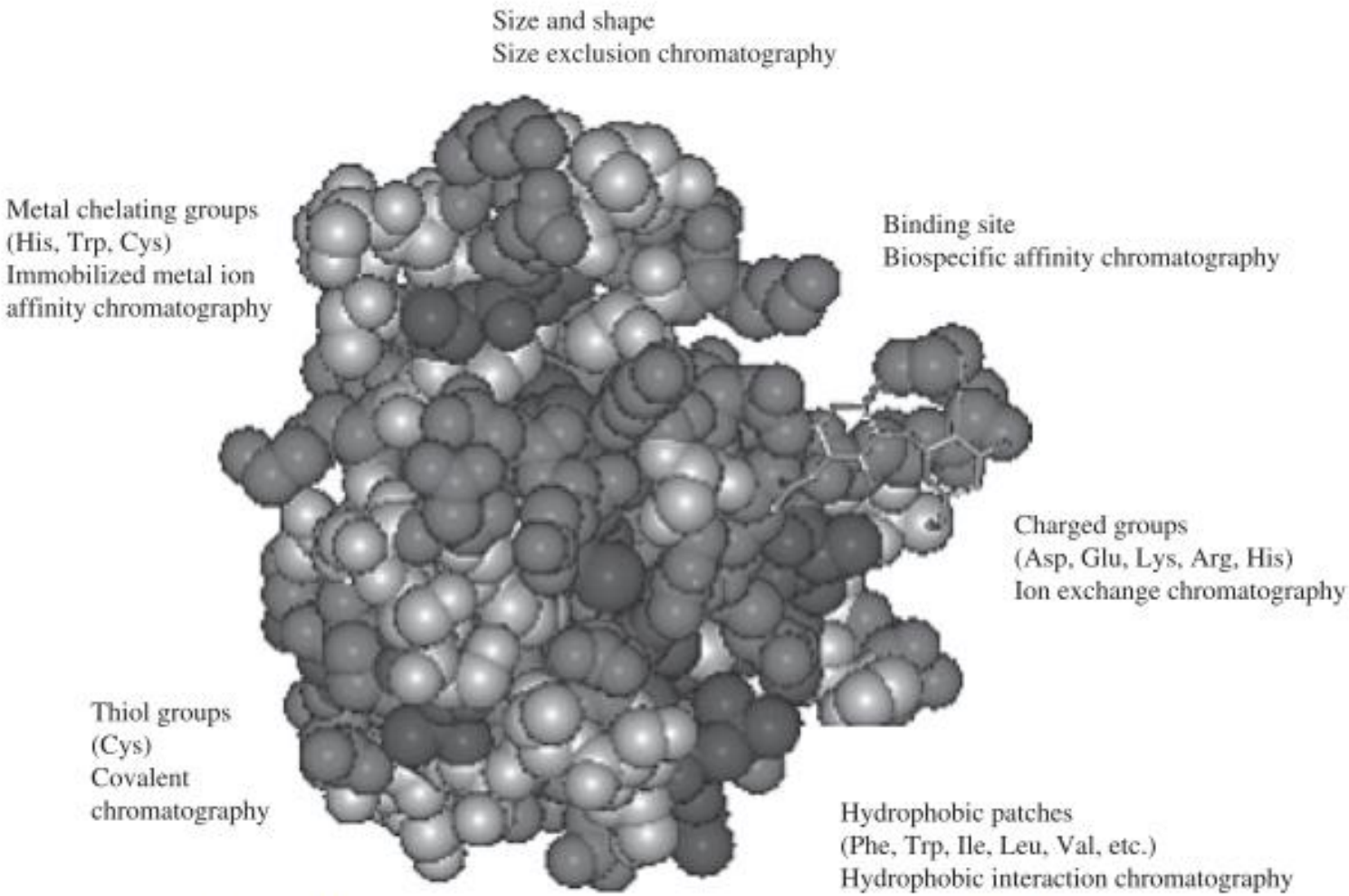
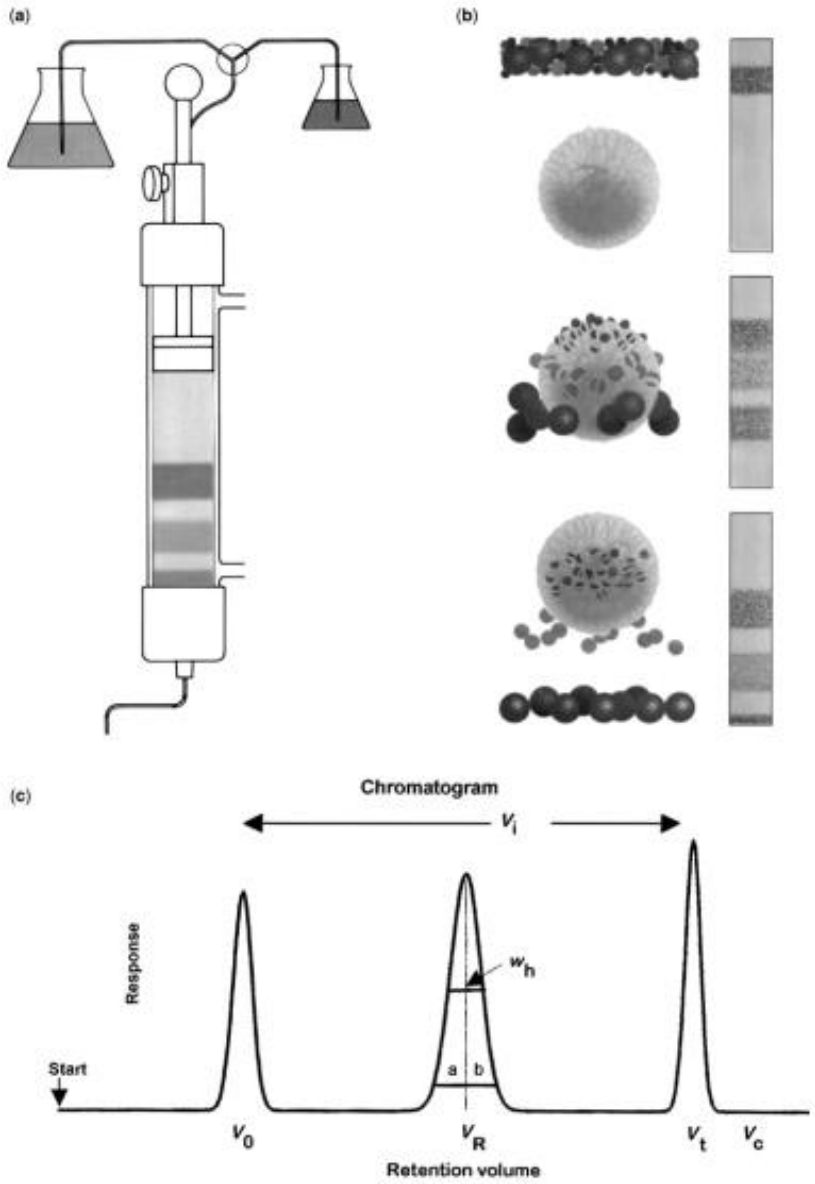


Figure 2.9 Separation principles and corresponding chromatographic techniques.

GEL FILTRATION: SIZE EXCLUSION CHROMATOGRAPHY

- The separation of solutes by their molecular size has, in addition to gel filtration and gel permeation, been given a variety of designations, and gel chromatography, exclusion chromatography, molecular sieving chromatography, steric exclusion chromatography, and size exclusion chromatography are synonymously used in older literature.

- Today, there seems to be a general consensus to use the term size exclusion chromatography as a general designation of the separation principle, because it is a mechanistically correct descriptive term of the process, in accordance with the proposal by Pedersen.


(a)
(b)
(c)
Chromatogram
V_i
Response
w_h
Start
a
b
V_0
V_R
V_t
V_c
Retention volume

- Gel filtration chromatography (sometimes referred to as molecular sieve chromatography) is a method that separates molecules according to their size and shape.
- The separation of the components in the sample mixture, with some exceptions, correlates with their molecular weights. In these cases, gel filtration can be used as an analytical method to determine the molecular weight of an uncharacterized molecule.
- Gel filtration is also an important preparative technique since it is often a chromatographic step in the purification of proteins, polysaccharides and nucleic acids.
- The basic components of the gel filtration experiment are the matrix, chromatography column and the elution buffer.
- The matrix is the material in the column that is actually the separation medium. It is the stationary phase of the chromatography.

- The column is a tube with a frit and elution spout fitted at the bottom. The frit is a membrane or porous disk that supports and retains the matrix in the column but allows water and dissolved solutes to pass.

- The elution buffer is the mobile phase of the chromatography and flows through the matrix and out of the column.

- The column, with the matrix and applied sample, is “developed” by the elution buffer.

- This means that the molecules in the sample are carried by the flow of buffer into the matrix where they are gradually separated.

- The separated zones of molecules then flow out of the column where they are collected for analysis.

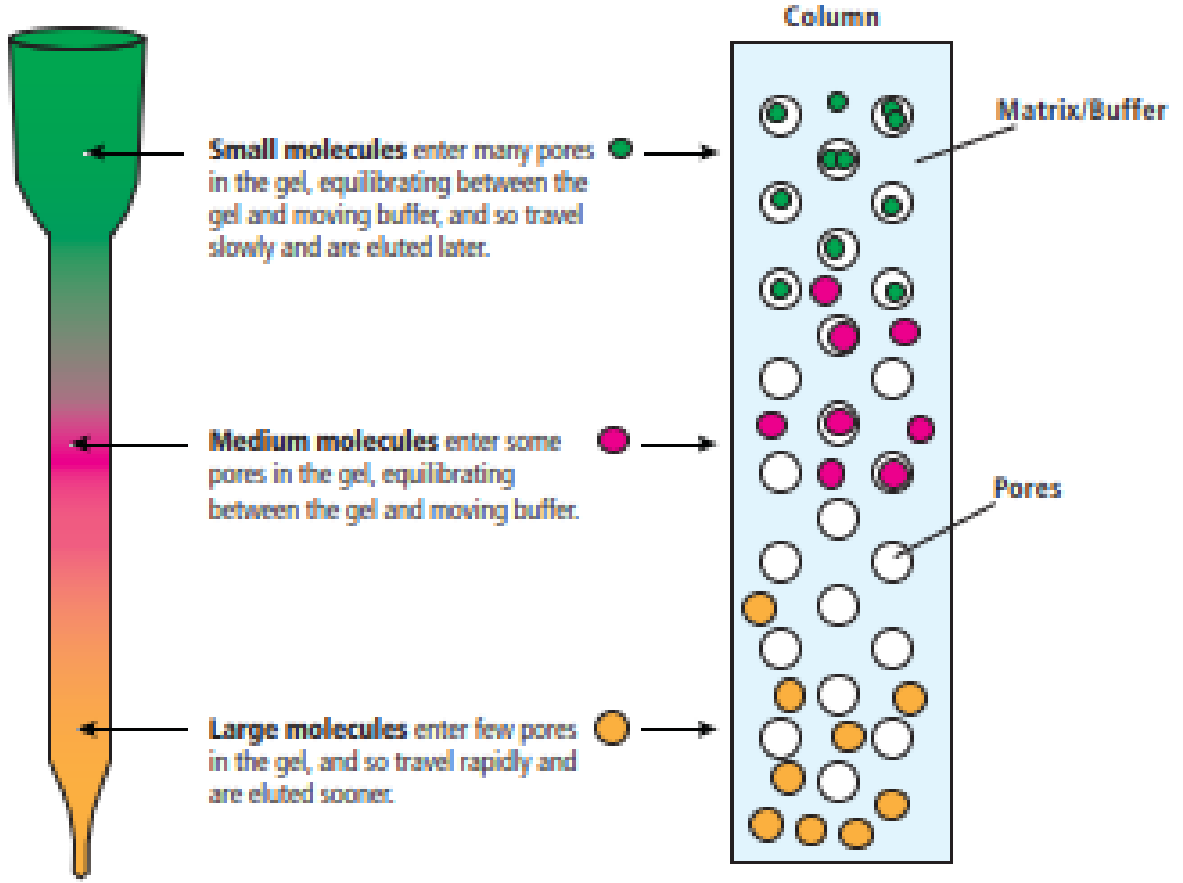
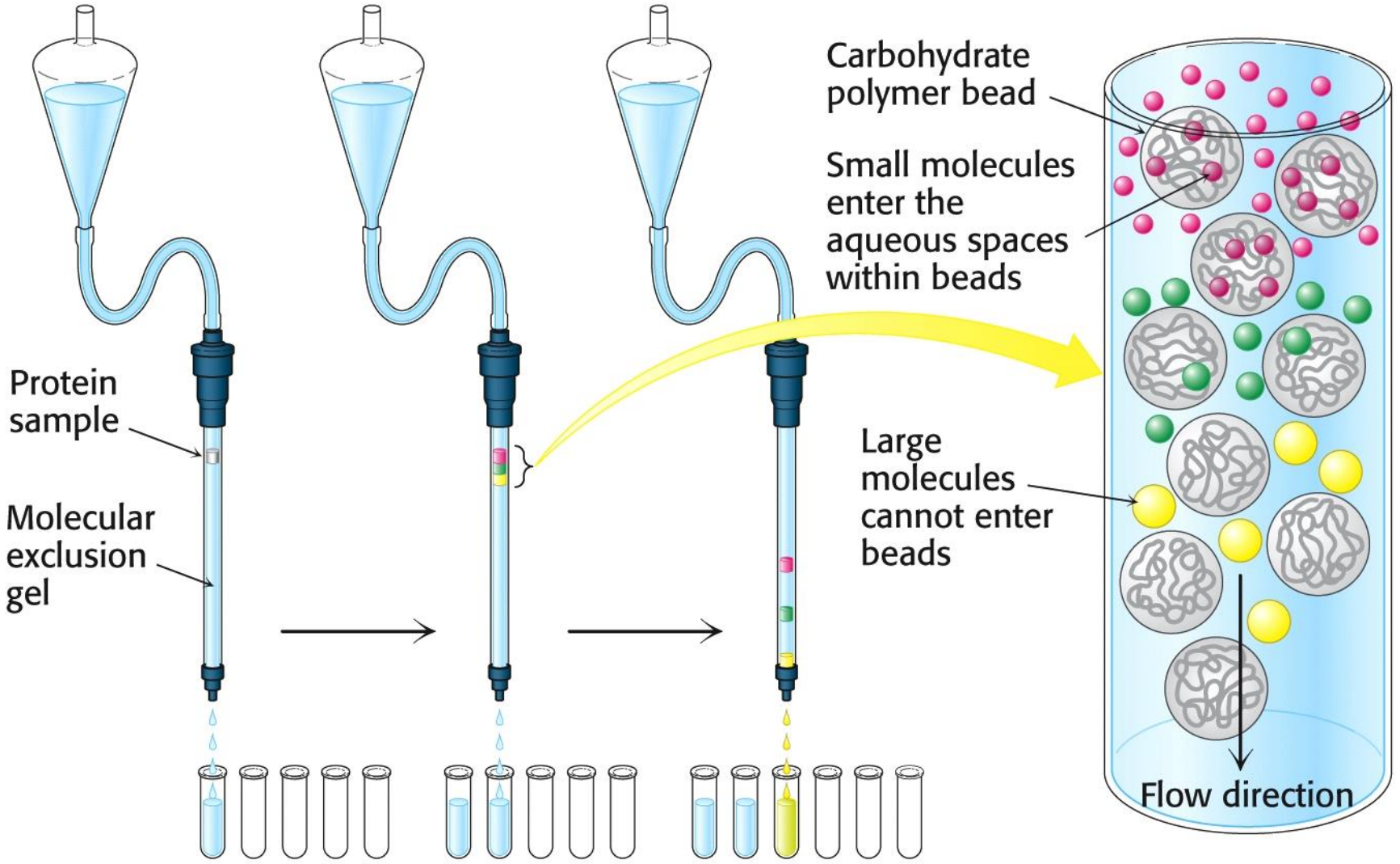
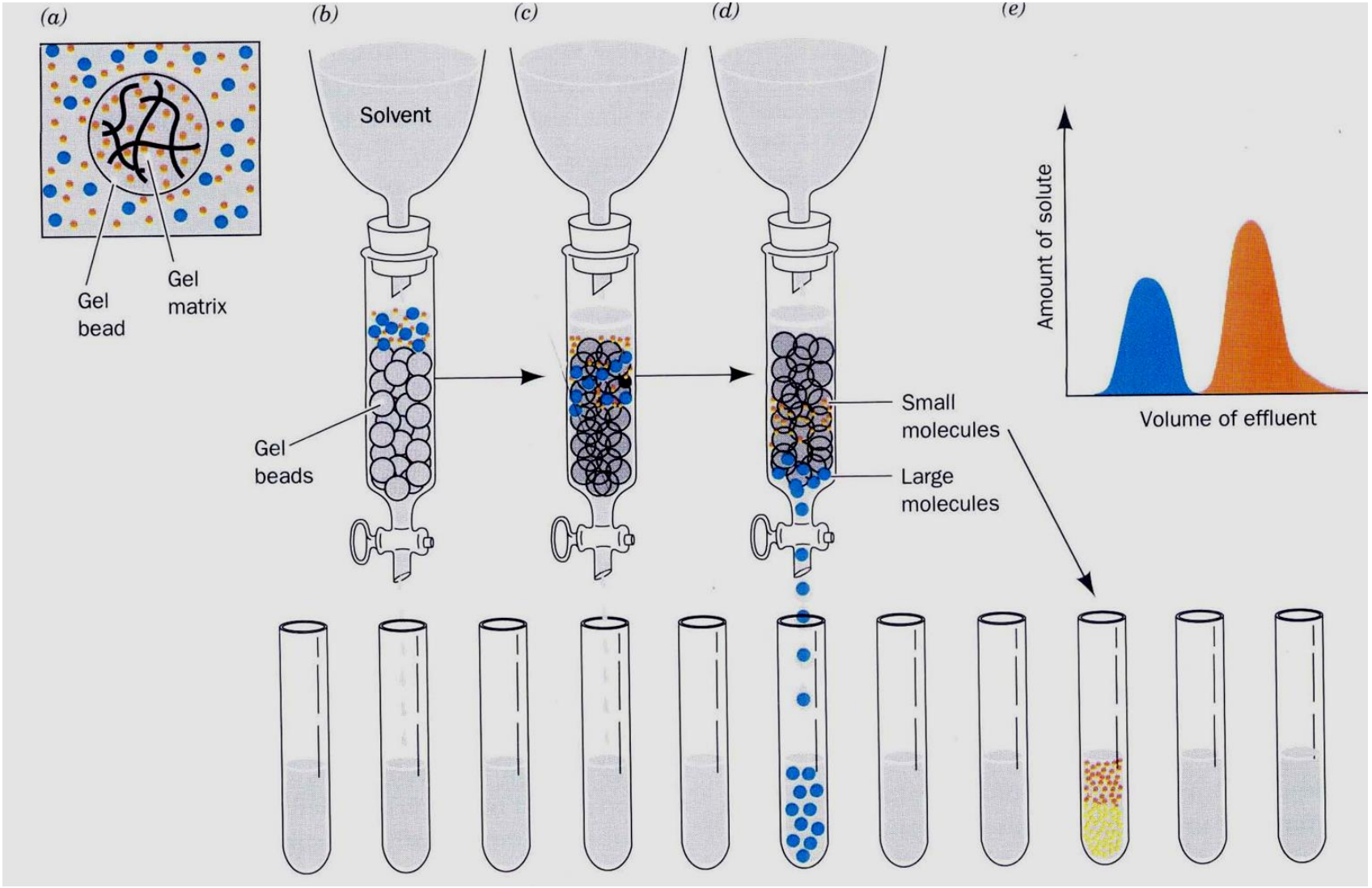


Figure 1:
How Different Sized Molecules Move Through the Matrix.


Protein sample
Molecular exclusion gel
Carbohydrate polymer bead
Small molecules enter the aqueous spaces within beads
Large molecules cannot enter beads
Flow direction


(a)
(b)
(c)
(d)
(e)
Gel bead
Gel matrix
Solvent
Gel beads
Small molecules
Large molecules
Amount of solute
Volume of effluent

Determination of Molecular Mass

- Analytical SEC is frequently used for the assay of molecular mass of proteins or molecular weight (i.e., mass) distributions (MWD) of hydrophilic macromolecules or polymers.

- It must be noted that calilbration of the column using the elution volume corresponding to the peak apex of a polymer requires that the molecular mass or molecular size corresponding to the peak apex is known.

- An example of calibration of an analytical SEC column is given in Figure 3.16.
- Here, a mixture of seven reference proteins were injected and the elution volume converted to *K*av (distribution coefficient available) for the calibration curve.
- The figure illustrates that the shape of the curve is fairly linear over the central portion of the fractionation range.
- Note that the calibration curve is, in practice, identical to the selectivity curve.

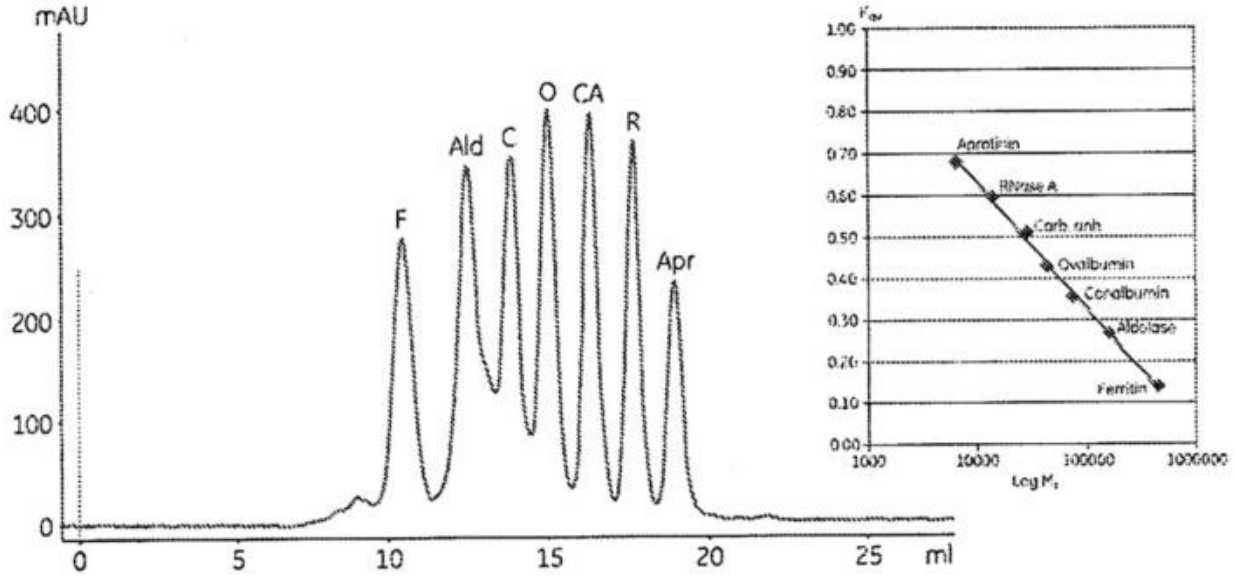
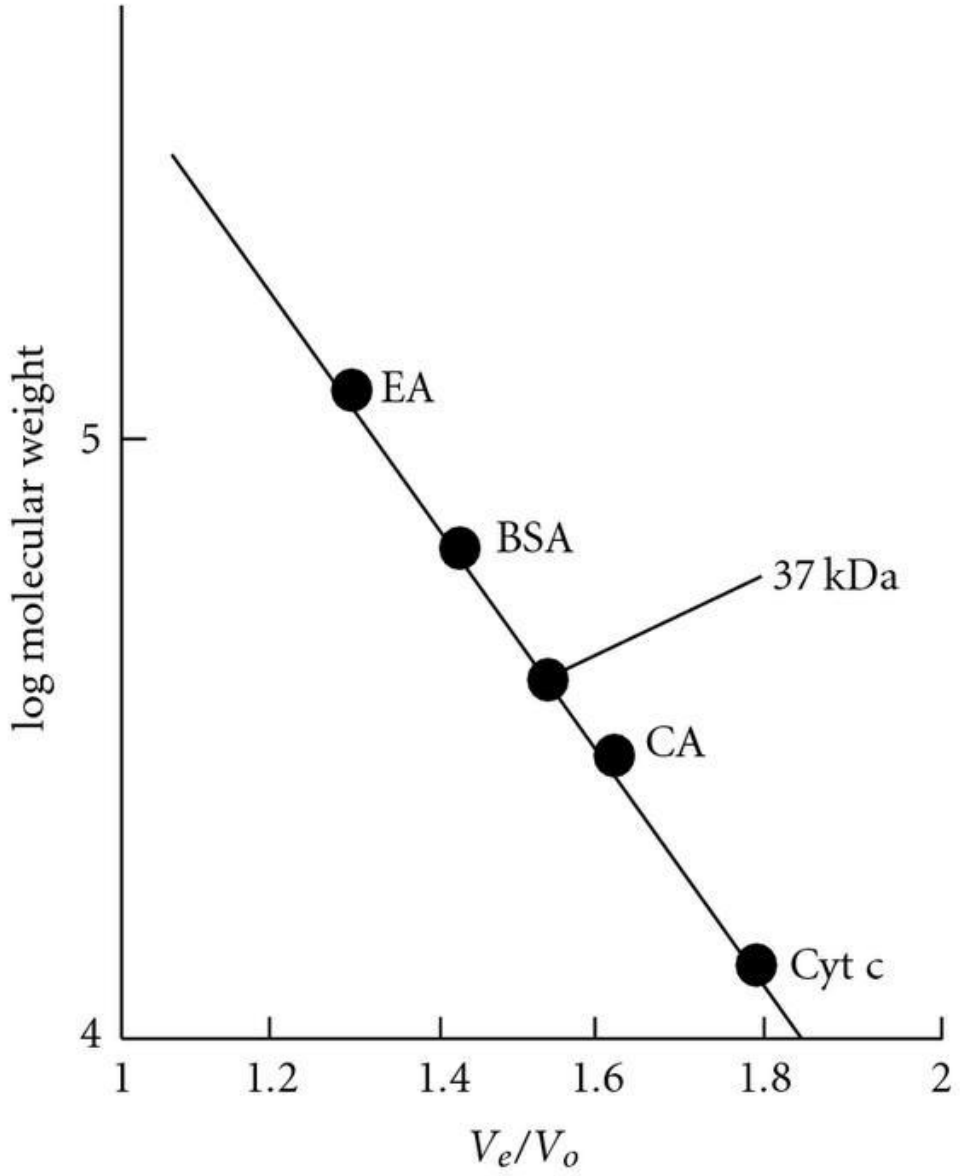


Figure 3.16 Example of a calibration curve for analytical SEC. Standard protein mixture of ferritin, aldolase, catalase, ovalbumin, carbonic anhydrase, ribonuclease A, and aprotinin applied on a Tricorn™ Superdex™ 200 10/300 GL column. Copyright General Electric Company – all rights reserved. Reproduced from Reference 177 with kind permission.


log molecular weight
5
4
EA
BSA
37 kDa
CA
Cyt c
1
1.2
1.4
1.6
1.8
2
V_e/V_o

Ion exchange chromatography (IEC)

- Ion exchange chromatography (IEC) has been in common use for more than 50 years for the separation and purification of proteins.
- EC has high resolving power, high protein binding capacity, and versatility (there are several types of ion exchangers). Also, the composition of the buffer and pH can be varied, and it is easy to use. Although IEC is an established technique in most biochemical laboratories, the fundamental mechanisms behind protein binding to charged surfaces are not fully understood.
- In the stoichiometric model of Boardman and Partridge, a number of charged groups of the protein bind to the same number of oppositely charged groups of an ion exchanger, and counter ions are released both from the protein and the ion exchanger. The charges are localized to special points on the protein molecule, and the number of charged groups binding to the ion exchanger is called the Z-value,

- The basis for IEC is the electrostatic attraction between proteins in solution and charged groups of the ion exchanger.
- The strength of the interaction depends upon the charge of the proteins and the ion exchangers, the dielectric constant of the medium, and competition from other ions for the charged groups of the ion exchanger and protein.
- When the concentration of competing ions is low, the proteins adsorb to the ion exchanger. When it is high, the proteins are desorbed.
- The most common technique in IEC is adsorption of target proteins from a buffer of low ionic strength and desorption with a more concentrated buffer

Factors other than the net charge that can influence IEC of protein include the following:

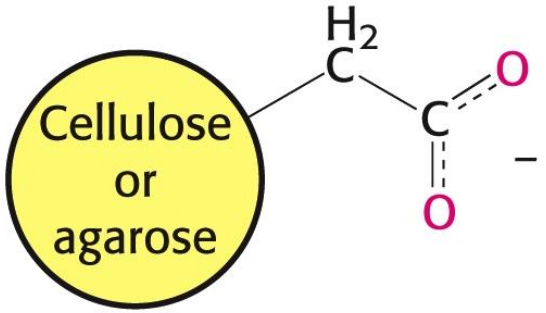
† charge distribution on the protein surface

† nature of the particular ions in the solvent

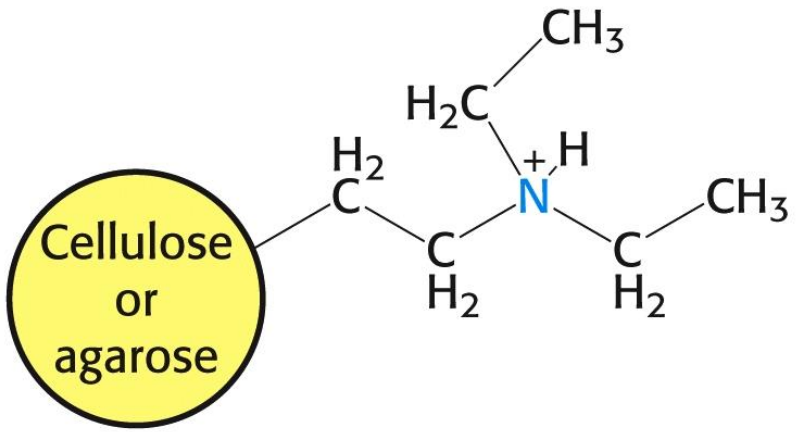
† nonelectrostatic interactions with the ion exchanger, hydrophobic interactions, and hydrogen bonding

† temperature

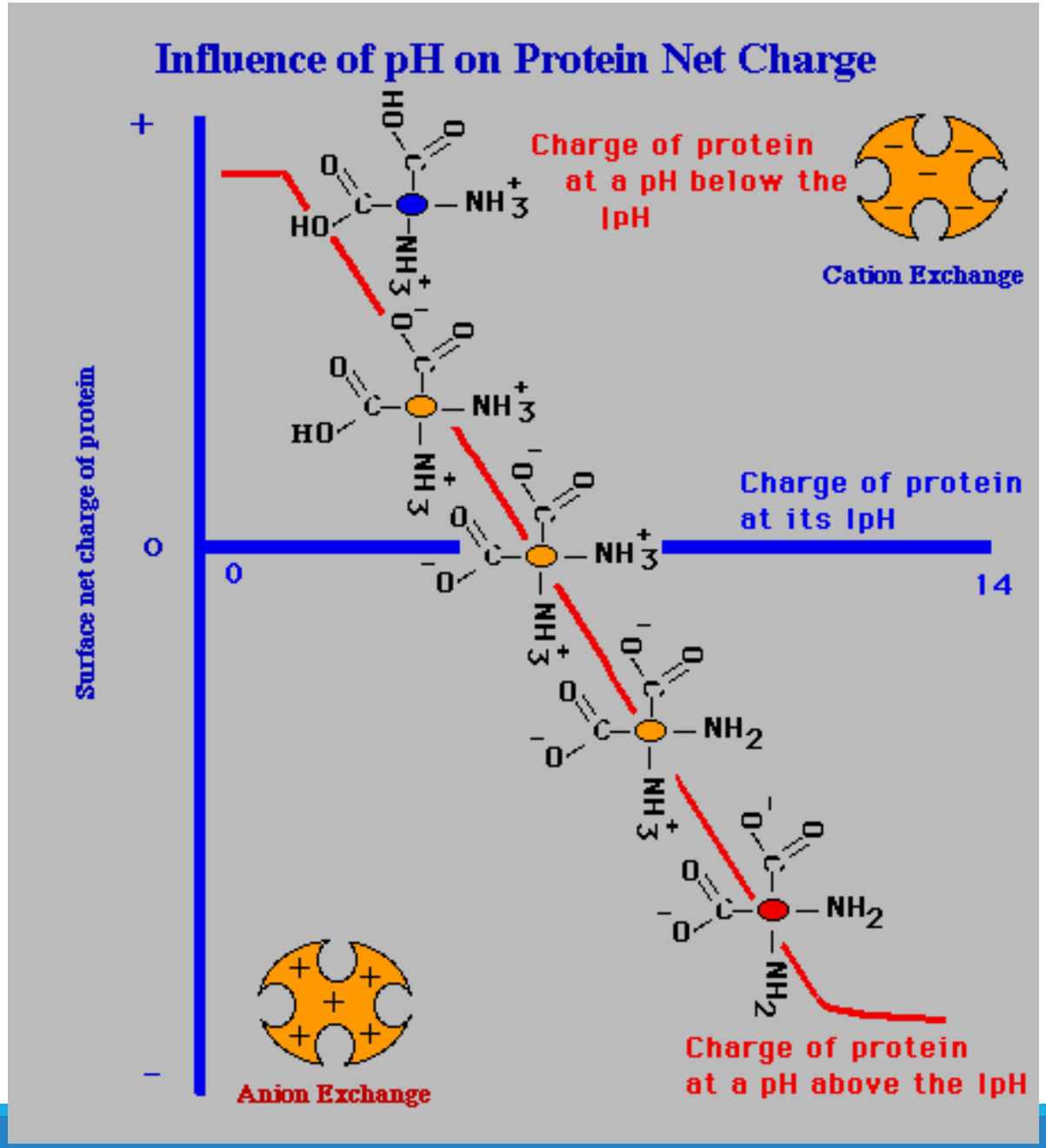
† additives such as organic solvents.

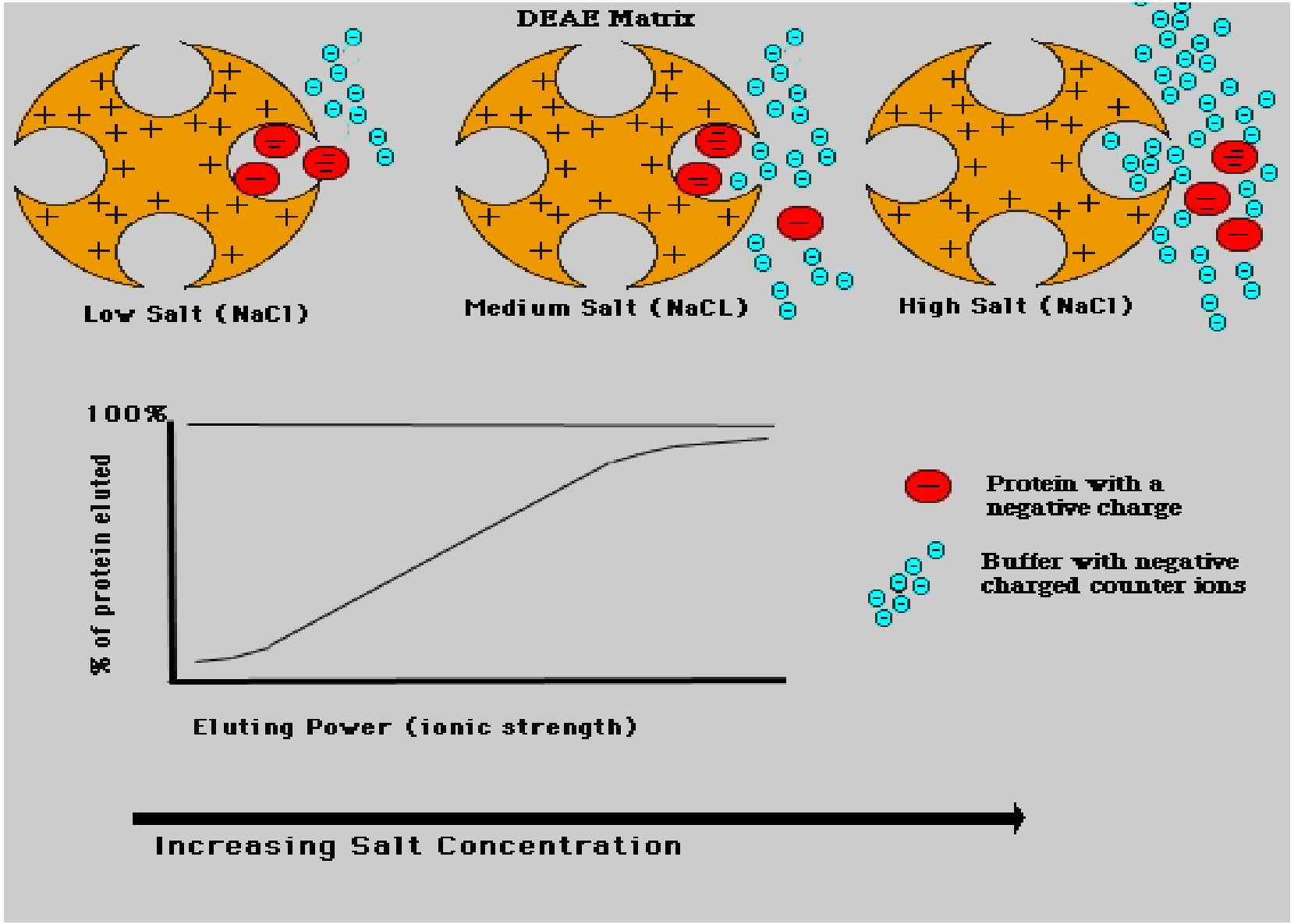


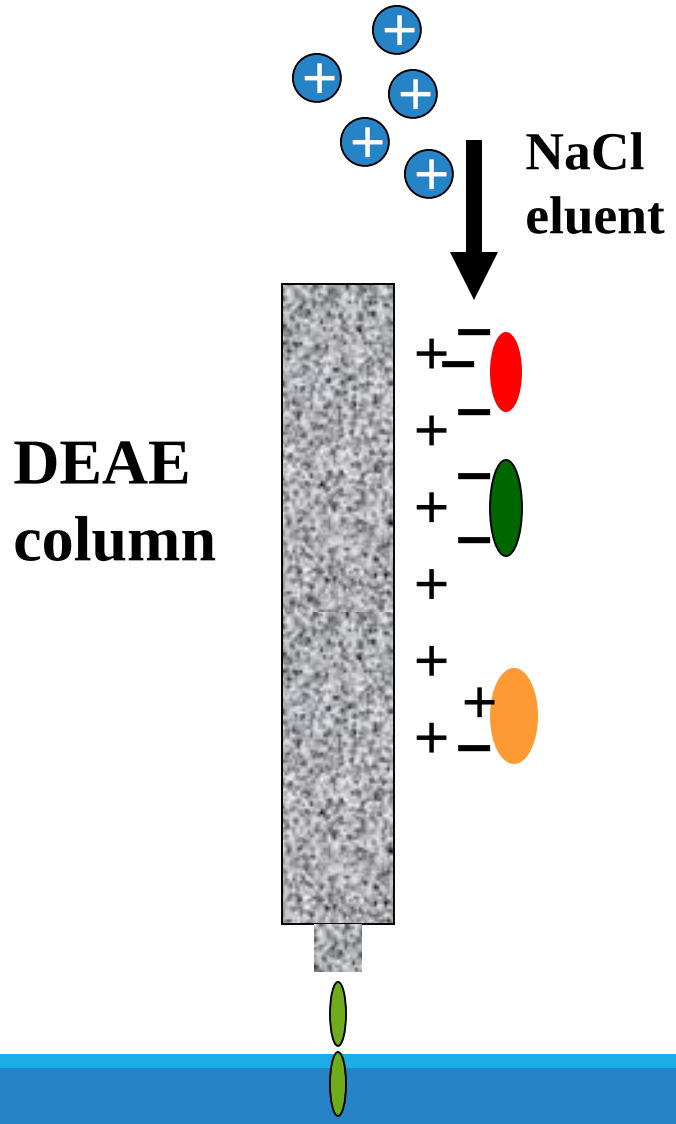
Carboxymethyl (CM) group
(ionized form)



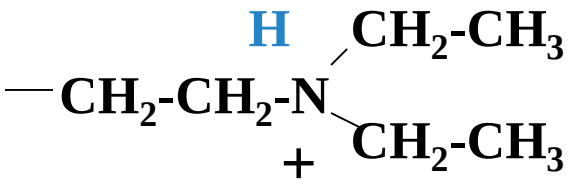
Diethylaminoethyl (DEAE) group
(protonated form)

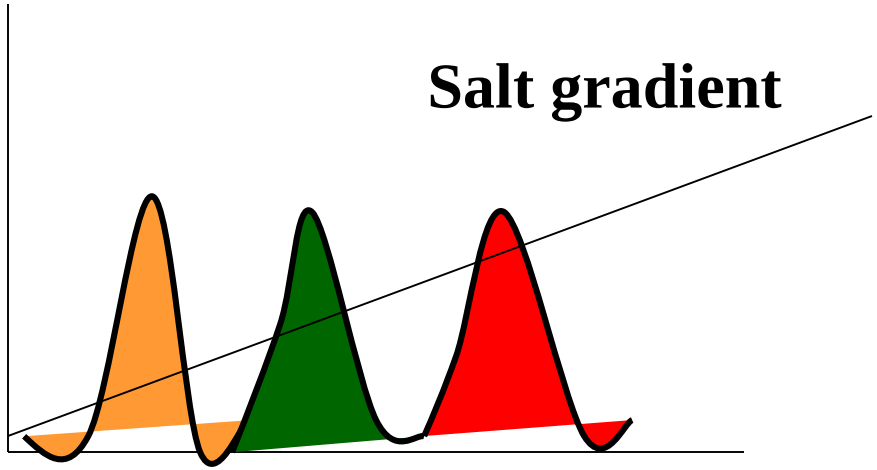

Influence of pH on Protein Net Charge
Surface net charge of protein
+
0
–
0
14
Charge of protein at a pH below the IpH
Cation Exchange
Charge of protein at its IpH
Anion Exchange
Charge of protein at a pH above the IpH


DEAE Matrix
Low Salt (NaCl)
Medium Salt (NaCL)
High Salt (NaCl)
100%
% of protein eluted
Eluting Power (ionic strength)
Protein with a negative charge
Buffer with negative charged counter ions
Increasing Salt Concentration


NaCl
eluent
DEAE
column

(DEAE)


H
CH2-CH3
CH2-CH2-N
CH2-CH3
+


Salt gradient

The first ion exchangers used in the separation of biological substances are cellulosic ion exchangers. "Sephadex", a modified dextran, "Sepharose", a cross-linked agarose, and "Sephacel", a cross-linked cellulose, are the first spherical ion exchangers with a high-porosity, cellulose.

TABLE 4.2 Functional Groups and pK_a Values of Ion Exchangers (Ionic Strength 0.1 M)

Name	Designation	pK_a	Structure
		Anion Exchangers	
Diethylaminoethyl	DEAE	5.8[a] and 9.1[a]	$-OCH_2NH(C_2H_5)_2$
Trimethylaminoethyl	TMAE	—	$-OCH_2CH_2N^+(CH_3)_3$
Dimethylaminoethyl	DMAE	~10	$-OCH_2CH_2NH(CH_3)_2$
Trimethylhydroxypropyl	QA		$-OCH_2CH(OH)NH(C_2H_5)_2$
Quaternary amino ethyl	QAE		$-OCH_2CH_2N^+(C_2H_5)_2$ $CH_2CH(OH)CH_3$
Quaternary amine	Q		$-OCH_2N^+(CH_3)_3$
Triethyl amine	TEAE	9.5[b]	$-OCH_2N^+(C_2H_5)_3$
		Cation Exchangers	
Methacrylate		6.5[c]	$-CH_2CH(CH_3)COOH$
Carboxymethyl	CM	3.5–4	$-OCH_2COOH$
Orthophosphate	P	3 and 6	$-OPO_3H_2$
Sulfoxyethyl	SE	2	$-OCH_2CH_2SO_3H$
Sulfopropyl	SP	2–2.5	$-OCH_2CH_2CH_2SO_3H$
Sulfonate	S	2	$-OCH_2SO_3H$

[a] See Section 4.4.2.
[b] The pK_a value does not refer to a quaternary amine.
[c] See Section 4.4.5 for pK_a values at different ionic strengths.