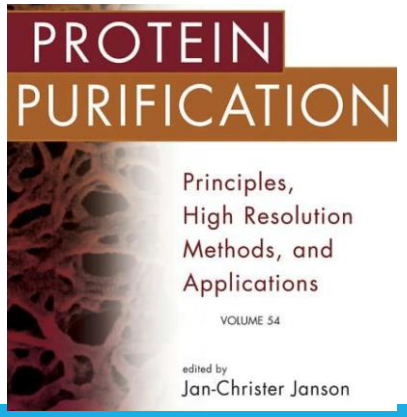
Protein Extraction and Purification Methods Chromatography-2

ASSOC. PROF. DR. GÖZDE AYDOĞDU TIĞ



HYDROPHOBIC INTERACTION CHROMATOGRAPHY

- HIC has been known by several names, including hydrophobic bonding, hydrophobic chromatography, hydrophobic affinity chromatography, non-ionic adsorption chromatography, salting out adsorption in amphiphilic adsorbents, hydrophobic interaction chromatography, and salt-promoted adsorption chromatography.

- In general, it is well appreciated that charge-based interactions involve much longer range forces than hydrophobic, hydrogen-bond or other van der Waals interactions. So, if you have an amphipathic surface the charge forces will tend to dominate initial interactions with amphipathic colloids such as proteins. Once the colloid is localized at the surface, the other forces will come into play and can even dominate.

- HIC media that have charged and hydrophobic groups are classic examples of this. Of course, many other interactions may also play a role, such as asymmetric partition of salt and solvent between the bulk and media localized liquid phase regions.

The main chromatographic components that affect HIC are as follows:

† nature of the adsorbent (type of base matrix, structure,
concentration of immobilized ligands, etc.)
† composition of the solvent (type and concentration of
salts in the equilibration buffer, pH, and temperature)
† characteristics of the solute and other components of the
sample.

Of these parameters, the type and concentration of ligand as well as the type and concentration of salt in the adsorption buffer are of paramount importance in determining the outcome of an HIC event. In general, the type of immobilized ligand determines its adsorption selectivity towards the proteins in a sample, and its concentration determines its adsorption capacity.

- 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.

IMMOBILIZED METAL ION AFFINITY CHROMATOGRAPHY (IMAC)

- 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.

- Most proteins can form complexes with metal ions. Many of these are multidentate complexes (chelates) that allow for the purification of the proteins by IMAC. The strength of the complexes formed varies from protein to protein, which, in many cases, gives rise to the high specificity of IMAC.

- The chromatographic sorbent used in IMAC (see scheme in Fig. 7.1) consists of a suitable chromatographic support to which a metal-chelating substance (B) has been attached by a leash or linkage group (A).

- The structure of the complex formed when metal ions are added must be such that some coordination sites are left free for the binding of solvent or solute molecules (ligands). Alternatively, the complex should be able to rearrange itself to allow incoming ligands to participate. in the formation of chelates or complexes with the metal ion.

- Solvent or buffer molecules will occupy “free” coordination sites of the metal in the absence of ligands with higher affinity for the metal ion.

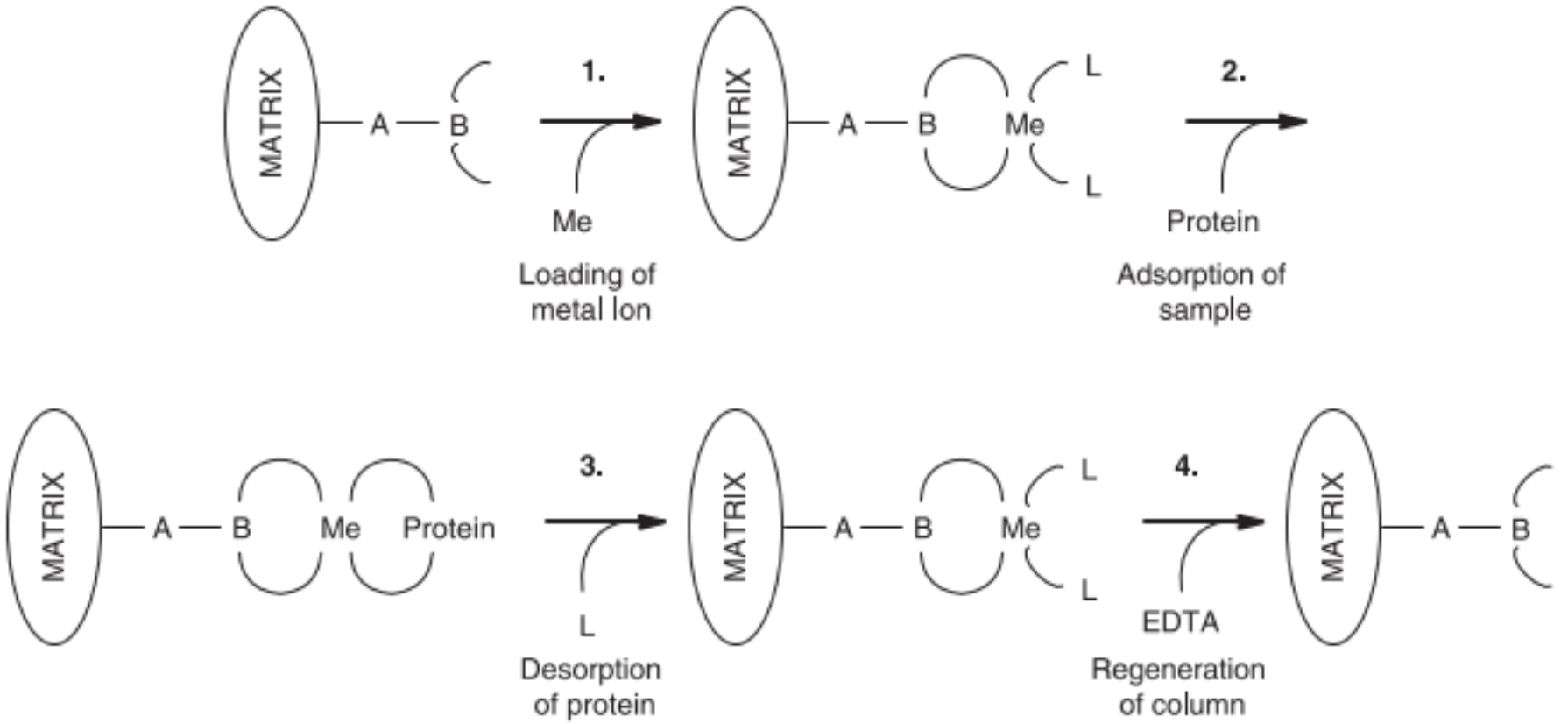


Figure 7.1 Principle of immobilized metal ion affinity chromatography (IMAC). A = linkage (spacer) group, B = chelating group, Me = metal ion, L = solvent or buffer molecule.

Some of the special features of IMAC of proteins can be summarized as follows:

† Exposure of certain amino-acid residues (histidine, cysteine, tryptophan) on the “surface” of the proteins is required for the adsorption of proteins.

---

† The steric arrangement of the protein chain plays an important role, which means that molecules with closely similar properties with respect to charge, molecular size, amino-acid composition, but with differences in their secondary and tertiary structure, can be separated.

† Simple ionic adsorption and other complicating factors can be suppressed or modified by buffers of high ionic

strength.

† Binding is influenced by pH. Low pH causes elution of adsorbed substances.

† The technique can be made very efficient and simple in performance by the use of histidine tags.

† Several elution techniques are available (pH gradient, competitive ligands, organic solvents, chelating agents).

† IMAC is a general technique for purifying proteins. Metalloproteins do not bind specifically at their metal coordination sites but rather through amino-acid residues exposed at the protein surface.

Basically, the requirements of the support in affinity chromatography of biological molecules apply also to IMAC

Ideally the support should have the following features:

† It should be easy to derivatize.

† It should exhibit no unspecific adsorption.

† It should have good physical, mechanical, and chemical stability.

† It should be of high porosity to provide easy ligand accessibility.

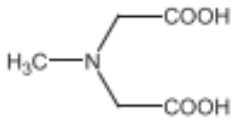
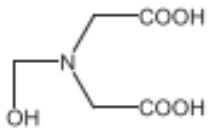
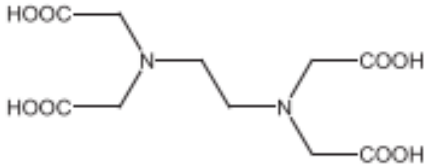
† It should permit high flow rates.

† It should be stable to eluents, including, for example, denaturing additives.

† It should allow regeneration of the column without deterioration of the gel bed.

† It should provide a stable gel bed with no shrinking/ swelling during the chromatographic process.

TABLE 7.1 Formation Constants[a] (log *K*) for 1:1 Complexes of Chelating Compounds[b] and Metal Ions. I, *N*-methyliminodiacetic Acid; II, *N*-(hydroxymethyl)iminodiacetic Acid; III, *N*-(hydroxyethyl)ethylenediaminetriacetic Acid

	Chelating Compound	Metal Ion						
No.	Formula	Ca^{2+}	Fe^{2+}	Fe^{3+}	Co^{2+}	Ni^{2+}	Cu^{2+}	Zn^{2+}
I		3.8	6.3		7.6	8.7	11.1	7.6
II		4.8	6.8		8.1	9.4	11.7	8.5
III		8.2	12.2	19.5	14.5	17.1	17.5	14.6

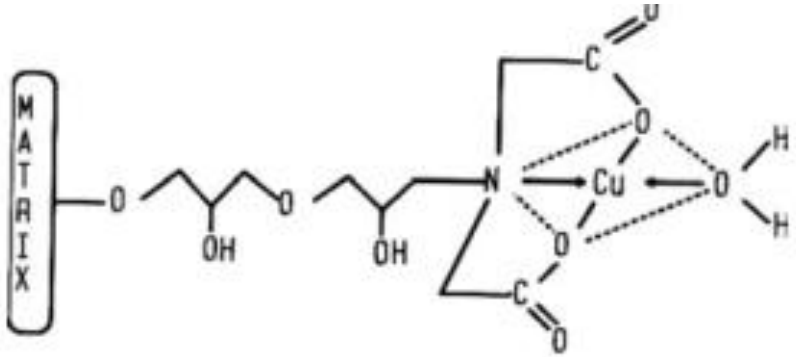


Figure 7.3 Postulated planar Cu^{2+} chelate with iminodiacetic acid as chelating group.

TABLE 7.3 Protein Structure and Choice of Metal Ion

Presence of Histidine or Tryptophan on "Surface" of Protein	Metal Ions Providing Adsorption
No His/Trp	—
One His	Cu^{2+}
More than one His	Cu^{2+} (stronger adsorption), Ni^{2+}
Clusters of His	Cu^{2+}, Ni^{2+}, Zn^{2+}, Co^{2+}
Several Trp, no His	Cu^{2+}

TABLE 7.4 Behavior of Cohn IV Fraction Proteins from Human Serum in IMAC

Metal Ion	Buffers in Sample and Starting Buffer	Behavior of Proteins			
		Albumin	Transferrin	α_1-HS Glycoprotein	α_1-Lipoprotein
Zn^{2+}	50 mM Tris-HCl pH 8.0 150 mM NaCl	No retention	Retained	Retained	Retained
Cu^{2+}	100 mM Na acetate pH 7.7 500 mM NaCl	Complete retention	Retained	Retained	Retained
Cu^{2+}	20 mM phosphate pH 7.7 500 mM NaCl	Complete retention			
Cu^{2+}	50 mM Tris-HCl pH 8.0 150 mM NaCl	Partial retention			
Cu^{2+}	50 mM Tris-HCl pH 8.0 150 mM NH_4Cl	Major part unretained[a]	Retained	Retained (partial?)	Retained

[a]Albumin oligomers and minor part of albumin monomer were retained.

- The predominant use of IMAC to date has been in the isolation and purification of proteins and peptides, as discussed in this review. The chromatography of nucleotides, dinucleotides, and related compounds on metal chelates has also been shown to be possible.

- Pyrimidines show little interaction with the metal ions, but purines are resolved on Cu^{2+} chelates. The technique is potentially very useful for the large scale purification of these compounds.

AFFINITY CHROMATOGRAPHY

All biological processes depend on specific interactions between molecules. These interactions might occur between a protein and low molecular weight substances (e.g., between substrates or regulatory compounds and enzymes; between bioinformative molecules—hormones, transmitters, etc.—and receptors, etc.), but biospecific interactions occur even more often between two or several biopolymers, particularly proteins. Examples can be found from all areas of structural and physiological biochemistry, such as in multimolecular assemblies, effector-receptor interactions, DNA-protein interactions, and antigen–antibody binding.

One of the members of the pair in the interaction, the ligand, is immobilized on the solid phase, while the other, the counter ligand (most often a protein), is adsorbed from the extract that is passing through the column. Examples of such affinity systems are listed in Table 9.1.

TABLE 9.1 Examples of Biological Interactions Used in Affinity Chromatography

Ligand	Counterligand
Antibody	Antigen, virus, cell
Inhibitor	Enzyme (ligands are often substrate analogs or cofactor analogs)
Lectin	Polysaccharide, glycoprotein, cell surface receptor, membrane protein, cell
Nucleic acid	Nucleic acid-binding protein (enzyme or histone)
Hormone, vitamin	Receptor, carrier protein
Sugar	Lectin, enzyme, or other sugar binding protein

A field that has been so successful that it is often treated separately is affinity based on antigen–antibody interactions, called immunosorption. Sometimes this is the only available route to the purification of a protein and is especially attractive when there is a suitable monoclonal antibody at hand.

A good affinity ligand should possess the following characteristics:

† The ligand must be able to form reversible complexes with the protein to be isolated or separated.

† The specificity must be appropriate for the planned application.

† The complex constant should be high enough for the formation of stable complexes or to give sufficient retardation in the chromatographic procedure.

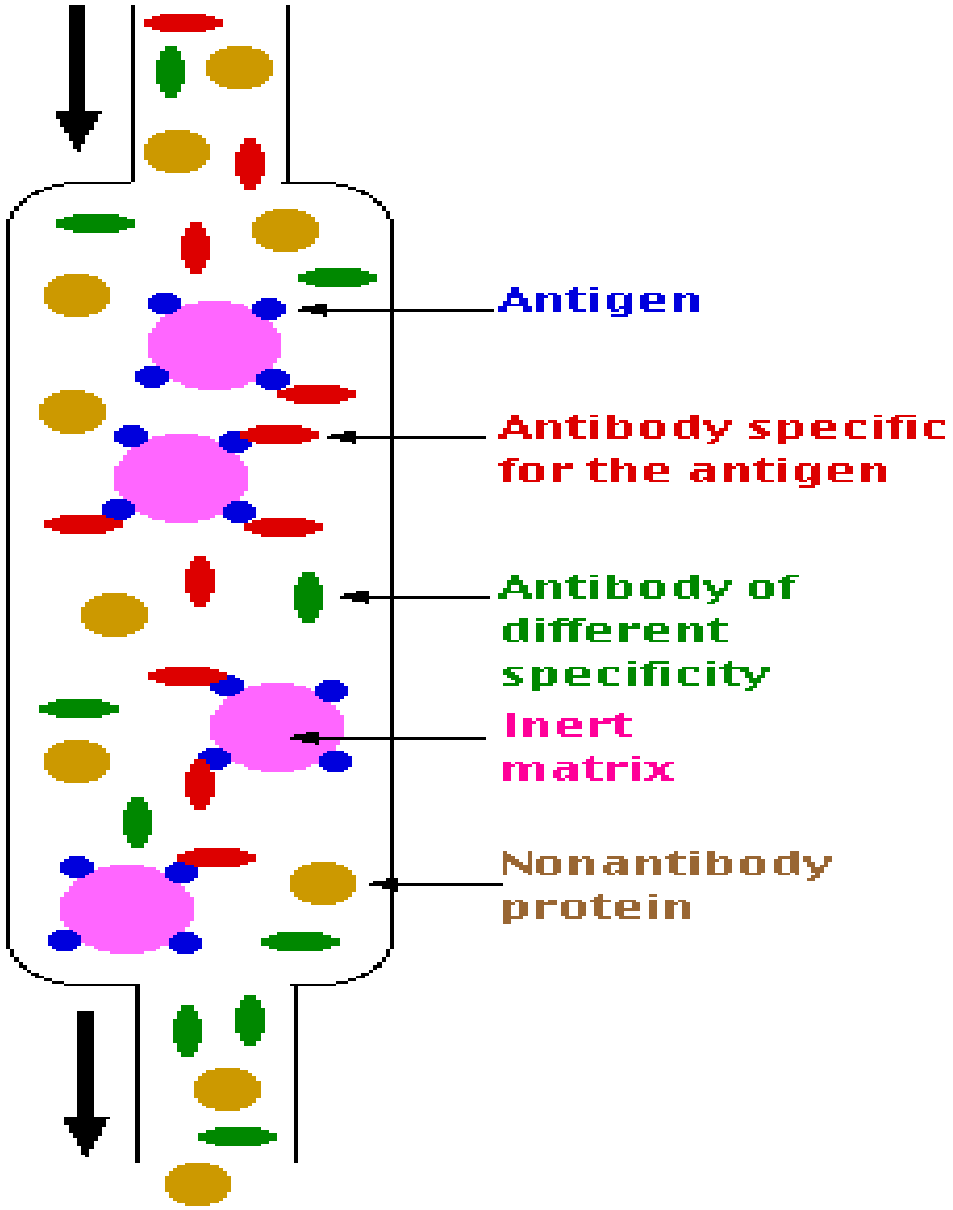
† It should be easy to dissociate the complex by a simple change in the medium, without irreversibly affecting the

protein to be isolated or the ligand.

† It should have chemical properties that allow easy immobilization to a matrix.

To adsorb a protein counterligand to an affinity gel, the binding constant K_A for the interaction needs, for most practical purposes, to exceed or be equal to 10^5 –10^6 M.

Generally, ligands may be classified as either monospecific or group-specific, each of which in turn may be divided into low molecular weight or macromolecular.


Antigen
Antibody specific for the antigen
Antibody of different specificity
Inert matrix
Nonantibody protein

Monospecific Low Molecular Weight Ligands

- This group includes ligands such as steroid hormones, vitamins, and certain enzyme inhibitors. The term monospecific refers to the fact that these ligands bind to a single or a very small number of proteins in any particular cell extract or body fluid.
- Generally, monospecific ligands bind more strongly and require harsher eluents than group-specific ligands, which can usually be eluted under mild conditions.
- example of a monospecific low molecular weight ligand with a very high binding constant to its counterligand is biotin, which binds avidin with a K_A of 10^{15}.

Group-Specific Low Molecular Weight Ligands

This is the largest group of ligands containing a wide variety of enzyme cofactors and their analogs. This group also includes biomimetic dyes, boronic acid derivatives, and a number of amino acids and vitamins. A representative list of group-specific ligands and their target proteins is given in Tables 9.2 and 9.3. The target proteins are most often enzymes and the most thoroughly studied are the NAD^+ and $NADP^+$ dependent dehydrogenases and kinases.

A large number of affinity chromatography adsorbents are based on group-specific ligands coupled to a variety of carrier matrices commercially available from several sources.

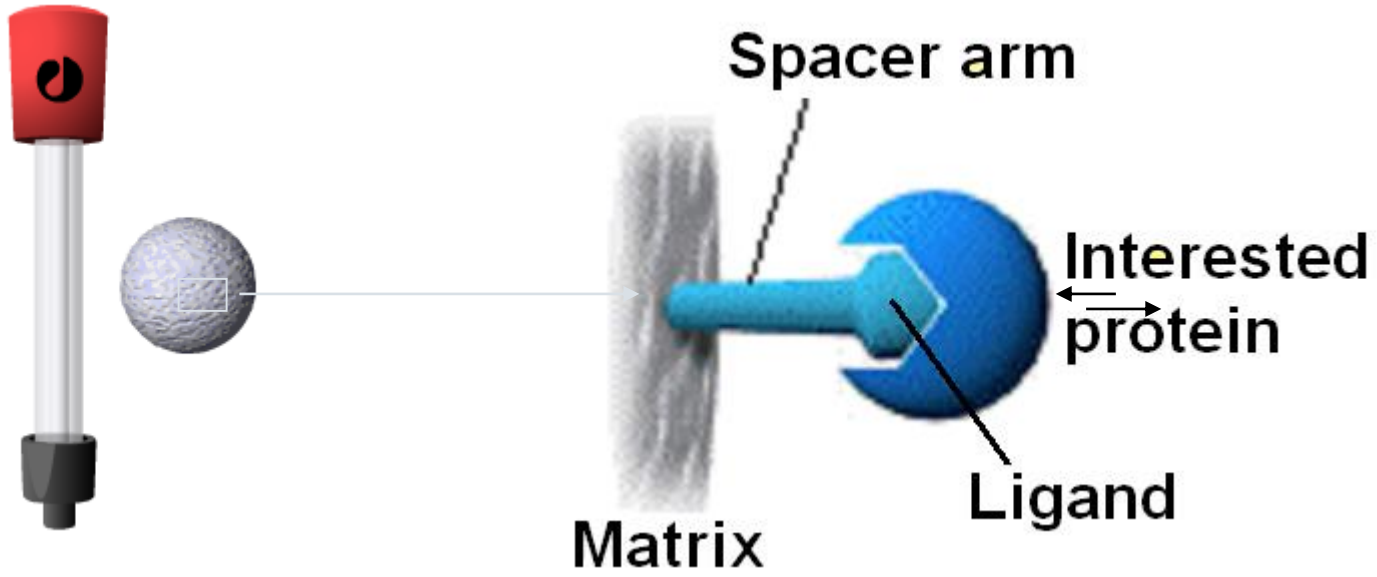
TABLE 9.2 Examples of Group-Specific Low Molecular Weight Ligands and their Target Proteins

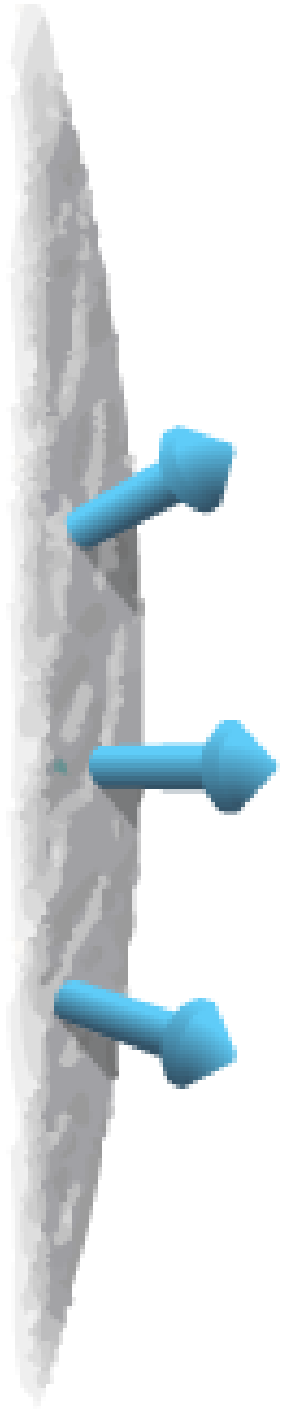
Ligand	Target Proteins
5′-AMP	NAD^+-dependent dehydrogenases ATP-dependent kinases
2′,5′-ADP	$NADP^+$-dependent dehydrogenases
ATP	ATP-dependent kinases
NAD^+	NAD^+-dependent dehydrogenases
$NADP^+$	$NADP^+$-dependent dehydrogenases
Benzamidine	Serine proteases
Phenylboronic acid	Glycoproteins
Cibacron Blue F3G-A	See Table 9.3
Procion Red HE-3B	See Table 9.3

TABLE 9.3 Examples of Proteins with Affinity for Two Broadly Specific Dye Ligands, Cibacron Blue F3G-A and Procion Red HE-3B (from Reference 9)

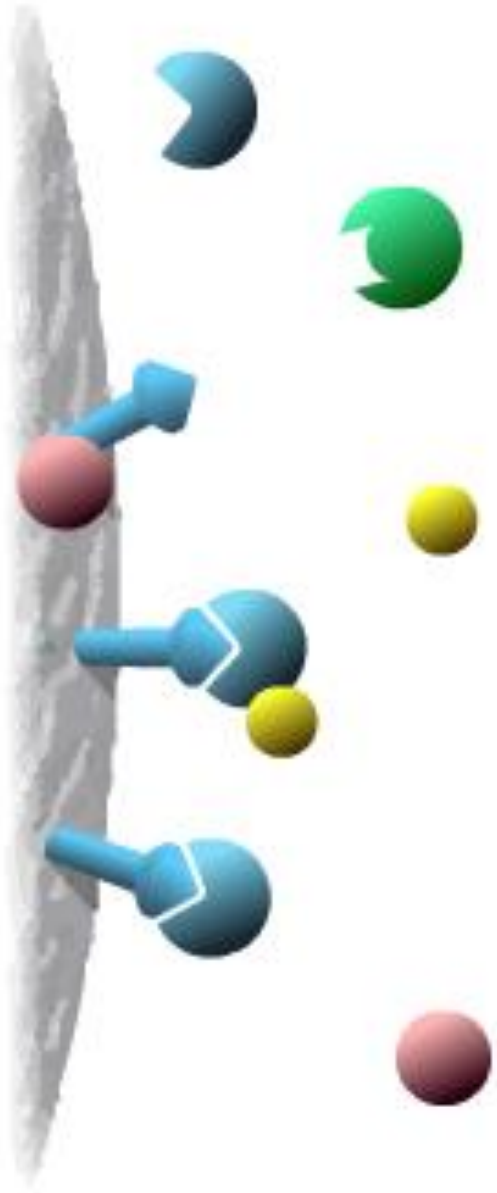
Cibacron Blue F3G-A	Procion Red HE-3B
Kinases and Phosphatases	*Dehydrogenases*
Adenylate cyclase	Aldehyde reductase ($NADP^+$)
Adenylate kinase	Dihydrofolate reductase ($NADP^+$)
Amino acyl tRNA synthetase	Glucose-6-phosphate dehydrogenase ($NADP^+$)
cAMP-dependent protein kinase	Glutamate dehydrogenase ($NADP^+$)
Creatine kinase	Glutathione reductase ($NAD^+/NADP^+$)
DNA polymerase	3-Hydroxybutyrate dehydrogenase (NAD^+)
Fructose diphosphatase	Isocitrate dehydrogenase (NAD^+)
cGMP-dependent protein kinase	Lactate dehydrogenase (NAD^+)
Nucleoside kinase	Malate dehydrogenase (NAD^+)
Phosphofructokinase	6-Phosphogluconate dehydrogenase ($NADP^+$)
Phosphoglycerate kinase	
Phosphorylase A	*Other Proteins*
Protein kinase	Carboxypeptidase G
Restriction endonucleases	Dopamine β-monooxygenase
Succinyl-CoA transferase	Inhibin
	Interferon
Dehydrogenases	3-Methylcrotonyl-CoA carboxylase
Alcohol dehydrogenase (NAD^+)	Plasminogen
Glutathione reductase	Propionyl-CoA carboxylase
Hydroxysteroid dehydrogenase	
Isocitrate dehydrogenase (NAD^+)	
Lactate dehydrogenase (NAD^+)	
Malate dehydrogenase (NAD^+)	
Phosphogluconate dehydrogenase	
Other Proteins	
Albumin	
Blood coagulation factors II, IX	
Interferon	

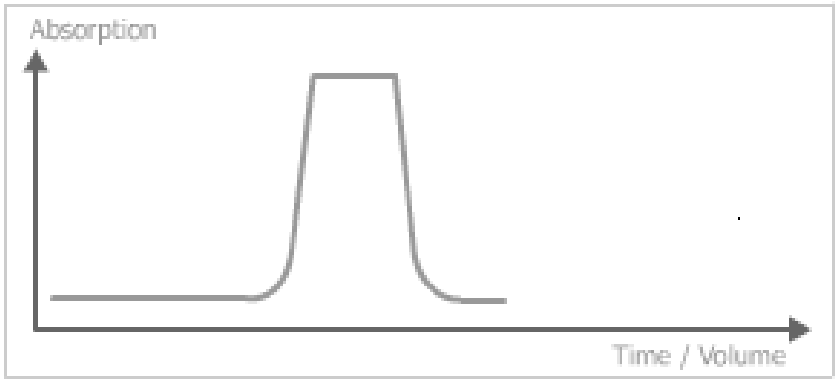
Principle

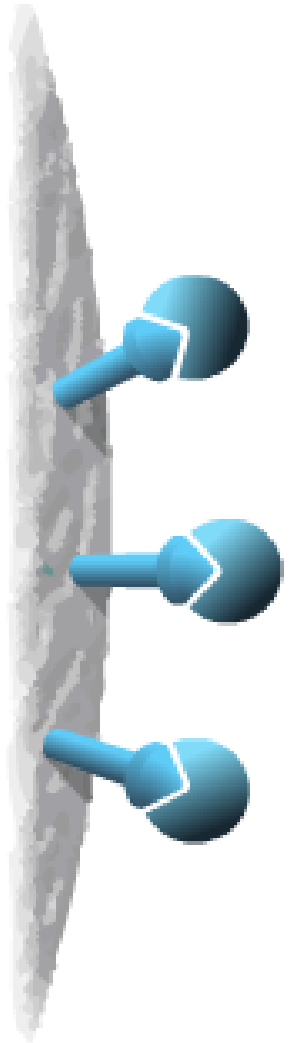


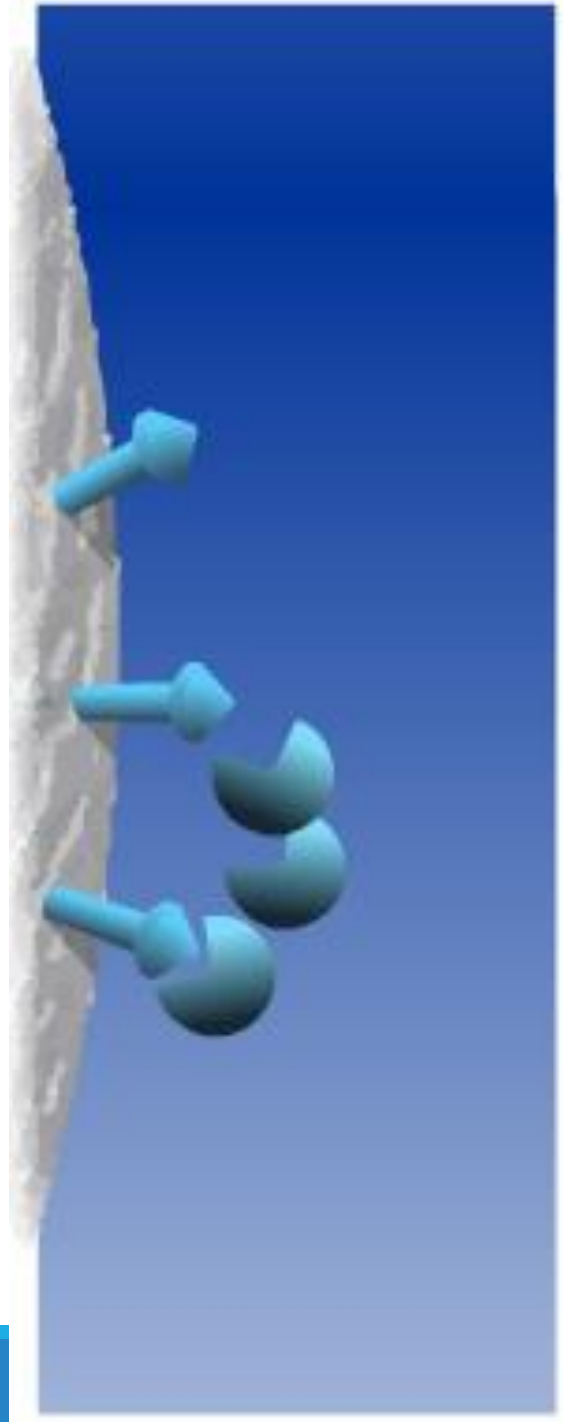



Absorption
Time / Volume




Absorption
Time / Volume





Elution