第五章 蛋白质纯化、鉴定及结构与功能分析-1

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第一节 蛋白质纯化的策略和思路

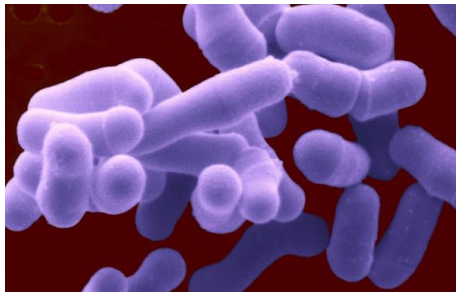
- A black art (proteins have personality)
- Requires knowledge of protein
  - What kind of cell is it coming from
  - What part of cell
  - What does it do
- Particularly helpful
  - Size
  - Composition

Strategy

- Move from organism to pure protein in as few steps as possible with as little loss of activity (assayable quality) as possible
  - Time and temperature are factors

Protein Purification

- Molecular weight
- Charge
- Solubility
- Affinity

	微生物	植物	动物
			
细胞裂解	高压细胞破碎仪、超声破碎等方法，多用5~10倍体积的裂解液	研磨、高压细胞破碎仪、匀浆等方法，使用1~3倍体积的裂解液。注意对细胞壁的破碎	匀浆法，使用2~5倍体积的缓冲液，或绞碎并用裂解液搅拌
去除杂质	核酸等	酚类等次级代谢物等	组织中的脂肪等
蛋白质的分离	不含去污剂的裂解液充分溶解，离心		含去污剂的裂解液充分溶解，离心
	上清液（起始提取物）	沉淀	上清液（细胞内的大多数蛋白质）
			沉淀（难溶性蛋白质）

蛋白质分级分离

上清液

分级分离第一步：使用盐分分级分离、有机溶剂沉淀、亲和法和两相分配

分级分离第二步：使用离子交换层析、疏水层析、亲和法、其他吸附剂或凝胶过滤

进行精制步骤：使用HPLC-反相、HPLC-离子交换或等点聚焦

纯化的蛋白质

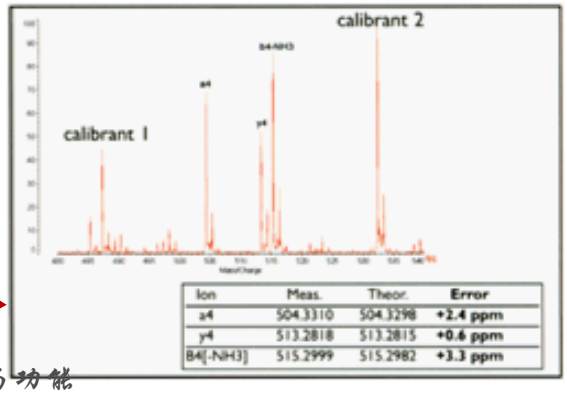
沉淀

重悬，用其他溶剂（含去垢剂）充分溶解，离心

纯化的蛋白质

不溶的沉淀

肽质谱图

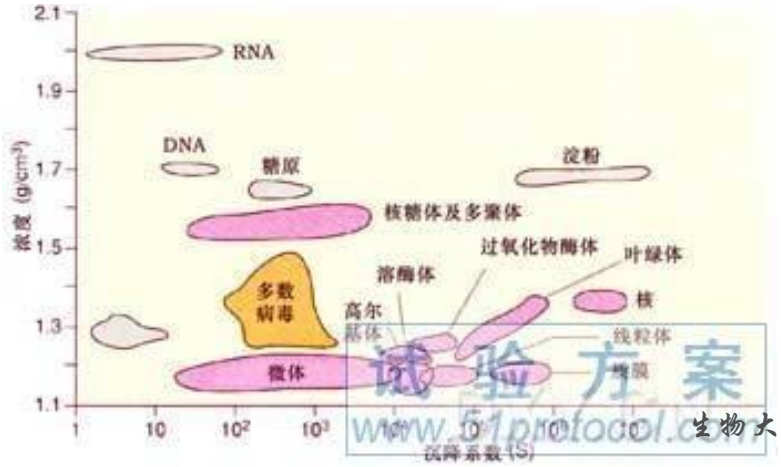


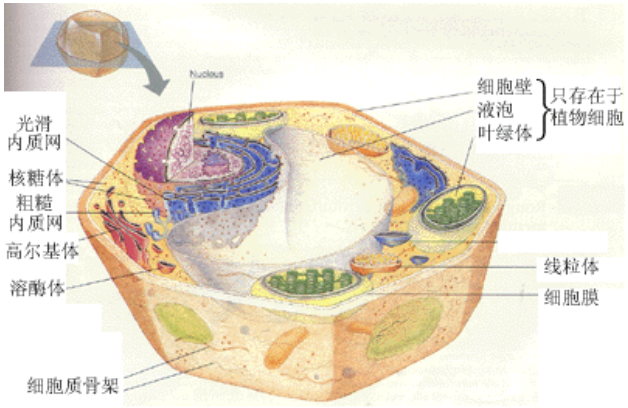
蛋白质特性分析功能研究等

- 细胞破碎
  - 温和的方法：渗透法、冻融法、裂解液法、酶裂解
  - 比较强烈的方法：超声波法、研磨法、匀浆法、压力杯法
- 蛋白质沉淀：
  - pH值=等电点
  - 盐析硫酸铵、硫酸镁、硫酸钠、氯化钠、磷酸钠
  - 有机试剂：三氯乙酸(TCA)、丙酮、乙醇、丁醇
  - 高盐与有机溶剂结合：乙酸铵、甲醇、丙酮
- 蛋白质裂解
  - 变性剂：尿素、硫脲
  - 去污剂：阴离子(SDS)，非离子(Triton X-100、 NP-40)，两性离子(CHAPS、OBG、ABS-14)
  - 还原剂:β-巯基乙醇、DTT

亚细胞器蛋白质样品制备

- 细胞破碎：温和的方法，例如组织细胞匀浆，等渗匀浆介质（0.25%蔗糖，0.003mol/L氯化钙）
- 分离细胞器：
  - 差速离心：细胞核、线粒体、溶酶体与过氧化物酶体、高尔基体与内质网、核蛋白
  - 密度梯度离心（蔗糖、Ficoll、葡萄糖-聚乙二醇）
- 纯度鉴定：电子显微镜、免疫化学法、标志酶活性测定





修饰蛋白质样品的制备

- 原则：简单富集后接续质谱鉴定的方法确定修饰位点信息
- 磷酸化蛋白质
  - 固相金属离子亲和色谱法(IMAC)、金属氧化物和金属氢氧化物富集法、抗体富集、强阳离子交换色谱(SCX)，强阴离子交换色谱(SAX)
  - 磷酸酯酶抑制剂
- 糖基化蛋白质：凝集素亲和技术，层析法等
- 泛素化蛋白质
  - 亲和标记

Molecular Weight

- Ultracentrifugation
- Dialysis
- Gel filtration separates by the native molecular weight
- SDS PAGE separates by the subunit molecular weight

SDS PAGE

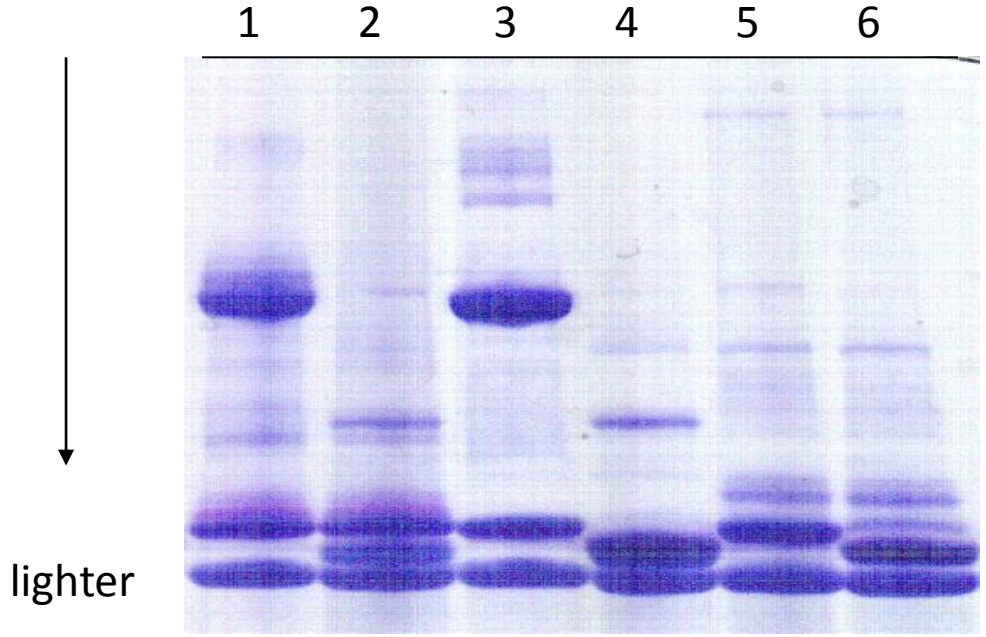
- This technique involves loading a sample of your mixture onto a polyacrylamide gel (PAGE). Polyacrylamide works like agarose except the matrix has smaller pores and so polyacrylamide gels separate smaller molecules (like proteins).

SDS PAGE

- Unlike DNA and RNA, proteins do not have a nice constant charge to mass ratio and can have any charge at a given pH, depending on their sequence, hence pI.
- To overcome this problem proteins are coated with a detergent, SDS, which makes them negatively charged.
- They then separate by molecular weight.

SDS PAGE

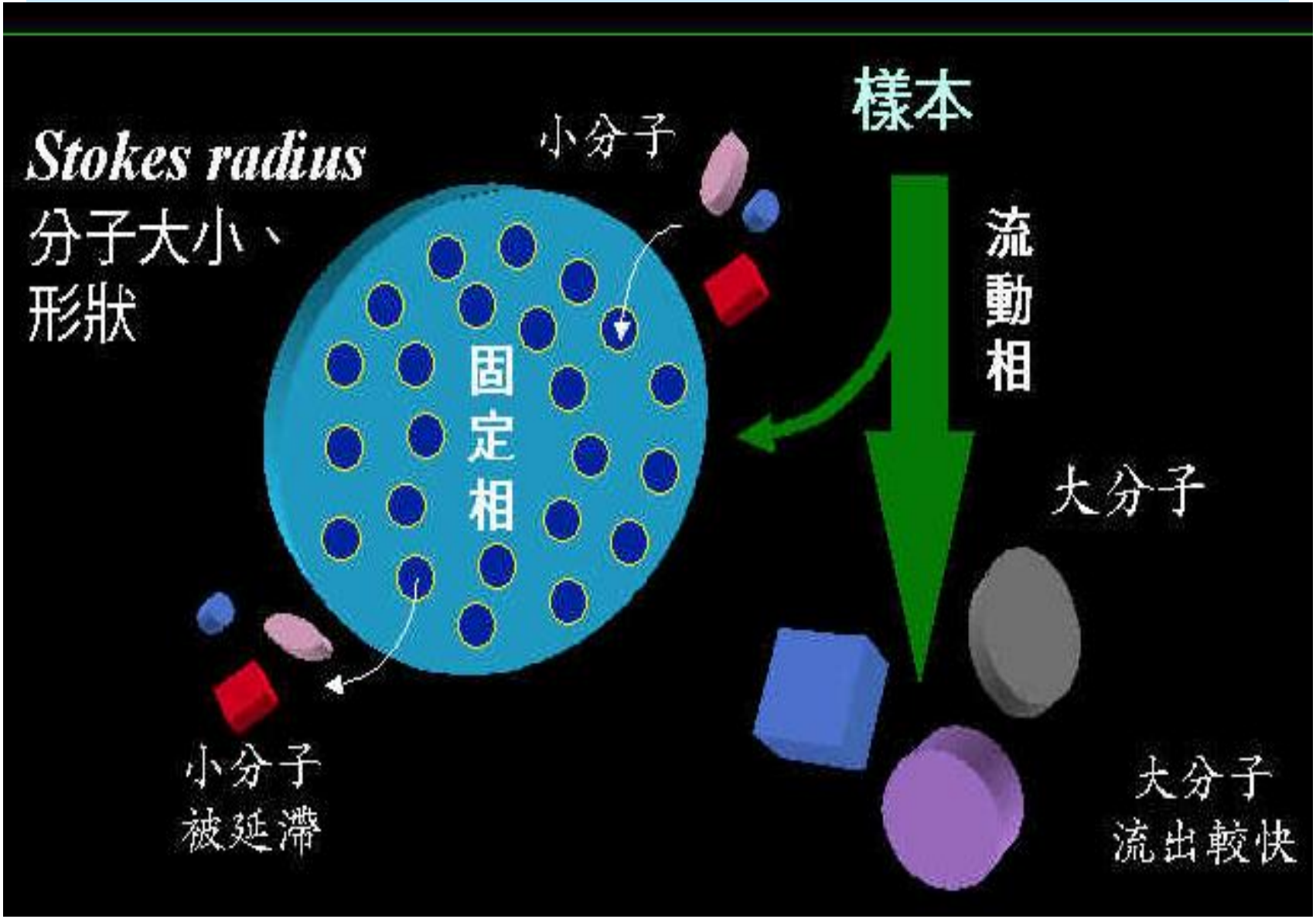
- They then separate by molecular weight.
- The SDS will disrupt the secondary, tertiary and quaternary structure so the subunits will separate. For this reason SDS-PAGE separates by subunit molecular weight.

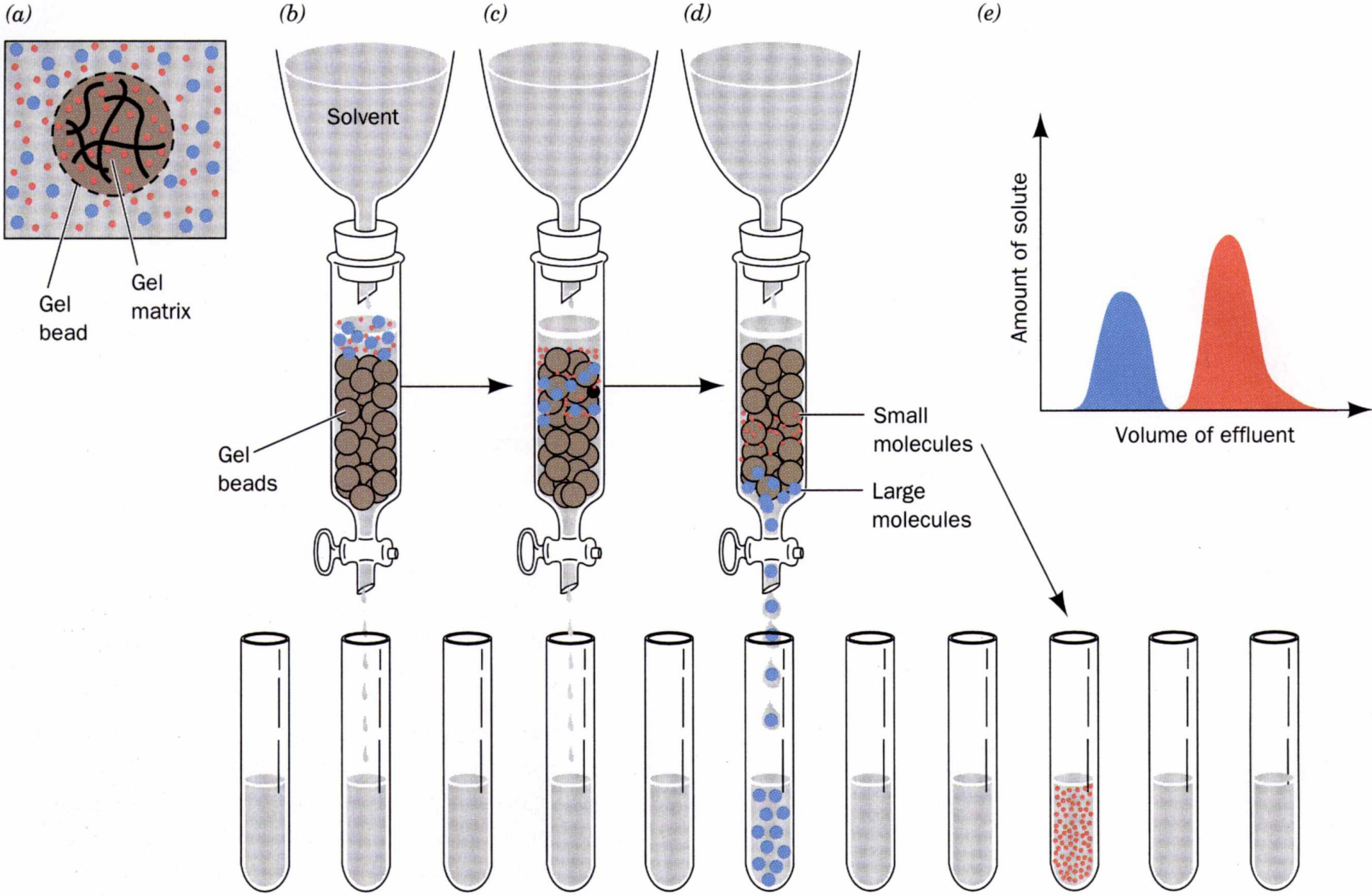


Gel Filtration

- This method relies on a column of beads of a specified pore size. This is known as a molecular sieve.
- Proteins (and other macromolecules) above a certain cut-off size cannot fit into the pores and so migrate down the outside of the beads. They will elute first.
- Smaller molecules below the cut-off can permeate the pores and so take longer to travel down the column.

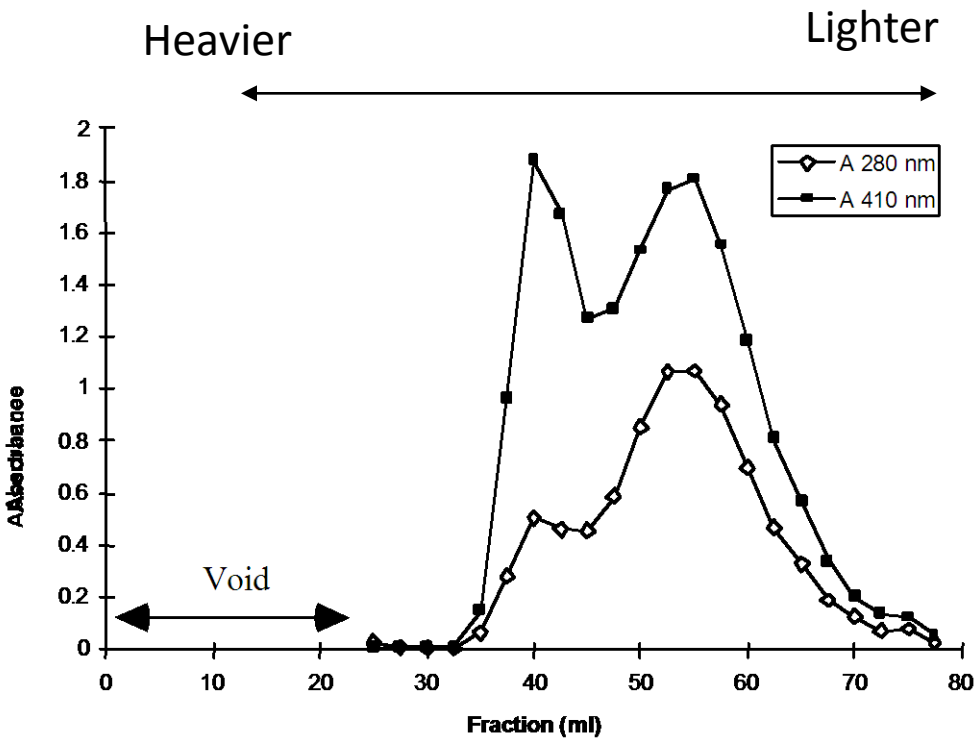
Gel Filtration 凝胶层析




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

2008-01-05

An elution profile



- **Gel-filtration of the protein mixture.** 1.2 ml of protein mixture (10 mg/ml) was loaded onto a 25 cm X 2.5 cm diam. Sephadex G-50 column equilibrated with buffer (50 mM Tris HCl, pH 7.5). The column was eluted with buffer at ~1 ml/min, collecting 2.5 ml fractions. The absorbance of each fraction was measured at 280 nm.

常见问题分析和解决办法

- 流速低: (a) 气泡; (b) 连接管堵塞; (c) 沉淀物聚集在凝胶顶部; (d)微生物污染; (e)有蛋白质等堵塞在柱子里头; (f)凝胶压的过紧;
- 异常峰形: (a)蛋白质被吸附在凝胶上; (b)缓冲液中的盐离子强度过高或者含有去垢剂;(c)蛋白的疏水作用-降低离子浓度;
- 蛋白质峰分辨能力差: (a)流速太快; (b)层析柱太短; (c)柱底死体积太大, (d)上样体积太大; (e) 层析柱填装不好, 导致缓冲液流动异常; (f)选错了凝胶类型; (g)凝胶颗粒度不合适。
- 样品回收率低: (a) 样品发生沉淀; (b)上样前已经丢失; (c)蛋白质被吸附在凝胶上; (d)洗脱条件不当; (e)蛋白质被降解; (f)微生物滋生在树脂中;
- 洗脱条件不能重复: (a)实验条件不一致; (b)样品发生沉淀; (c)样品保存过程中变化。
- 蛋白质失活: (a)辅助因子失活; (b)实验缓冲液中蛋白质不稳定; (c)微生物导致蛋白质变性。

Charge

- Ion Exchange Chromatography
- Native gel electrophoresis
- Isoelectric focusing

Charges on proteins

- Different proteins have different native charges.
- The overall charge on a protein will depend on:
  - The sequence
  - The pH

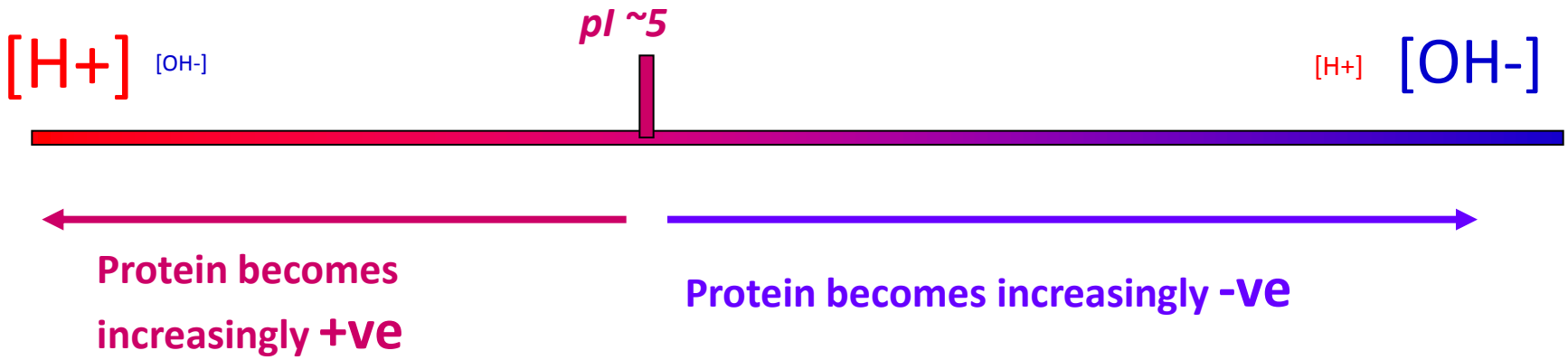
Determining the *pI* of a protein

- It can be predicted from the difference between the sum of the acidic side chains (asp + glu) and the sum of the basic side chains (lys + arg + his).
- It is determined experimentally by techniques such as isoelectric focusing. The protein is placed in a pH gradient and subjected to an electric field. The protein moves to its pI.

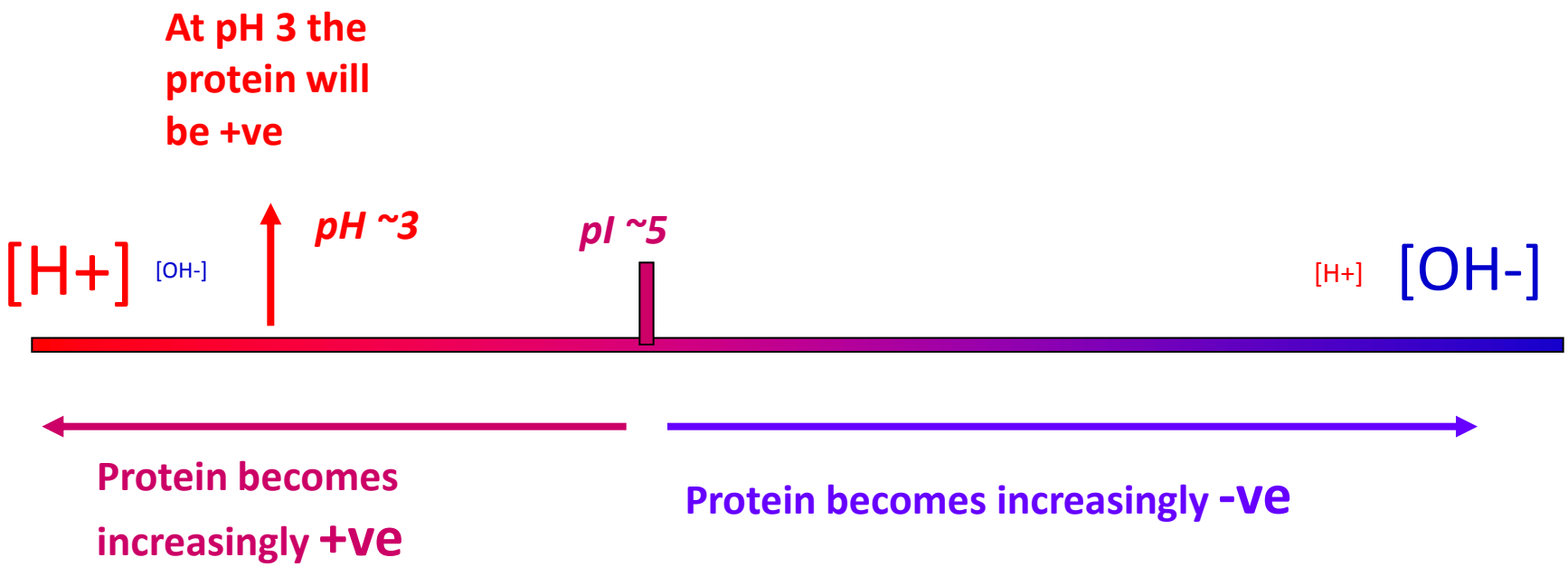
蛋白质可以解离的基团

- 带电基团的来源：来自于特定的氨基酸和蛋白质在修饰过程中引入的。
- 很多氨基酸的侧链带有可解离基团，其中有的能进行酸性解离而带上负电荷，如Asp和Glu的侧链羧基、Tyr的酚羟基、Cys的巯基；
  - 有的能进行碱性解离而带上正电荷，如Lys的侧链氨基、Arg的胍基、His的咪唑基等。
  - 肽链的N端的游离氨基，C末端的游离羧基。
  - 结合蛋白质的辅基也可能有可解离基团。
- 蛋白质翻译后修饰过程中引入的可解离基团，如磷酸化引入的磷酸基、凝血因子中的γ羧基、糖蛋白寡糖链上的唾液酸残疾等。Lys和His的甲基化作用会增加这些侧链的碱性。
- 翻译后修饰也可能消除原先解离基团的酸碱性质。例如N-末端的Glu发生环化生成焦谷氨酸。

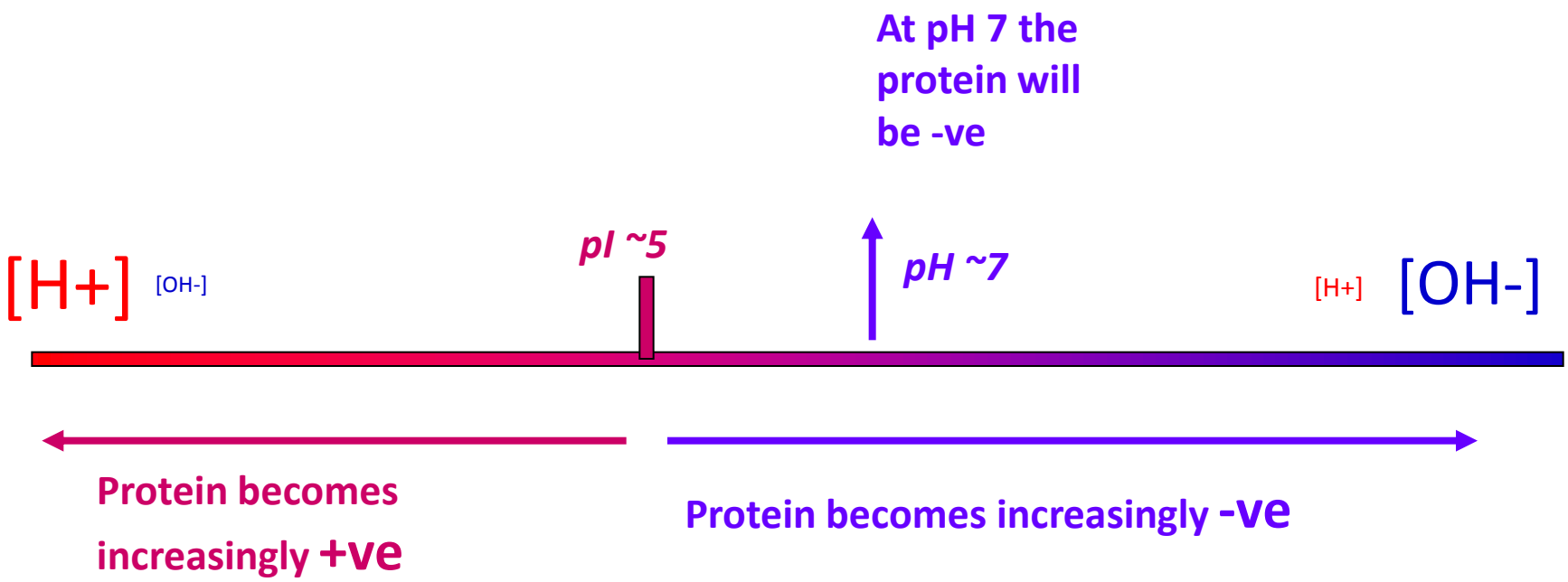
Estimating the charge of a protein



Estimating the charge of a protein



Estimating the charge of a protein

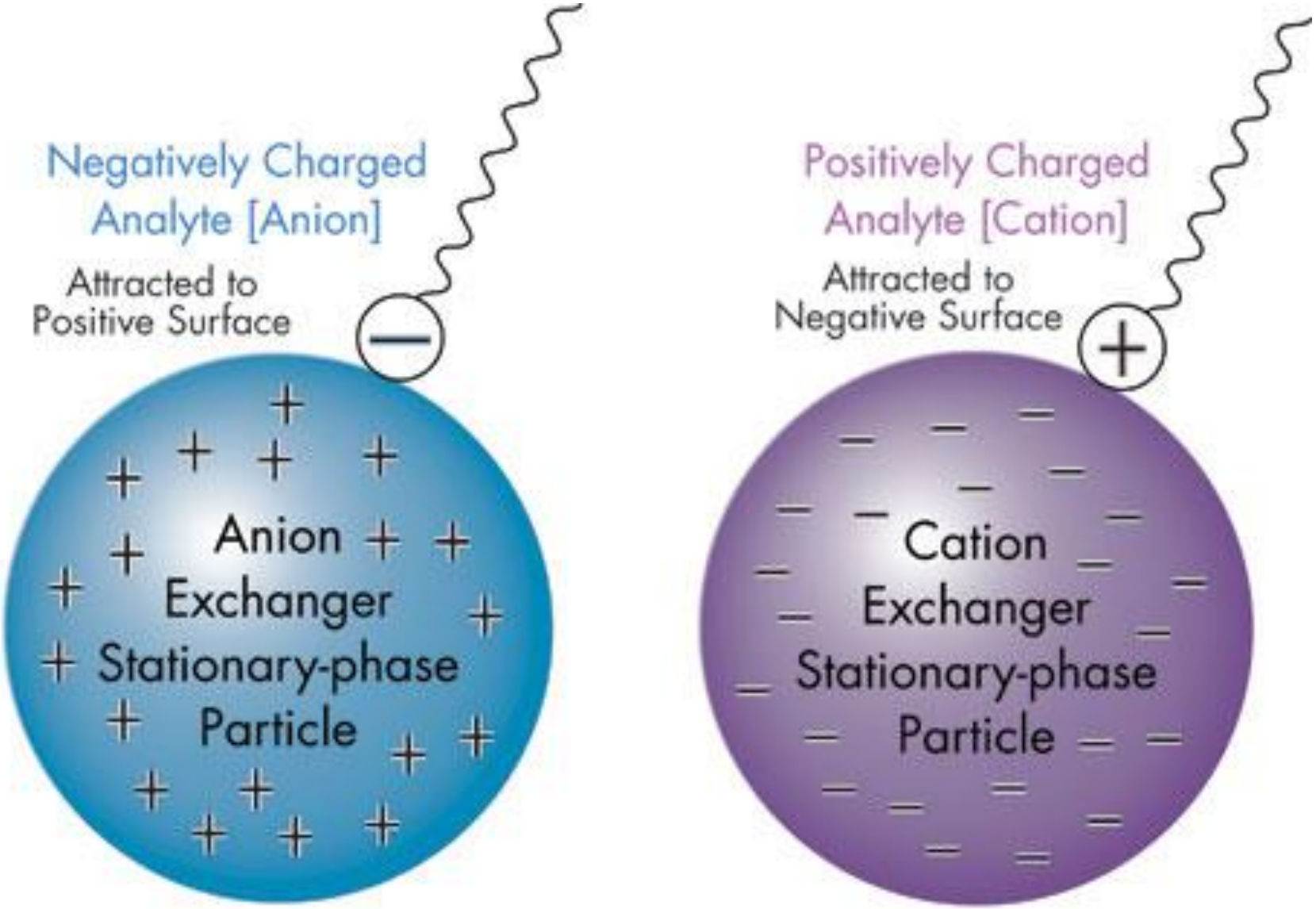


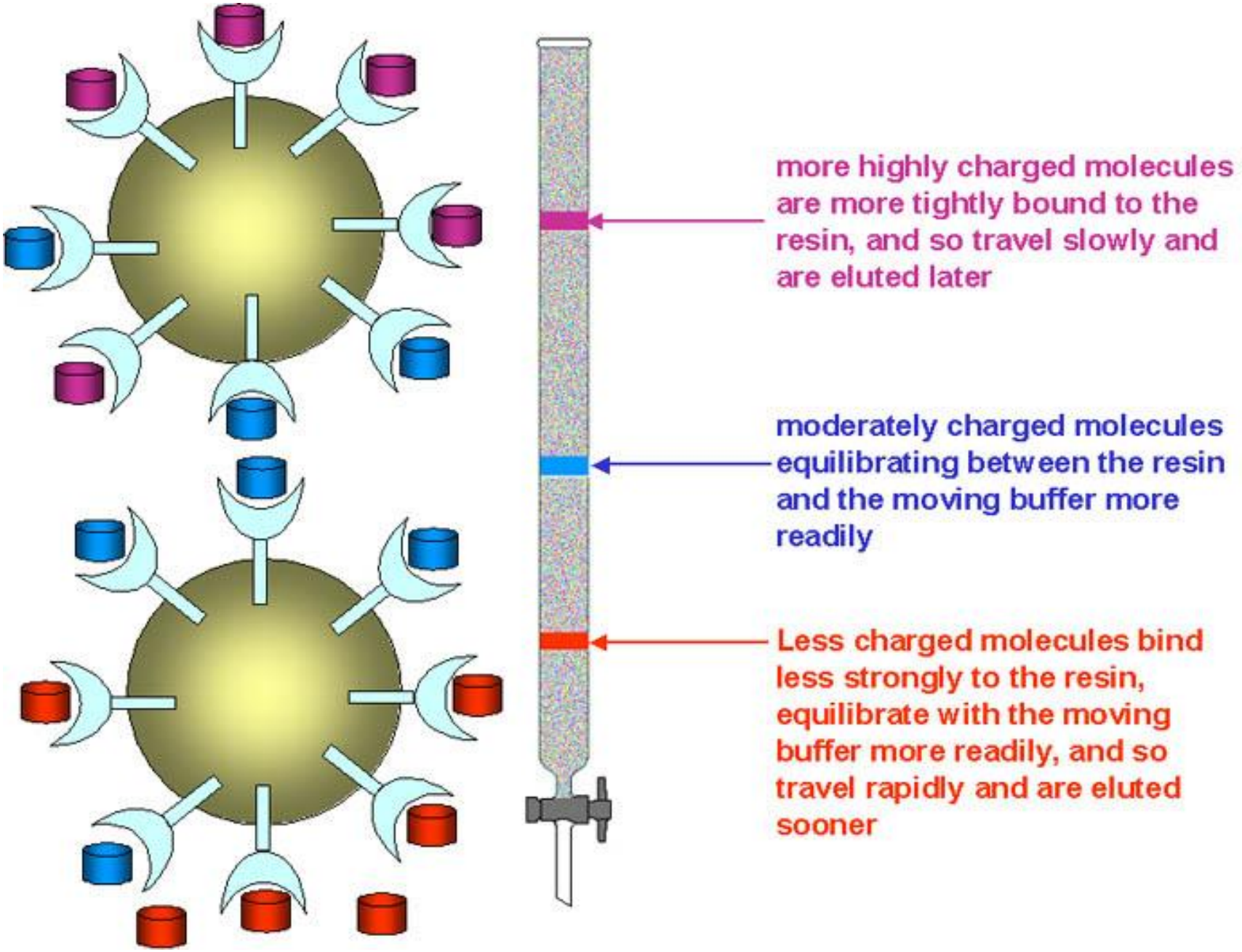
Ion Exchange Chromatography

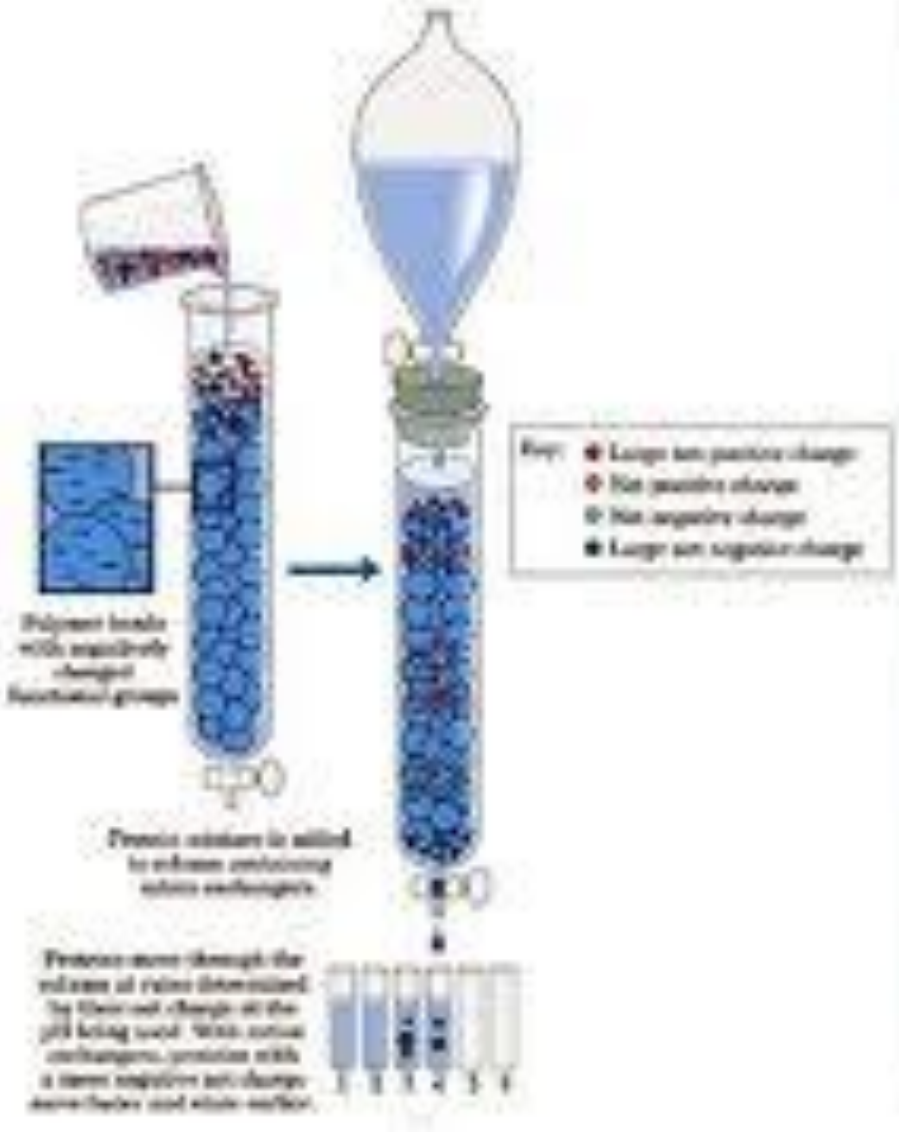
- If the column is positively charged i.e. DEAE then….
- Proteins with pIs < the pH of the buffer will be negatively charged and bind to the column.
- Proteins with pIs > the pH of the buffer will be positively charged and will not bind to the column but elute.

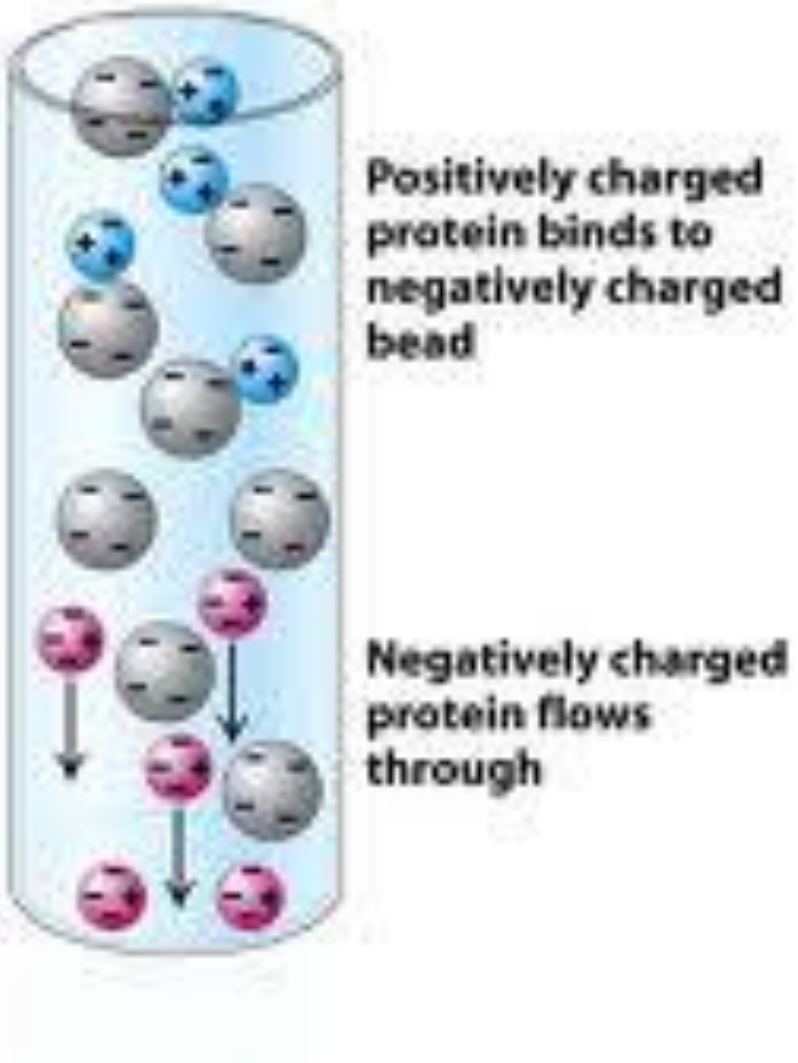
Ion Exchange Chromatography

- If the column is negatively charged charged i.e. carboxymethyl then….
- Proteins with pIs l < the pH of the buffer will be negatively charged and not bind to the column but elute.
- Proteins with pIs > the pH of the buffer will be positively charged and will bind to the column.


Negatively Charged
Analyte [Anion]
Attracted to
Positive Surface
Anion
Exchanger
Stationary-phase
Particle
Positively Charged
Analyte [Cation]
Attracted to
Negative Surface
Cation
Exchanger
Stationary-phase
Particle


more highly charged molecules are more tightly bound to the resin, and so travel slowly and are eluted later
moderately charged molecules equilibrating between the resin and the moving buffer more readily
Less charged molecules bind less strongly to the resin, equilibrate with the moving buffer more readily, and so travel rapidly and are eluted sooner




Positively charged protein binds to negatively charged bead
Negatively charged protein flows through

	functional group	support medium
weak anion exchangers		
DEAE-Sephacel	diethylethylaminoethyl	Sephacel
DEAE-Sephadex	diethylethylaminoethyl	Sephadex
PEI-cellulose	polyethyleneimine	cellulose
weak cation exchangers		
CM-Sephacel	carboxymethyl	Sephacel
CM-Sephadex	carboxymethyl	Sephadex
Bio-Rex 70	carboxylic acid	acrylic polymer

影响分离效果的因素

(1) 层析树脂：

(2) 平衡缓冲液：

(3) 上样：

(4) 洗脱缓冲液：

(5) 洗脱速度：

(6) 上样之前的对样品的预处理：

(7) pH和离子强度：

(8) 疏水作用和氢键：

聚焦层析chromatofocusing

(1)在等点聚焦基础上发展起来的一种离子交换层析。

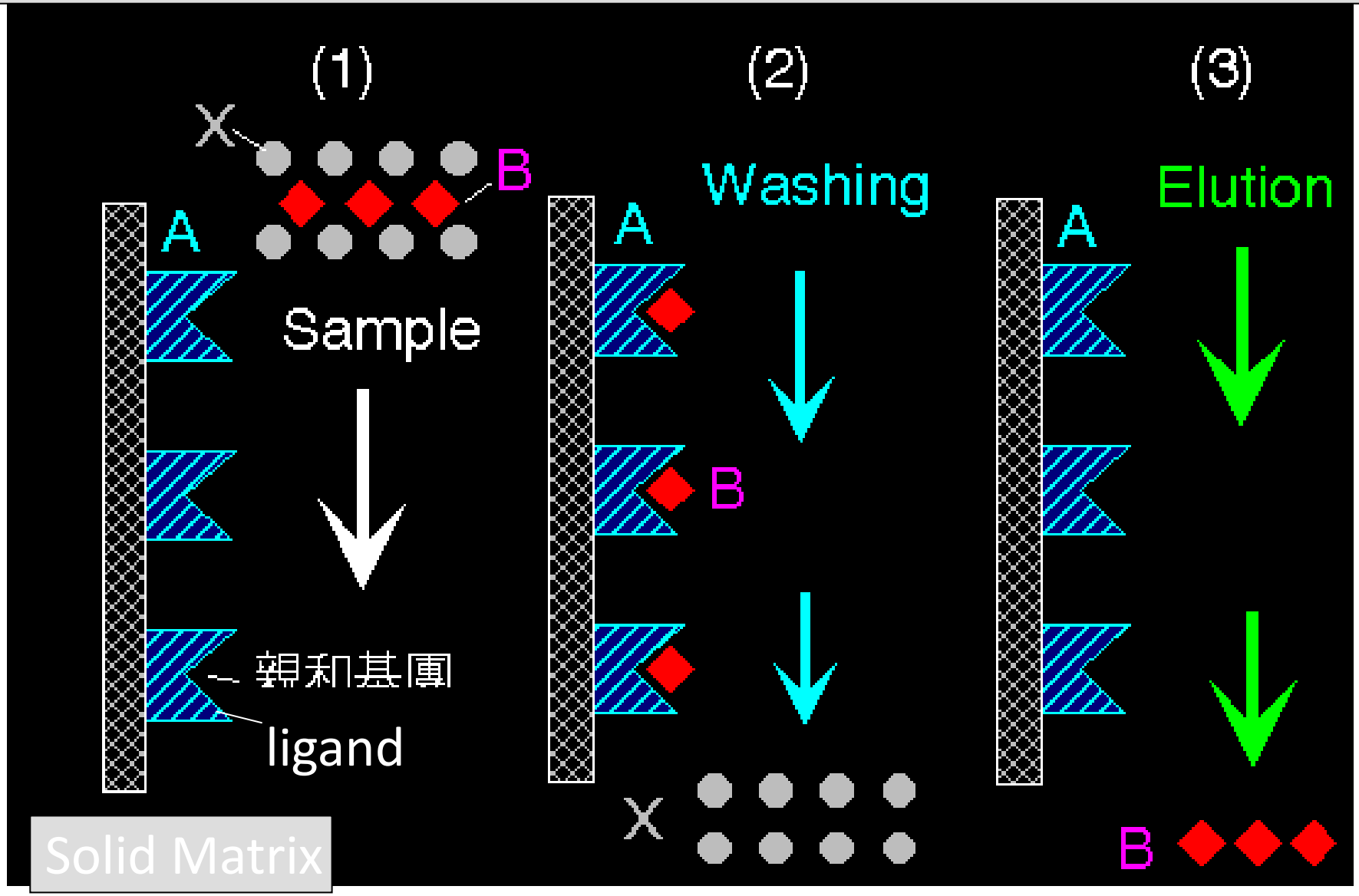
(2)流动相为多缓冲剂，固定相为多缓冲交换剂。

(3) 多缓冲剂：一系列精选的物质构成，在一定pH 范围内具有相似的、较强的缓冲能力。如多缓冲剂polubuffer96和polybuffer74分别在pH6-9和4-7范围内有较强的缓冲能力。

(4)多缓冲剂以sepharose 6B为基质，通过化学方法偶联上多种类型电荷基团的配体，所以他们也有相当的缓冲能力。

(5) 交换剂携带具有缓冲能力的电荷基团，故pH梯度溶液可自动形成。

Affinity chromatography 亲和层析



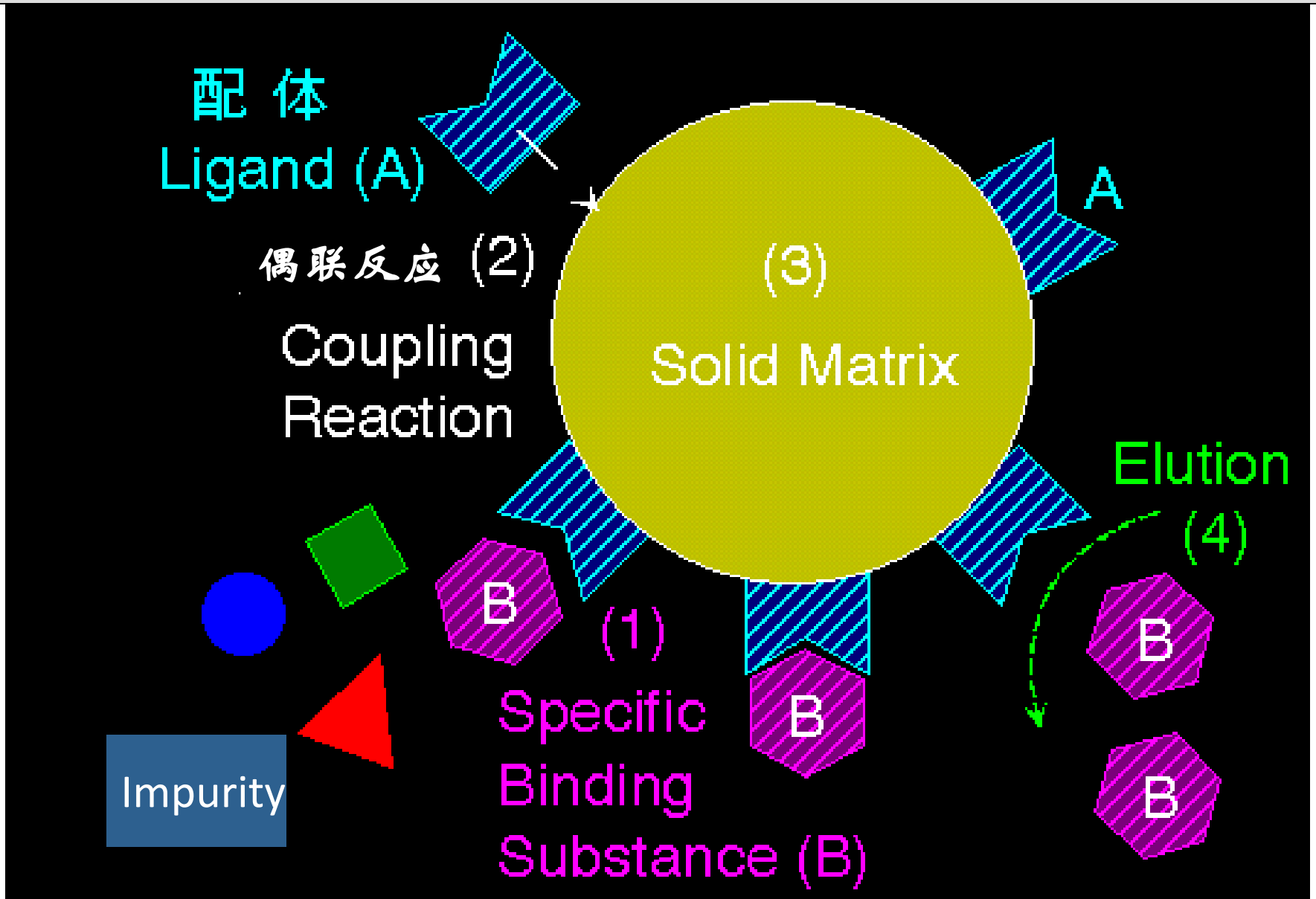
•配体和分析物的多种方式:

(1) GST/MBP/Ni-NTA等;

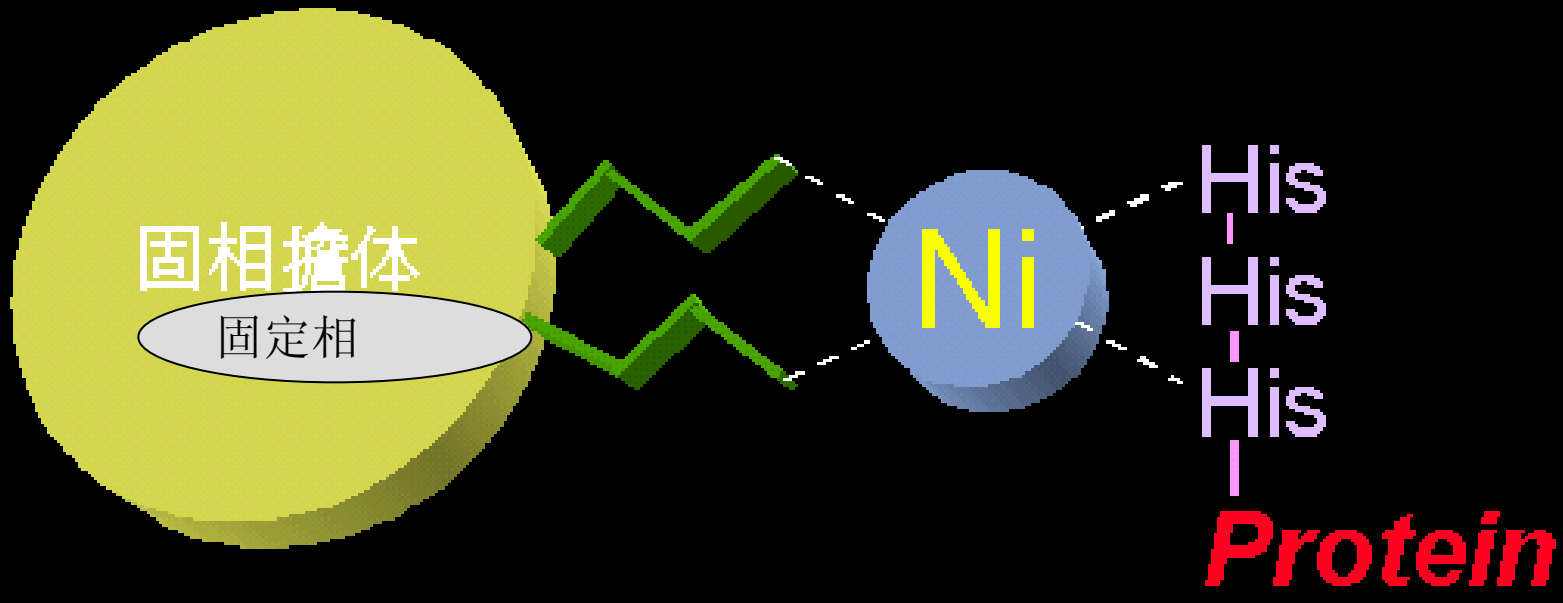
(2) Biotin-avidin; 糖蛋白与凝聚素结合等;

(3) 抗体-抗原; 激素与受体; 酶的活性中心或别构中心通过次级键与专一性底物、辅酶、激活剂或者抑制剂结合等;

Affinity chromatography 亲和层析



金屬螯合层析法



Metal Chelate Affinity Chromatography