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# Chromatography Method

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Jingjing Li, Wei Han and Yan Yu

Additional information is available at the end of the chapter

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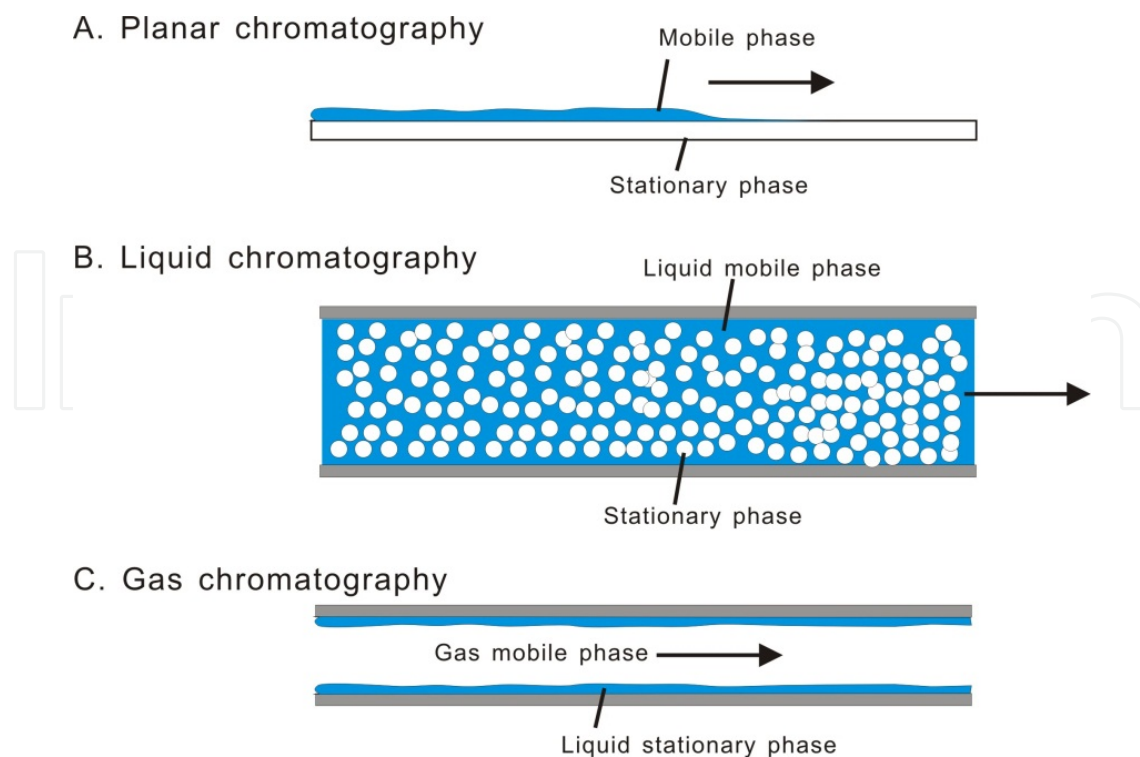
## 1. Introduction

Term 'chromatography' was firstly employed by Russian Scientist Mikhail Tsvet in 1900 to describe the phenomenon that a mixture of pigments was carried by a solvent to move on paper and separated from each other. Since the pigments have different colors, the phenomenon was termed by "chromato-graphy" literally means 'color writing' [1]. Now, it is generally refers to a series techniques for the separation of mixtures [2].

Each chromatography involves two phases, mobile phase and stationary phase. The mobile phase drives compounds to flow through the surface of the stationary phase and the movements of compounds are retarded by interaction with stationary phase. Compounds are retarded differentially according to the strength the interaction and finally are separated.

The chromatography was early performed on papers or thin layers to separate small molecule compounds, termed planar chromatography (Figure 1A). Later, the column chromatography was developed, in which the stationary phase is manufactured into porous particle media and parked in a column and the mobile phase flows through thin channels among media [3]. If the mobile phase is gas and stationary phase is liquids, the technique is termed gas chromatography [4], which is used in separation of volatile compounds (Figure 1B). If the mobile phase is liquid and stationary phase is solid, it is termed liquid chromatography [5] and used widely in separation of small compounds or biological macromolecules (Figure 1C).

The liquid chromatography is the most popular technique in protein purification and analysis. The liquid mobile phase containing proteins flows through the column and is separated by interacted with media. The stationary phase composed by porous particles supplies much more surface compared with traditional planar chromatography. So the loading capacity is much more increased and could purify even grams of protein in one cycle. Furthermore, the column structure provides a possibility to employ high pressure to drive the mobile phase flowing much faster and complete a separation within short time, termed high pressure liquid



**Figure 1.** Different types of chromatography

chromatography [6]. At same time, uniform size of matrix benefited by exquisite quality gives the column chromatography much higher resolution than before. The high performance in high loading capacity, high flow rate, and high resolution made the column chromatography become the most rapidly developed protein separation technique in the last two decades.

Several basic types of chromatography had been developed based on different separation properties (Table 1). This chapter describes both principles and applications of these techniques.

Property	Technique
Net charge	Ion exchange chromatography
Hydrophobicity	Hydrophobic interaction chromatography and Reverse phase chromatography
Biorecognition	Affinity chromatography
Size	Size exclusion chromatography

**Table 1.** Different chromatography techniques and corresponding protein properties

## 2. Ion-exchange chromatography

Ion-exchange chromatography (IEXC) was introduced to protein separation in the 1960s and plays a major role in the purification of biomolecules [7]. IEXC separation is based on the

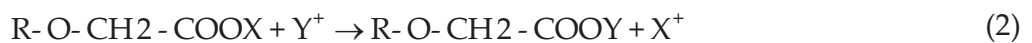
reversible electrostatic interactions between charged solutes and an oppositely charged medium. The technique is straightforward on its theory and operation, so that easily to be grasped by beginners.

Ion exchange refers to the exchange of ions between two electrolytes or between an electrolyte solution and a complex. For example:  $\text{NiSO}_4 + \text{Ca}^{2+} = \text{CaSO}_4 + \text{Ni}^{2+}$ . When one of the electrolytes was immobilized on resin, the exchange will happen between the interface of liquid phase and solid phase, termed exchanger, such as,



In which R indicates the base matrix portion of the resin, the ion  $\text{X}^+$  exchanges with  $\text{Y}^+$  and is adsorbed by resin.

The exchange reaction is reversible and the direction depends on the concentration and ionization constant of the electrolytes. In Equation 1, if concentration of ion  $\text{Y}^+$  increases,  $\text{X}^+$  will be desorbed.



The ion  $\text{Y}^+$  could be any cation, such as  $\text{Na}^+$ ,  $\text{H}^+$ . The two equations present the process of the binding and elution in IEXC.

According to the above two equations we know the binding of protein on exchanger is a kinetic equilibrium between adsorption and desorption. The equilibrium constant  $K_d$  is:

$$K_d = \frac{[\text{X}^+][\text{R-O-CH}_2\text{-COOY}]}{[\text{Y}^+][\text{R-O-CH}_2\text{-COOX}]} \quad (3)$$

With mobile phase moving, protein molecules in mobile phase are carried forward and adsorbed by downstream medium, at same time adsorbed proteins are released from stationary phase to mobile phase. Proteins remove forward accompanied with continuous adsorption and desorption. Under the same ionic strength, the higher  $K_d$  a protein has, the more fraction distributes in mobile phase and moves faster. Reversely, proteins having smaller  $K_d$  are more retarded than that having larger  $K_d$ . Actually, all kinds of adsorption chromatographies are base on the kinetic equilibrium mechanism.

## 2.1. Isoelectric point of protein

Proteins are amphoteric on which carboxyl groups and amino groups of side chains and two terminals could ionize and cause proteins being positively and negatively charged. The positive charges of proteins typically attribute to ionized cysteine, aspartate, lysines, and histidines. Negative charges are principally provided by aspartate and glutamate residues. At

a certain pH point, the total positive charges of a protein equal to the total negative charges, the net charge is 0 at this time and the pH is defined as the isoelectric point (pI) of this protein. When the solution pH higher than pI of a protein, more carboxyl groups ionized and the protein is negatively charged, vice versa (Figure 2). pI of a protein could be determined by several experiment methods, but an approximate value could be calculated by mathematics methods. Once a protein primary structure is given, the pI can be calculated by software or some concise websites such as:

[http://web.expasy.org/compute\\_pi/](http://web.expasy.org/compute_pi/)

<http://www.scripps.edu/~cdputnam/protcalc.html>

## 2.2. Selection of exchanger

The exchangers in IEXC are composed of base matrix and functional groups that coupled on surface of the matrix. The base matrix is nonporous or porous spherical particles with charge free surface on which different functional groups link. Porous matrix offers a large surface area for protein binding and so gives a high binding capacity, but sacrificed some resolution due to the diffusion between outside and inside of matrix. On the contrary, nonporous matrix is limited on binding capacity, but used to provide high resolution on micropreparative or analytical separations.

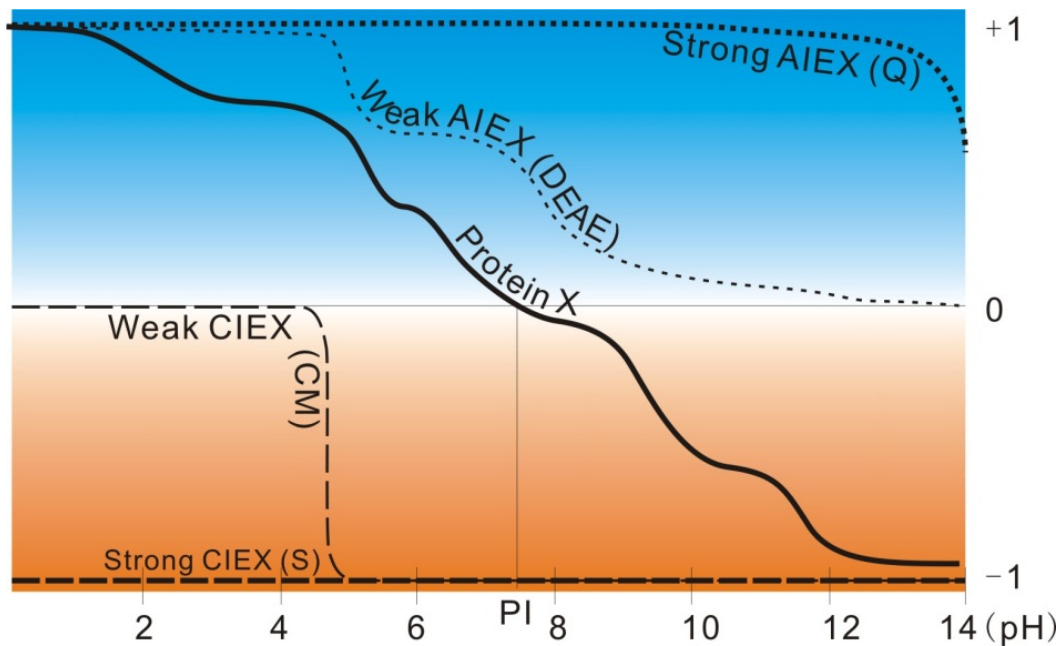
Similar to the effect of porosity, the size of particles also influences the resolution of all kinds of chromatography including IEXC. Even and small particle size facilitates the efficient transfer of molecules between the mobile and the stationary phases, and provides high resolution, but increases the resistance of the column so that needs higher pressure or longer separation time. Small size particles are preferable for analytical separations. On the contrary the large size particles are more used on large scale production.

The selectivity of ion exchange media depends briefly on the nature and substitution degree of the functional groups, or called ligands. The media are classified into anion exchangers and cation exchangers. Ligand of the anion exchangers can be positively charged and anions can bind and exchange on it. On the contrary, the cation exchangers can be negatively charged on which cations exchange. The commonly used exchangers named after the functional groups and list in Table 2.

Exchanger	Ligand	Charged group
Strong cation	Sulfopropyl (SP)	$-\text{CH}_2\text{CH}_2\text{CH}_2\text{SO}_3^-$
Weak cation	Carboxymethyl(CM)	$-\text{O}-\text{CH}_2\text{COO}^-$
Strong anion	Quaternary ammonium (Q)	$-\text{N}^+(\text{CH}_3)_3$
Weak anion	Diethylaminoethyl (DEAE)	$-\text{N}^+\text{H}(\text{C}_2\text{H}_5)_2$
	Diethylaminopropyl (ANX)	$-\text{N}^+\text{H}(\text{C}_2\text{H}_5)_2$

**Table 2.** Commonly used exchangers

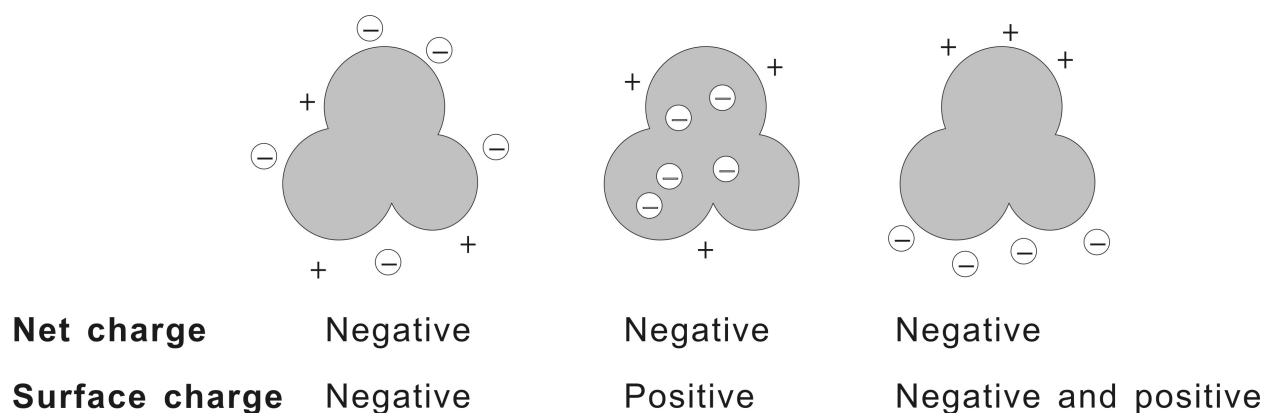
Ion exchangers are classified as weak or strong according to the ionization properties of ligands. The strong exchangers own ligands with high ionization coefficient (Figure 2). They are fully charged in pH range 1~13. In this range, pH change does not influence the charge of the ion exchanger. Thus the strong exchangers can wide used in almost all pH range. On the contrary, weak ion exchangers have weak electrolytes as functional ionic groups. The ionization of these groups is influenced by solution pH. So that they can offer a different selectivity compared to strong ion exchangers.



**Figure 2.** Charge property of the common types of ion exchangers and example protein with different pH value. (Modified from Ion Exchange chromatography & chromatofocusing, principle and methods, GE healthcare)

### 2.3. Surface charge of protein

The mobile phase in IEXC is aqueous solution with proper pH value and ionic strength. The pH value determines the charge property of protein. A pH value lower than a protein pI will causes a positive net charge of the protein and vice versa. It should be noted that IEXC is base on the electrostatic interaction. The interaction between a protein and an ion exchanger depends more on the charge distribution of the protein surface than the net charge (Figure 3). The distribution of the charge on surface and internal is not even, so a solution with pH value slightly different to protein pI could not insure the protein exhibit an expected charged surface. In practice, the pH is typically at least 1 unit higher or lower than pI of target protein to ensure the protein has an expected surface charge.



**Figure 3.** Different charge distributions of proteins.

## 2.4. Mobile phase

Mobile phase is composed of pH buffer system and neutral salt ions. Buffering ions in buffer should have the same charge with exchanger. Otherwise the buffering ions will bind to exchanger prior to eluent ions and cause significant pH fluctuation during elution. The commonly used buffers are given in table 3.

Buffers	pH range at 20 mM
<b>Buffer for cation exchange chromatography</b>	
Citric acid	2.6~3.6
Acetic acid	5.3~6.3
MES	5.8~6.8
Phosphate buffer	6.3~7.3
HEPES	7.1~8.1
<b>Buffer for anion exchange chromatography</b>	
Bis-tris	6.0~7.0
Tris-HCl	7.5~8.5
TEA	7.4~8.8
Ethanolamine	9.0~10.0
Piperidine	10.5~11.5

**Table 3.** Commonly used buffer for cation and anion exchange chromatography

Except pH value, the ionic strength also influences the binding of the protein. A typical IEXC experiment includes a binding stage and an elution stage. As indicated in Equation 1 and 2, proteins tend to be adsorbed by exchanger at low ionic strength and be desorbed at high ionic strength. So the ionic strength should be low enough in binding process to ensure protein adsorption and increased to elute proteins. The ionic strength in IEXC is usually modulated by adding high concentration of NaCl solution.

## 2.5. Operation

### 2.5.1. Binding process

All solutions used in column chromatography, including sample solution, should be degased and filtered (0.22 or 0.45  $\mu\text{m}$  membrane) to avoid the clogging of column by air bubbles or particles. Before sample loading the column should be equilibrated with 2 column volumes (CV) of initial buffer. And then sample is loaded with same flow rate. After that 3 CV of initial buffer should be run to wash off the unbound impurity proteins.

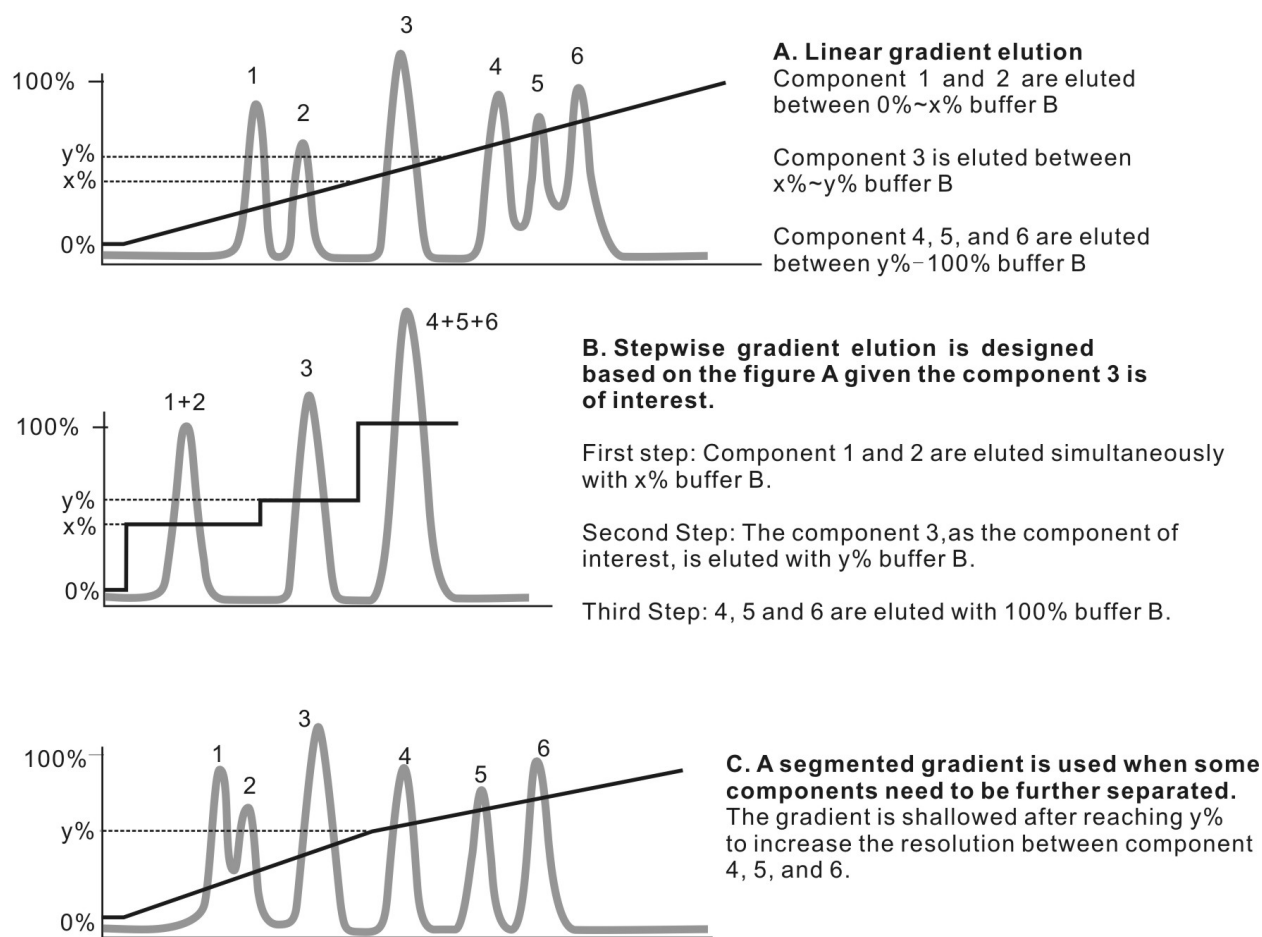
### 2.5.2. Elution

Although proteins could be separated under constant solvent composition, termed isocratic elution, for most tightly adsorbed proteins, it will take very long time to be eluted.

In practice, the mostly used strategy is to accelerate the exchange of protein by increasing ion strength in initial buffer. The most widely used agent is NaCl. It is convenient to increase the cation  $\text{Na}^+$  and anion  $\text{Cl}^-$  at same time and without significantly change pH value of solution. Proteins could be eluted by linear or stepwise gradient ion strength or combination of them (Figure 4). The stepwise gradient elution is used in group separation. In each step one group of proteins with similar charge property is eluted simultaneously. It is often used in large scale production. While, linear gradient could be seem as infinite number of tiny steps, in which protein was eluted and separated one by one. It is more used in preliminary experiments or analytical separations. In practice, the usual strategy is combination of linear and stepwise gradient. As show in figure 4C, a part of impurities are eluted first by a step elution, and then the target protein is separated from the similar charged protein by a linear elution.

Another elution method is to change the surface charge of proteins by changing pH value of the elution buffer. Typically, in cation IEXC, increased pH value decreases the surface positive charge and the interaction between proteins and exchangers is weakened. Reversely the pH value is decreased in anion IEXC to elute protein. Proteins are eluted at the pH value close to their pI. It should be noted that, change of pH could also alter the charge property of weak exchangers in certain ranges, so the weak exchanger possibly gives different resolution in these ranges. But pH elution is less used in practice because some proteins precipitate at pH value near to their pI and clog column. Additionally, it is hard to keep ion strength constant as changing pH value and present a worse reproducibility.





**Figure 4.** Different strategies of gradient elution.

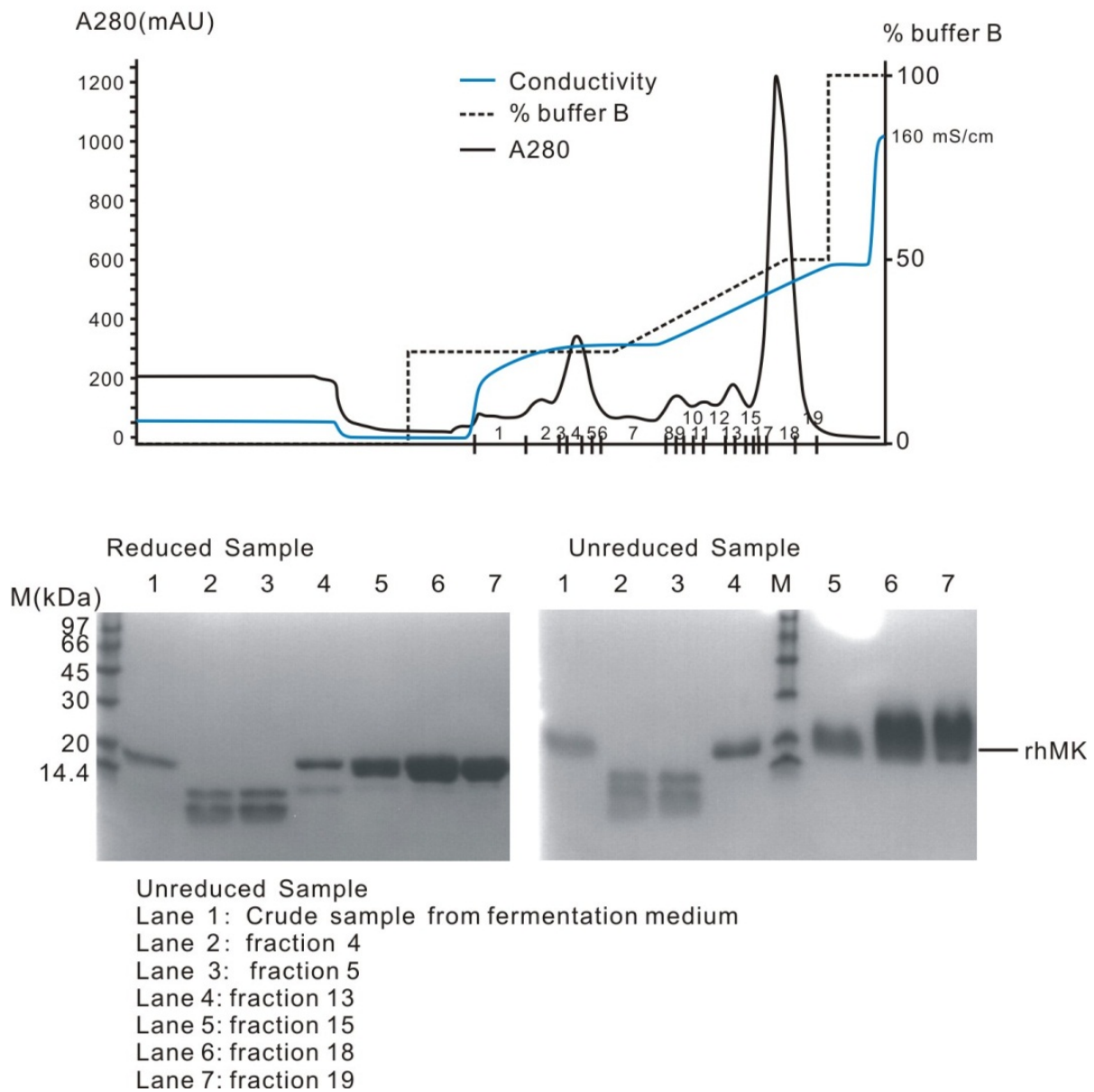
## 2.6. Feature and application

IEXC is one of the most frequently used chromatographic techniques for the protein separation. The adsorption and elution take place under mild condition so that the natural activities can be well maintained during chromatographic process.

### 2.6.1. Purification of recombinant human Midkine by SP column

A recombinant human Midkine,  $pI=9.7$ , was expressed by a yeast fermentation technology and separated by IEX chromatography using SP column. The fermentation culture with high potassium phosphate buffer (100 mM) was diluted by pure water, lowering the conductivity to  $<10$  mS/cm, and adjusted to pH 6.2 by  $\text{Na}_2\text{HPO}_4$  solution. 50 ml Sepharose FF column with maximum loading capacity of 70 mg/ml was used to capture total 200 mg proteins in sample solution. A fraction of non-target protein was eluted by stepwise elution using 0.5 M NaCl, and then a linear gradient from 0.5~1.0 M NaCl was used to separated the target protein from the other impurities.

**Sample:** 6000 ml Yeast X33 fermentation culture containing recombinant human Midkine (~0.04mg/ml)  
**Column:** SP FF column (50 ml)  
**Buffer A:** 20 mM sodium phosphate, pH 6.2  
**Buffer B:** 20 mM sodium phosphate, 2M NaCl, pH 6.2  
**Gradient:** 25~50%B in 200 ml(6CV) where 50%B=1.0 M NaCl  
**Flow rate:** 5 ml/min



**Figure 5.** Cation IEXC of rhMK (result of Shixiang Jia, Ping Tu et al. General regeneratives (shanghai) limited, Shanghai, PR China)

### 3. Hydrophobic interaction chromatography

Hydrophobic interaction chromatography (HIC) bases on the interactions between hydrophobic surface of proteins and hydrophobic ligands on the medium [8]. It is used in protein separation for more than a half century, although there is not a widely accepted theory to define the hydrophobic interaction.

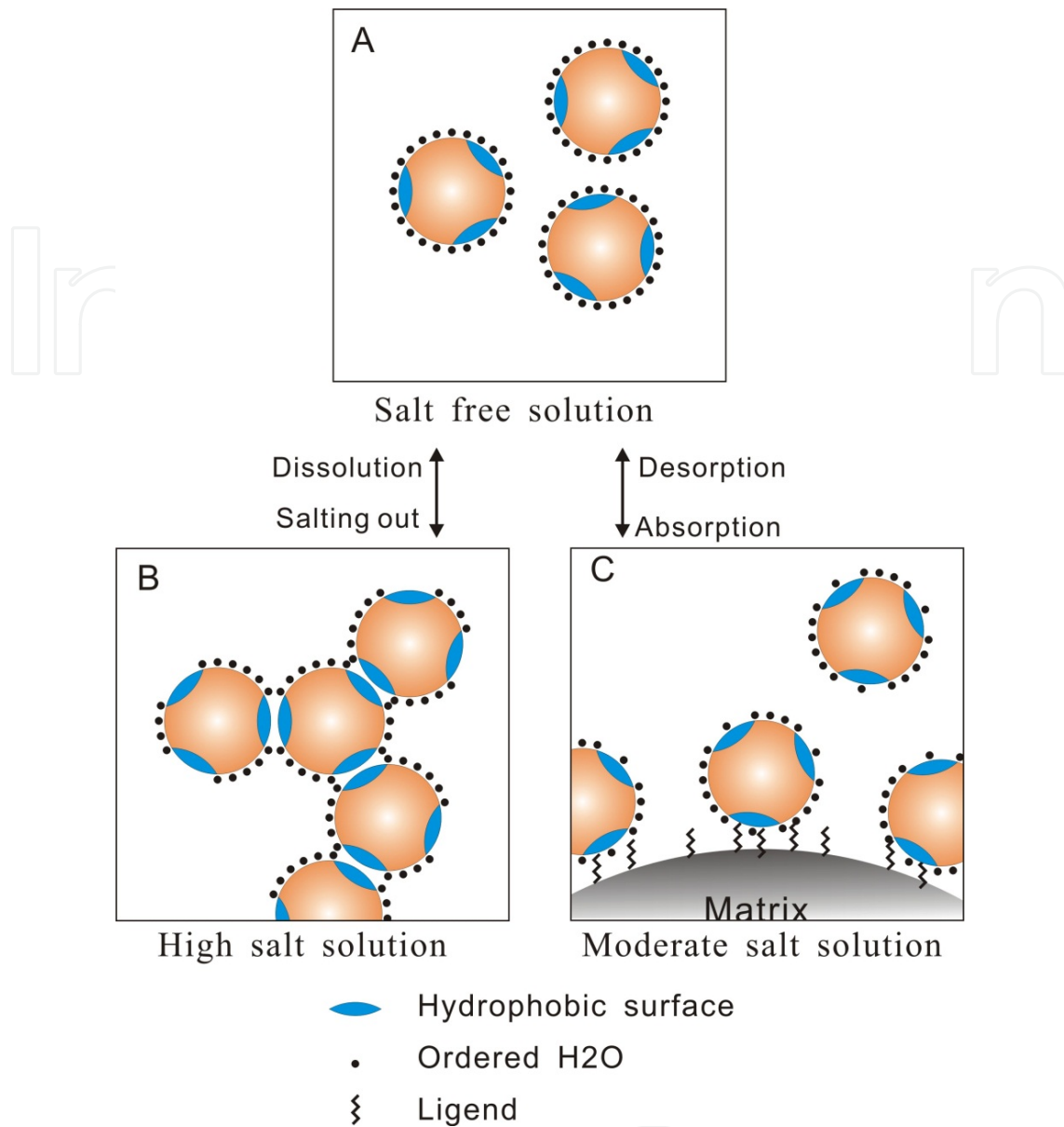
The principle of HIC is parallel to that of salting out. In aqueous solution, hydrogen bond is formed between water molecules and protein surface. By hydrogen bond, the side chains of protein molecules adsorb water molecules to form an ordered water film around them. The water film prevents protein molecules from aggregating and precipitating. Different amino acid side chains have variant abilities in forming hydrogen bond. Hydrophobic amino acids, such as isoleucine, valine, leucine, and phenylalanine, tend to loss their ordered water as solution ion strength increases. Relative hydrophobicity of amino acids was defined by the change of Gibbs free energy when amino acids are transferred from aqueous solution to non-polar solvent [9]. The distribution of hydrophobic amino acids on protein surface determines the hydrophobicity of the protein. As salt concentration increases, proteins associate each other and precipitate in the order of decreasing hydrophobicity. This process is termed fractional salting out (Figure 6B).

In HIC, the concentration of salt is controlled at an appropriate value, for example, 1 M  $(\text{NH}_4)_2\text{SO}_4$ . At this concentration, the hydrophobic interaction still not strong enough to cause proteins precipitate. However, the hydrophobic media, termed adsorbent, could adsorb proteins by high hydrophobic ligand coupled on it (Figure 6C). When protein solution flows through the HIC column, proteins having certain hydrophobicity will be adsorbed, and proteins with weak hydrophobicity will flow through with mobile phase. So, to adsorb proteins with weak hydrophobicity needs application of higher salt concentration or medium with stronger hydrophobicity to increase the hydrophobic interaction.

#### 3.1. Stationary phase

The media of HIC are composed of base matrix and ligand. Base matrix functions as a support on which the hydrophobic ligand is immobilized. To avoid the disturbance the hydrophobic interactions between proteins and ligand, the matrix should have an inert surface. Cross-linked agarose is one of the most widely used matrix, it has a porous structure, having high binding capacity, high flow rate, good physical and chemical stability. Except that silico or synthetic copolymer materials are also widely used matrix.

Hydrophobic ligands are attached to the surface of base matrix by covalent bonds, for example, by glycidyl-ether for agarose and silyl-ether for silico gel. Widely used ligands for HIC are linear chain alkanes and phenyl. The strength of the hydrophobicity increases with the increase of length of the carbon chain. Butyl (C4) and octyl (C8) are often used linear chain ligands. Another widely used ligand is phenyl, which not only has a same hydrophobicity with pentyl ligand, also has a potential for  $\pi$ - $\pi$  interactions with proteins rich in aromatic groups.



**Figure 6.** Salting out process and adsorption between protein and adsorbent. (A) A protein can disperse in salt free solution. (B) When salt concentration increases, the ordered water molecules are taken up. Proteins tend to aggregate and precipitates. (C) With a moderate salt concentration, the hydrophobic interaction between protein molecules is not strong enough to cause salting out, but can result in proteins adsorbed by hydrophobic matrix.

Before separating of each new protein, it is a good idea to screen different media by pretests on small prepacked column. The pretests should start from the medium with lowest hydrophobic. An ideal medium should firstly have an appropriate hydrophobicity by which the target protein could be adsorbed at a certain salt concentration. The lower hydrophobic a protein is, the higher hydrophobicity the medium should have in order to capture it. In addition the medium should be able to desorb the protein as the salt concentration decreases. Once proteins are captured too tightly to be eluted, organic solvent must be added to increase the elution power, which possibly causes the inactivation of proteins.

### 3.2. Mobile phase

Contrary to the IEXC, the initial buffer in HIC requires the presence of high concentration of salt ions, which preferentially take up the ordered water molecules from the protein surface and promote the hydrophobic interaction. The power is various among different ions. An ion that more increases the tension of water tend to more increase the strength of interaction between proteins and HIC media, although the internal nature is still not clear. Hofmeister series list the common ions according to the power to increase the water tension [10].

Anions:  $\text{HPO}_4^{2-} > \text{SO}_4^{2-} > \text{C}_2\text{H}_3\text{O}_2^- > \text{F}^- > \text{Cl}^- > \text{Br}^- > \text{I}^- > \text{ClO}_4^- > \text{SCN}^-$

Cations:  $\text{N}(\text{CH}_3)_4^+ > \text{Cs}^+ > \text{Rb}^+ > \text{NH}_4^+ > \text{K}^+ > \text{Na}^+ > \text{Li}^+ > \text{Ca}^{2+} > \text{Mg}^{2+}$

Molal surface tension of salts is listed as below.

$\text{MgCl}_2 > \text{Na}_2\text{SO}_4 > \text{K}_2\text{SO}_4 > (\text{NH}_4)_2\text{SO}_4 > \text{MgSO}_4 > \text{Na}_2\text{HPO}_4 > \text{NaCl} > \text{LiCl} > \text{KSCN}$

This series is not consistent for every protein, since except for the effect on water tension, the specific interaction between ions and proteins also appears to be another parameter on hydrophobic interaction. It seems that the hydrophobic interaction is more effected by anions than by cations. For example, the  $\text{MgCl}_2$  is weaker than  $(\text{NH}_4)_2\text{SO}_4$  on the promotion of hydrophobic interaction.

In practice,  $(\text{NH}_4)_2\text{SO}_4$  is one of the most used salt, 1~1.5 M of  $(\text{NH}_4)_2\text{SO}_4$  solution could satisfied most protein separations. If could not obtain the ideal effect, altering concentration or changing other salt ions, such as  $\text{Na}_2\text{SO}_4$  or  $\text{NaCl}$ , should be considered. The disadvantage of  $(\text{NH}_4)_2\text{SO}_4$  is that the  $\text{NH}_4^+$  tend to form ammonia gas under high  $\text{OH}^-$  concentration, so it should be used under  $\text{pH} < 8.0$ . As adding high concentration of salt into sample, some high hydrophobic proteins likely precipitate. Therefore ever remember to filter or centrifuge sample solution to remove particles after unstable proteins sufficiently aggregate.

Solution pH value also has complex effect on strength of hydrophobic interaction. The mechanism is not very clear. In general, an increase in pH weakens hydrophobic interaction [11], possible due to an increase of surface net charge. But a research of Hjerten et al. revealed that increase in pH, on the contrary, increased the retention of some protein [12].

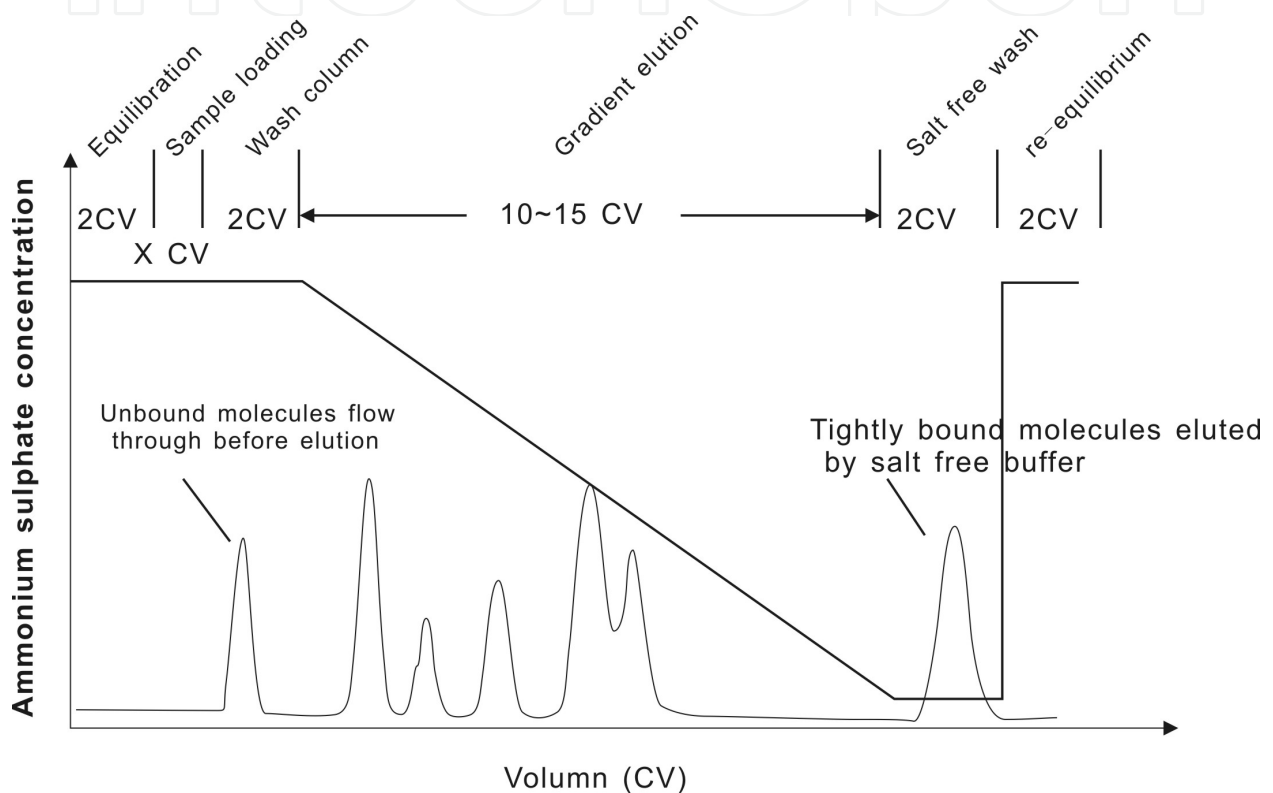
The effect of temperature on hydrophobic interaction is also complex. An increase in temperature could promote the hydrophobic interaction for some proteins, but weaken it for some others. The effect still can not be predicted efficiently on theory.

### 3.3. Elution

Similar with IEXC, isocratic elution with constant solvent composition can not elute protein efficiently. Gradient decrease of ion strength is the mostly used method in elution process of HIC. By decrease of ion strength, proteins are desorbed in the order of increasing surface hydrophobicity.

As decrease of salt concentration, proteins again obtain ordered water molecules and are eluted in the order of increasing hydrophobicity. A linear or stepwise gradient decrease of

salt concentration is employed in elution of protein in IHC. Similar to the strategies of IEXC, simple linear gradient elution presents even resolution to universal gradient range, which always used in the screening experiment or analytical separation, but takes more time. Stepwise gradient elution is preferred in large scale preparative separation. It is advantageous in time-saving and solution-saving and obtaining more concentrated product. But this strategy usually can not be performed until an appropriate elution condition is found out through preliminary works of linear gradient elution. A typical linear gradient elution spectrum is show in Figure 7.



**Figure 7.** A typical linear gradient elution spectrum of HIC

Additionally, adding neutral nonpolar solution, such as detergents, to the elution buffer could promote the elution of higher hydrophobic protein, such as membrane proteins or apolipoproteins. But nonpolar solution possibly causes irreversible inactivation, so should avoid to be used in IHC. If the target protein could not be eluted in salt free aqueous solution, changing of a lower hydrophobic medium should be considered. While, high concentration of organic solution could be used in column regeneration, by which tightly bound compounds will be washed away.

pH and temperature are two important factors on retention of proteins, but they are usually not used as variable parameters in elution since their effects are hardly controlled. So that, the pH and temperature condition should be consistent between patches in order to present a good reproducibility.

### 3.4. Features

HIC separates proteins based on different hydrophobicity of proteins. It combines the reversibility of hydrophobic interaction and the precision of column chromatography to yield excellent separation. With certain medium, HIC could capture almost all proteins at certain conditions and suit to capture, concentrate, or polish proteins.

The selectivity of HIC is orthogonal to that of IEXC and SEC, because it works base on hydrophobicity of proteins, a totally different property from the net surface charge used in IEXC and molecular size in SEC. So HIC is an orthogonal separation dimension when combining with IEXC or SEC. So using two of them in series will yields much better separation rather than using one.

## 4. Reversed-phase chromatography

Reversed-phase chromatography was named due to a reversed polarity between mobile phase and stationary phase compared with normal phase chromatography [13]. In normal phase chromatography, the mobile phase is organic solvent and stationary phase is hydrophilic resin. Reversely RPC uses hydrophobic adsorbents as stationary phase, which is the same with HIC in theory. However, in practice, the two methods have many differences. It is mainly due to the different degree of substitution of hydrophobic ligands on the medium surface. As shown in table 4, the density of ligand in RPC is an order of magnitude higher than that of HIC. It means that a protein molecule could bind more ligands when it is adsorbed. The huge forces could extract proteins from aqueous solution without help of neutral salt, so that the adsorbed proteins could not be eluted until using nonpolar solvents. Therefore, RPC is less used in preparation of activity proteins. However, the excellent resolution makes this technique to be the most important analytic chromatography. Liquid Chromatography-Mass Spectrometry is an important extended application of the technique.

	RPC	HIC
Interaction	Hydrophobic interaction	Hydrophobic interaction
Ligand	C2~C8 alkyl or aryl	C4~C18 alkyl
Substitution degree	10~50 mmoles/ ml gel	several hundred mmoles/ml gel
Capture condition	Salt free solution	High salt solution
Elution	Increase nonpolarity	Decrease ion strength
Application	Protein analysis Preparative separation of poly peptide or oligonucleotide	Preparative separation of protein

**Table 4.** Comparison between RPC and HIC

## 4.1. Stationary phase

Similar with HIC, the media of RPC is composed of inert base matrix and hydrophobic ligands on surface.

The base matrix for reversed phase media is generally composed of silica or a synthetic organic polymer such as polystyrene. Silica was the first material used as base matrix for RPC, which has an excellent mechanical strength and chemical stability under acid condition. However the disadvantages of silica base matrix is its chemical instability in aqueous solutions at high pH. Silica matrix could be dissolved at high pH, so it is not recommended for prolonged exposure above pH7.5. Additionally, due to incomplete substitution or long term usage, some underivatized silanol groups are exposed to mobile phase, which will be negatively charge at high pH value, and cause ionic interaction with proteins. The mixed chromatography always causes decreased resolution with significant broadening and tailing of peaks. Therefore, RPC using silica matrix is often performed at low pH values (<3).

The loading capacity and resolution are determined by size of resin, in general, smaller resin give the higher resolution but lower loading capacity. The resin with 3~5  $\mu\text{m}$  in diameter is preferable for analytic separation. Due to small size, it is hard to be packed well. So it is often offered in the form of prepacking columns. With increasing of diameter, the loading capacity increase, but resolution decrease simultaneously. Generally media with 15  $\mu\text{m}$  or larger diameter are used in preparative separation.

The porous structure is employed to increase the loading capacity of PRC media. In general the pore size is 10~30 nm. Media with pore sizes of 10 nm are used predominately for small peptides or molecules. Media with pore sizes of 30 nm or greater are used in purification of large peptide or proteins.

Ligands used in RPC are linear alkyl with different length of carbon chain, which is the main factor on selectivity of media. In general, a medium with longer chain ligands gives stronger hydrophobicity. Oligonucleotide and organic moleculars, having less hydrophobicity, needs more hydrophobic media to supply sufficient adsorbability, such as C18 media. On the contrary, large peptides or proteins generally have more hydrophobic sites and need less hydrophobic adsorbents, such as C4 or C8. Selectivity and loading capacity are also influenced by the substitution degree. For large peptides or protein, the effect of increase in substitution degree is equal to increase in length of carbon-chain.

## 4.2. Mobile phase

### 4.2.1. Organic solvent

Typically, sample was loaded onto the column in aqueous solution and eluted by decreasing solution polarity. The elution power increases as polarity decreases. Although a large part of organic solvents have enough elution power, only a few of them could be used in RPC because of the requirement on viscosity and ultraviolet (UV) transparency. High solution viscosity influences the diffusion of solutes between mobile and stationary phases, therefore high viscous solvent reduces resolution. UV absorption of solvent will disturb the detection of solute



UV absorption. Acetonitrile and methanol are two most widely used organic modifiers due to their moderate viscosity and perfect UV transparent. Although isopropanol and normal propanol have higher elution power, they are only used to clean and regenerate column because of their high viscosity.

It should be noted, all solvent used in RPC should be HPLC grade to minimize the damage of impurities to resin or samples.

#### 4.2.2. *pH*

pH value could influence protein hydrophobicity by possibly changing the charge property of proteins [14]. In practice, two proteins with the same retention time are likely separated by just changing the solution pH value, and *visse versa*. At present, there is not effective method to predict the effect, trying different pH value is the only way to optimize the resolution.

However, as described above, media base on silica matrix are not suit to work at high pH value because of uncovered silanol groups. So silica-based RPC should works at low pH value, in general between 2 to 3. Strong acids, such as trifluoroacetic acid (TFA) or ortho-phosphoric acid are typically used to just the pH.

#### 4.2.3. *Ion-pairing agent*

The retention time of solutes, such as proteins, peptides, or nucleotides can be modified by adding ion pairing agents to solution [15]. An ion-pairing agent could ionize and release positive or negative ions, which will bind to the sample molecules by ionic interactions and results in the modification of hydrophobicity. For example, at a very acid condition most proteins are positively charged. The negative ion pairing agent will bind to positive charge group. The effect of neutralization always increases the hydrophobicity of proteins. TFA is not only used in pH control but is the most commonly used negative ion pairing agent. Additionally, triethylamine is used as positive ion pairing agent in neutral and alkaline condition.

### 4.3. Elution

A simple linear gradient elution is often used in RPC. The eluent is a mixture of buffer A and buffer B by a mix pump. The buffer A generally is the start buffer, in which 0.1~0.5% TFA is added to control pH and functions as an ion pairing agent. The Buffer B typically is 0.1~0.5% TFA in pure organic solvent, such as acetonitrile or methanol. A gradient increase of buffer B from 0% to 90% or more in 30~60 min is often used.

### 4.4. Application

The application of RPC on protein separation is mainly focus on the analytic separation and purity check. Because, on one hand, RPC has the highest resolution compared with the other relative techniques, on the other hand, the harsh binding and desorption condition in RPC usually leads to protein denaturation and not suit to preparative separation. A good reproducibility on retention time and low limit of detection make it be the most favored method in

protein purity check. Additionally, RPC is the only one chromatography that can be used in association with mass spectrometry analysis, since the high resolution of RPC is the only one chromatography can separate a complex sample, such as serum, into single components and immediately analyzed by mass spectrometry.

## 5. Size exclusion chromatography gel filtration chromatography

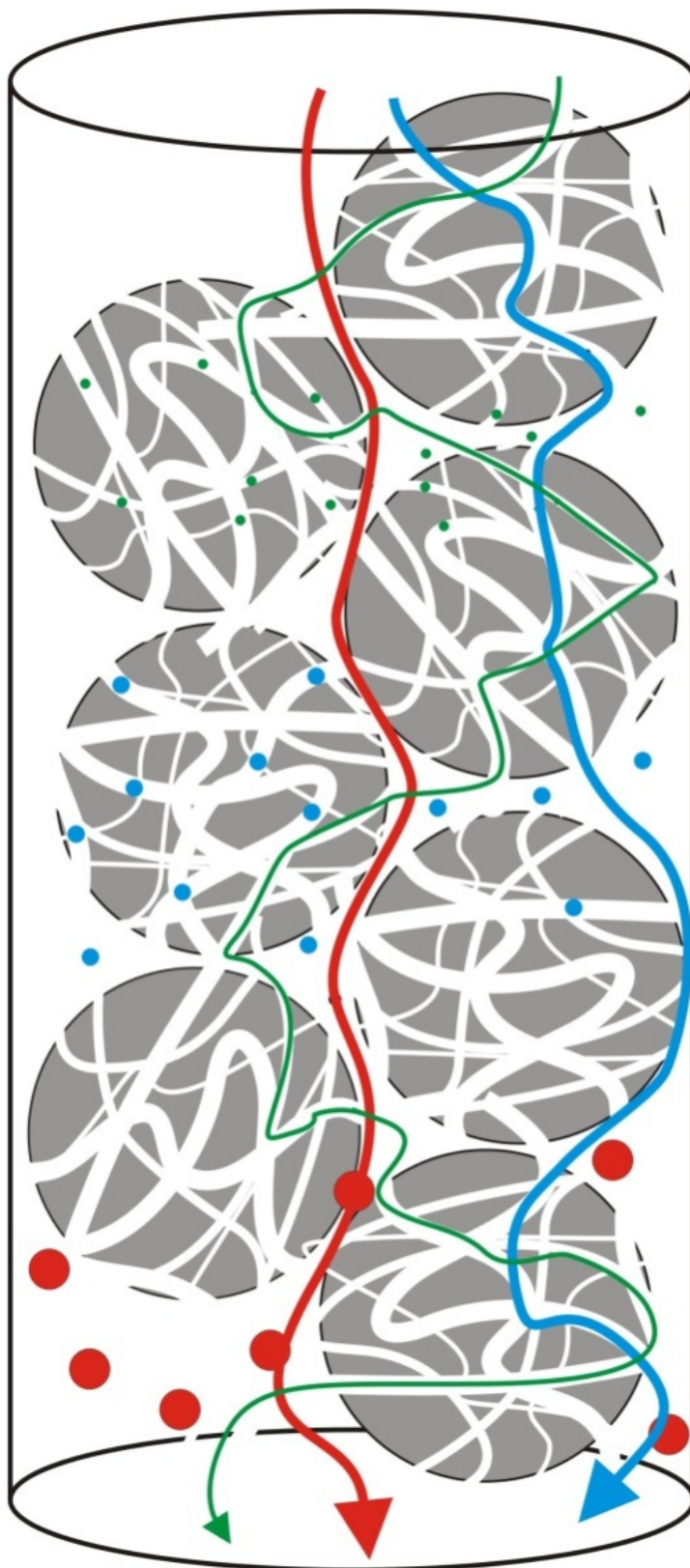
Size exclusion chromatography (SEC), or termed gel filtration chromatography, separates protein according to the difference on molecular size [16]. Different to those chromatography techniques based on adsorption, molecules do not bind to the surface of media in SEC, but are retarded by the porous structure of media. As shown in Figure 8, media of SEC are composed of porous material. However the pore size is much smaller than the pore size of the matrix used in adsorption chromatography and not uniform. The pore size of adsorption chromatography is big enough to allow entries of all molecules without selectivity. Comparatively, the pore sizes of SEC are smaller and selectively allow molecules with appropriate size enter and exclude the bigger molecules outside. Smaller molecules run longer and more winding paths in media rather than running straight paths outside the media as larger molecules do. So that smaller molecules are more retarded than larger ones.

### 5.1. Stationary phase

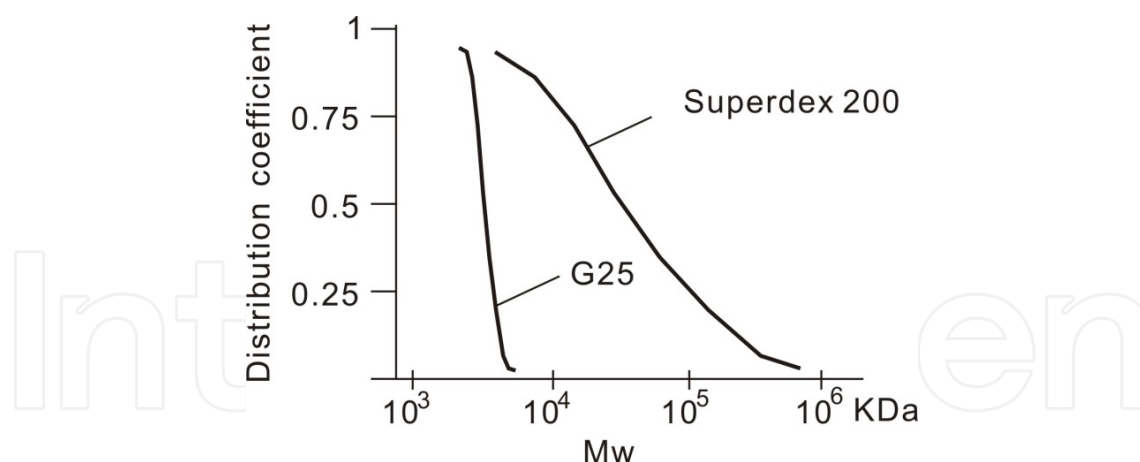
Resolution of SEC is influenced by many parameters of stationary phase, including, column volume, particle size, pore size distribution [17].

The matrix of SEC are often composed of polymers by cross-linking to form a three-dimensional network. The matrix is manufactured in small spherical particles. On the surface and the inside of the particles, small channels and pores are formed with different sizes by controlling different degree of cross-linking. The selectivity of a medium depends on the distribution of pore sizes and can be described by a selectivity curve (Figure 9). For example, the medium superdex 200 (by GE company) has a linear selectivity range of  $1 \times 10^4 \sim 6 \times 10^5$ , that means solutes having molecular mass ( $M_w$ ) in this range could be differentially retarded. The molecules larger than the upper limit are completely excluded from the inside space of the medium because no pores are big enough to allow them enter. At this time, the distribution coefficient ( $K_d$ ) reaches to 0. On the contrary, those molecules smaller than the lower limit are free to enter any channel, therefore they are maximally retarded without selectivity and has a  $K_d=1$ . Those solutes with  $M_w$  between the two extremes could enter channels with different degree,  $K_d$  is between 0 and 1, are retarded differentially.

The media with narrow linear range often employed in group separations, by which solutes are simply separated into two groups. A typical application is protein desalting by a G25 column (Figure 9). On the contrary, the media with wide linear range usually used to separate similar components (Figure 9), such as using superdex 200 to separate IgG ( $M_w=1.5 \times 10^5$ ) and albumin ( $M_w=6.6 \times 10^4$ ).



**Figure 8.** In SEC large molecules run through the space between media with a shorter pathway, while the smaller molecules run through the channels inside the medium with a longer pathway.



**Figure 9.** Selectivity curves of Superdex 200 and G25 media.

The height of packing bed affects both resolution and the separation time. Larger bed height often gives a better resolution with same sample volume, but takes more time to run a separation (Figure 10C).

The size of particle also is a parameter affecting resolution and the separation time. Smaller resin particles supply more efficient mass transfer between mobile and stationary phase, therefore present higher resolution. But simultaneously smaller particles increase the flow resistance and generally cause prolonged separation time.

## 5.2. Mobile phase

An unparalleled advantage of SEC in all chromatography is the wide compatibility to various solutions. Because SEC separates proteins depends on molecular size rather than interactions between solutes and media, so pH value and polarity of mobile phase generally have slight influence the retention of compounds.

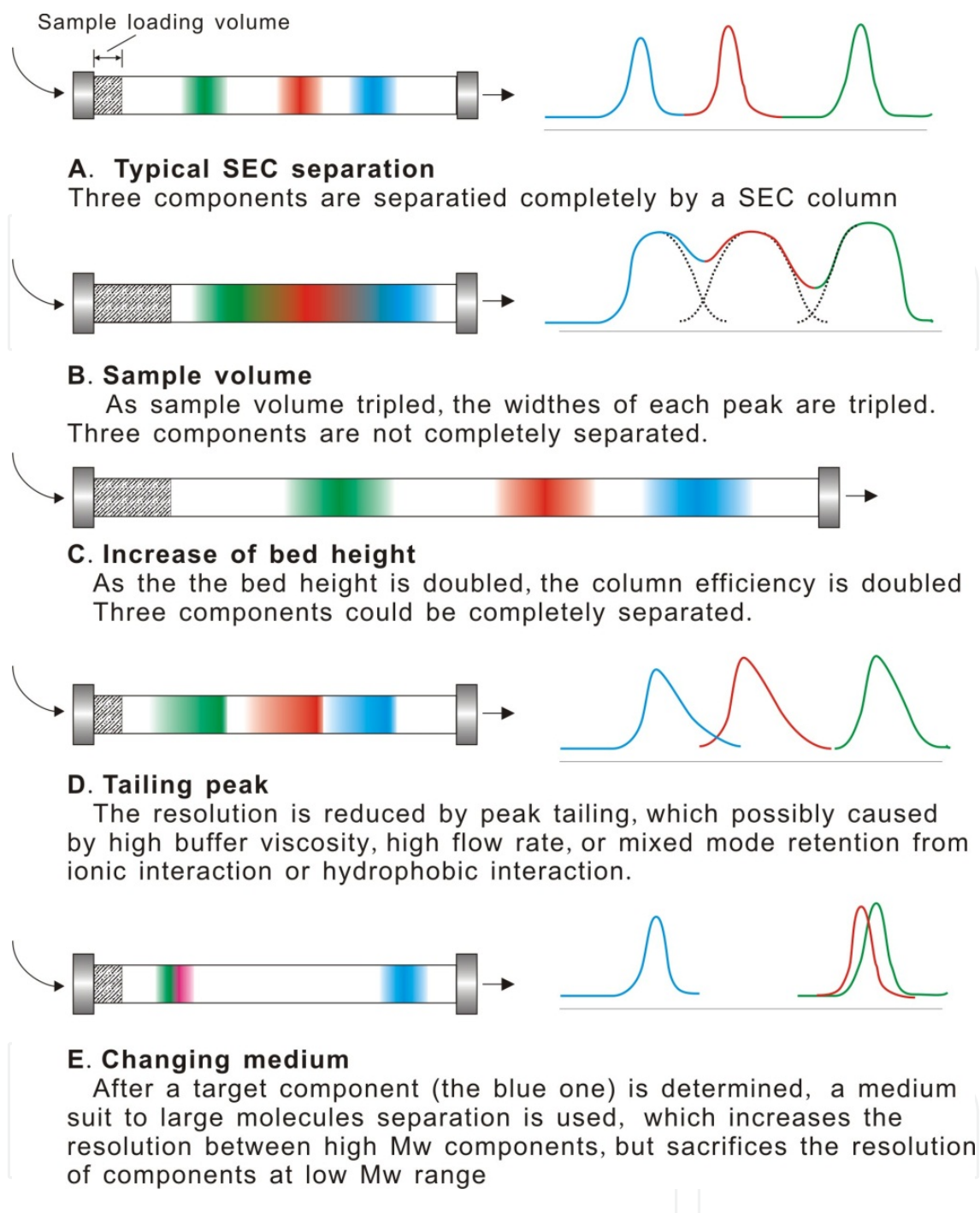
Since SEC has no concentration effect on elutes, so volumes of elution peak of each components are proportional to the sample volume. Increased sample volume will decrease the resolution (Figure 10B).

High viscosity in mobile phase has a certain effect on resolution by influence on the mass transfer between the mobile and the stationary phases, so that will cause broadening and tailing peaks (Figure 10D).

It should be noticed that the ionic interaction between proteins and the resin possibly takes place at a low ionic strength, so generally 0.15 M NaCl is added to avoid it.

## 5.3. Elution

SEC has no a definite elution step, since molecules are not adsorbed by media. After sample is loaded, a buffer usually same to the initial buffer is pumped with two column volumes until all solutes are eluted.



**Figure 10.** The factors affecting resolution of SEC.

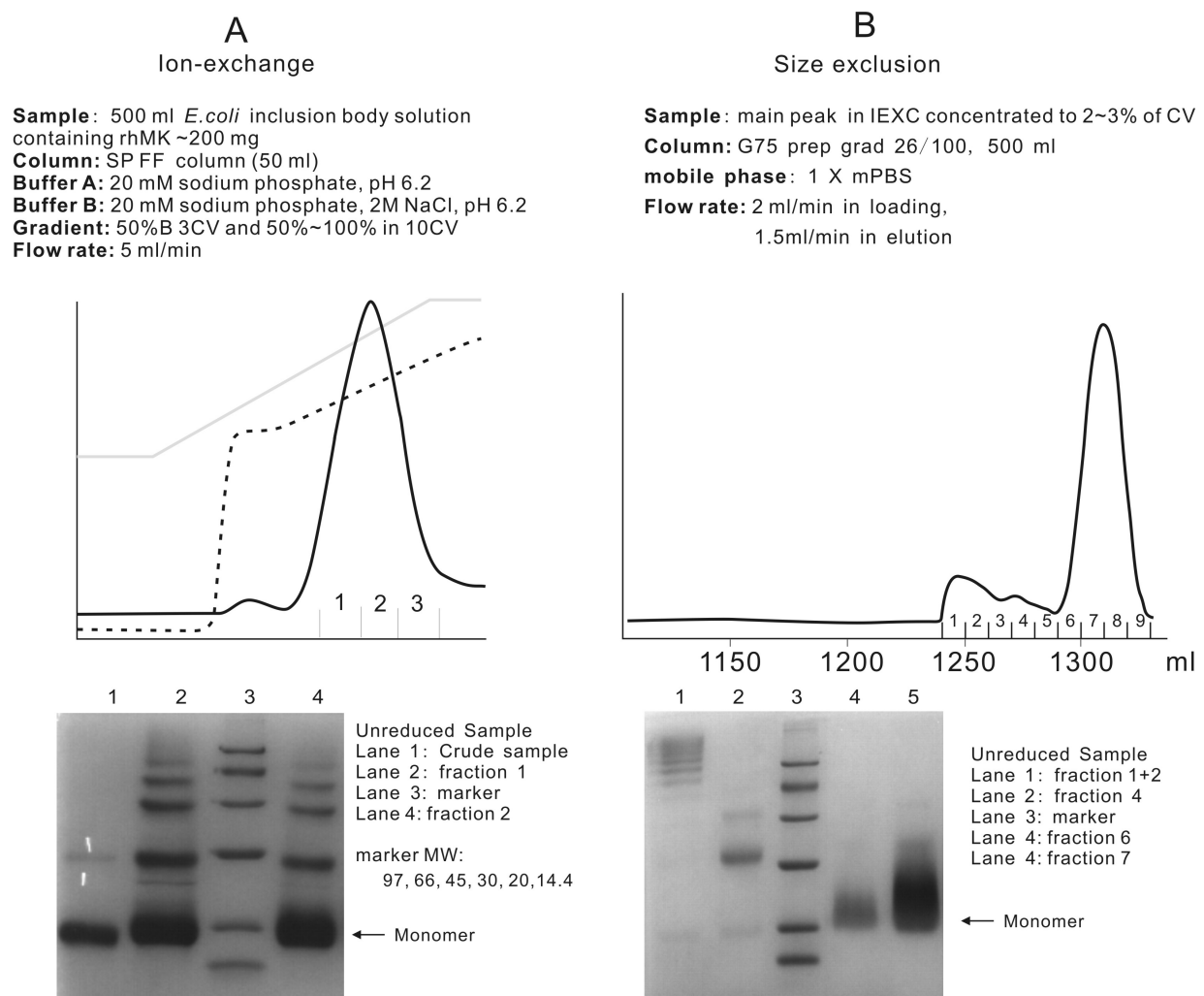
#### 5.4. Application

SEC has the most mild separation condition, since in the whole process the composition of mobile phase needs no change. This is a good property for separating proteins that are unstable to alterations of pH value, ionic strength or polarity. SEC is often used in polish step after a sample has been crudely separated by other chromatography, especially in separation of the monomer and polymers. Since monomer and polymers usually could not be separated by IEXC

and HIC due to the similarities in charge and hydrophobic property. But fortunately SEC can well separate them by different molecular size.

#### 5.4.1. Purification of recombinant human Midkine by SP column and SEC column

A recombinant human Midkine (Mw=14 kDa) was expressed by an *E.coli* BL21 strain as inclusion body form. The inclusion body was denatured by 6 M guanidinium chloride and renatured through 10-fold dilution in renature buffer. The renatured protein was separated by IEXC and SEC (figure 11). Since the incorrect formation of intermolecular disulfide bond, a fraction of the rhMK molecules formed different polymers, which could not be separated from monomers by IEXC and were eluted as a mixture (Figure 11A). To separate bioactive monomers, a Sephadex G-75 column, which owns a fractionation range of 3000~80,000 dalton, was used to separate monomers from polymers. Non-reduced SDS PAGE demonstrated the purity of monomers reached 95% in the target peak.



**Figure 11.** Purification of *E. coli* rhMK by IEXC and SEC. (result of Shixiang Jia, Ping Tu et al. General regeneratives (shanghai) limited, Shanghai, PR China)

## 6. Affinity chromatography

Affinity chromatography (AC) extensively refers to a series of techniques that separate proteins on the basis of a reversible interaction between proteins and their specific ligands coupled to a chromatography matrix [18]. The affinity interactions derive from a wide range of biorecognition, briefly including interactions between (1) enzymes and substrate analogues, inhibitors, cofactors [19], (2) antibodies and antigens [20], (3) membrane receptors and ligands [21], (4) nucleic acid and complementary sequence, histones, or nucleic acid polymerase, nucleic acid binding proteins, (5) biological small molecules and their receptors or carrier proteins [22], (6) metal ions and proteins having polyhistidine sequence.

Affinity interactions are always a result of a combination of different types of interactions, including electrostatic interactions, hydrophobic interactions, van der Waals' forces, or hydrogen bonding. The interactions of high specificity always supply extremely high selectivity, by which a target protein could easily be separated in one step with thousands fold of increase in purity and high recovery.

### 6.1. Media

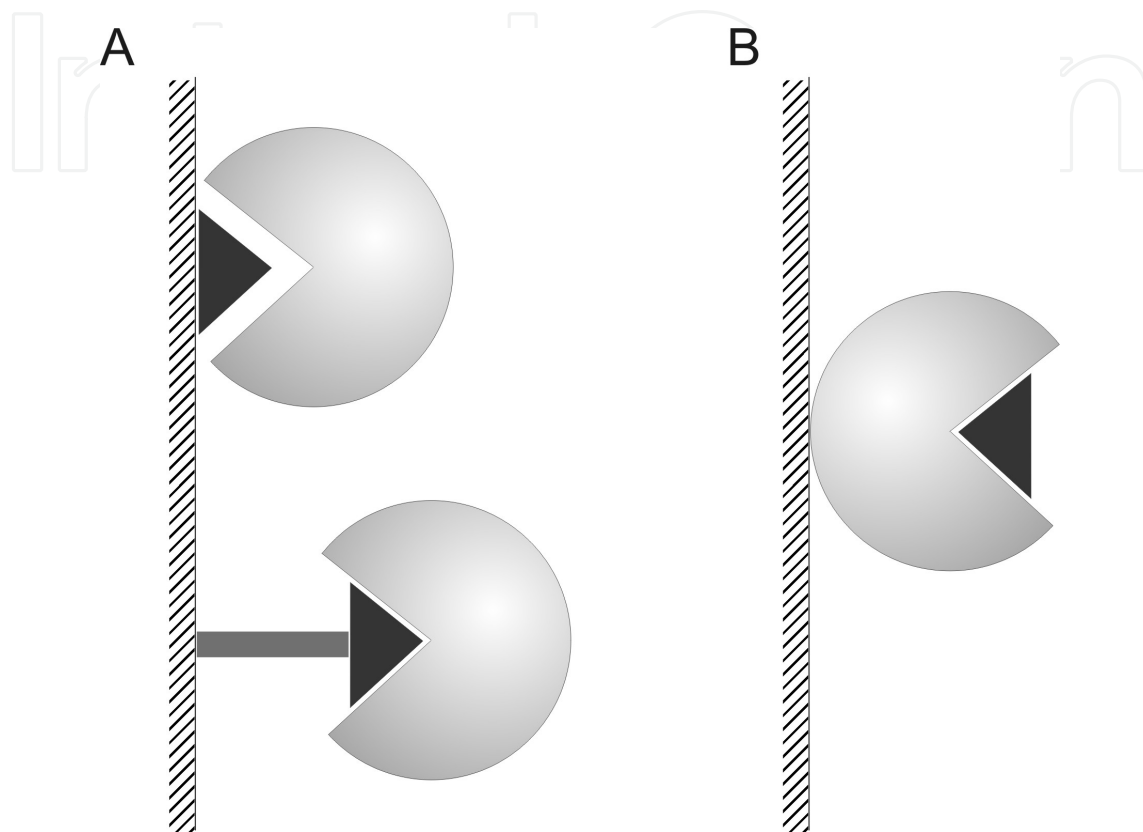
Development of an AC media is much more complex than that other chromatography. It needs not only a specific ligand, but also complex coupling process to couple the ligand to the matrix without reducing its binding activity significantly. Therefore more and more ready-to-use matrices, which already have active ligands coupled to, were developed commercially to satisfy different separation. If no suitable ligand is available, it can be considered to develop a specific affinity medium or use alternative purification techniques.

#### 6.1.1. Base matrix

The mostly used material is agarose or cross-linked agarose. The hydroxyl groups on the sugar residues are easily derivatized for covalent attachment of a ligand or spacer arms and the porous structure also supplies ideal flow rate and high capacity.

#### 6.1.2. Spacer arms

The binding site of a target protein often locates deep within the molecule. Due to steric interference, a small ligand directly coupled to the matrix always shows a lower affinity with the target protein than in their free state. To overcome this situation, spacer arms, typically linear molecules with different chain length, are used to bridge ligands and matrix. In general a spacer arm is necessary in coupling ligands  $M_w < 1000$ , and not need for larger ligands (Figure 12). An ideal spacer arms should have active groups at two ends by which it can be covalently coupled with matrix and ligand respectively. After coupling with matrix and ligand, the arms should be chemically stable to avoid reaction with other solutes and be hydrophilic to avoid the hydrophobic interaction with proteins.



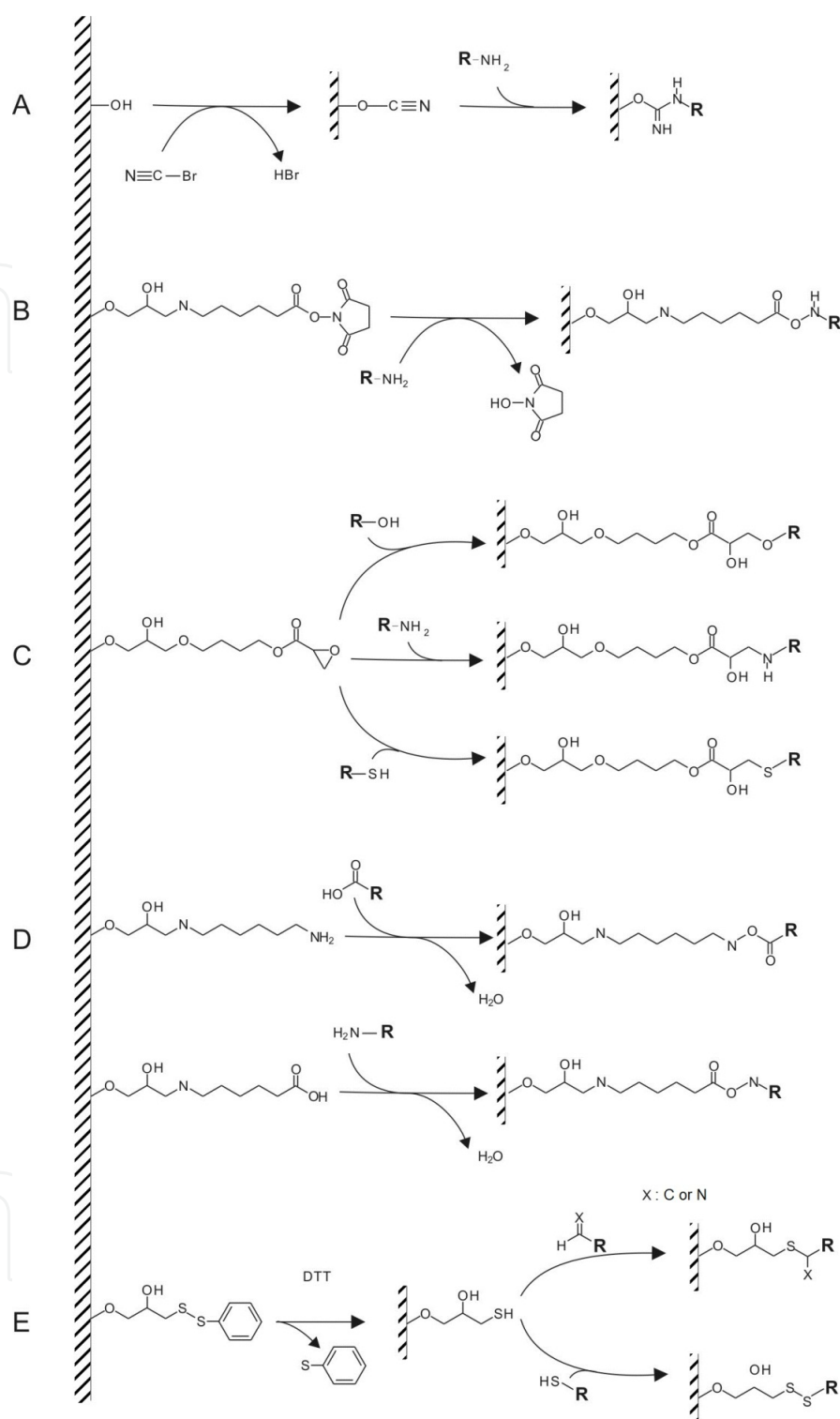
**Figure 12.** The influence of spacer arms on small or large ligands. A spacer arm is often necessary for coupling small ligands, which ensure an efficient binding between ligands and target proteins (A), but not necessary for large ligands (B).

The atom number of commonly used spacer arms varies from 4 to 12. They often couple with agarose matrix by stable ether links at one end and with ligand by other chemical bonds at the opposite end.

### 6.1.3. Ligand coupling

A coupling procedure of ligand is generally composed of three steps. First a group on matrix or spacer arm is activated by an activating agent. And then the activated group reacts with a functional group on ligand molecules. Finally, residual unreacted groups are blocked by blocking agent [23]. A matrix can be coupled with a ligand by a chemical group on itself or by groups on spacer arms. A variety of spacer arms are available to couple with functional groups on ligands such as amino, hydroxyl, carboxyl, thiol groups (Figure 13).

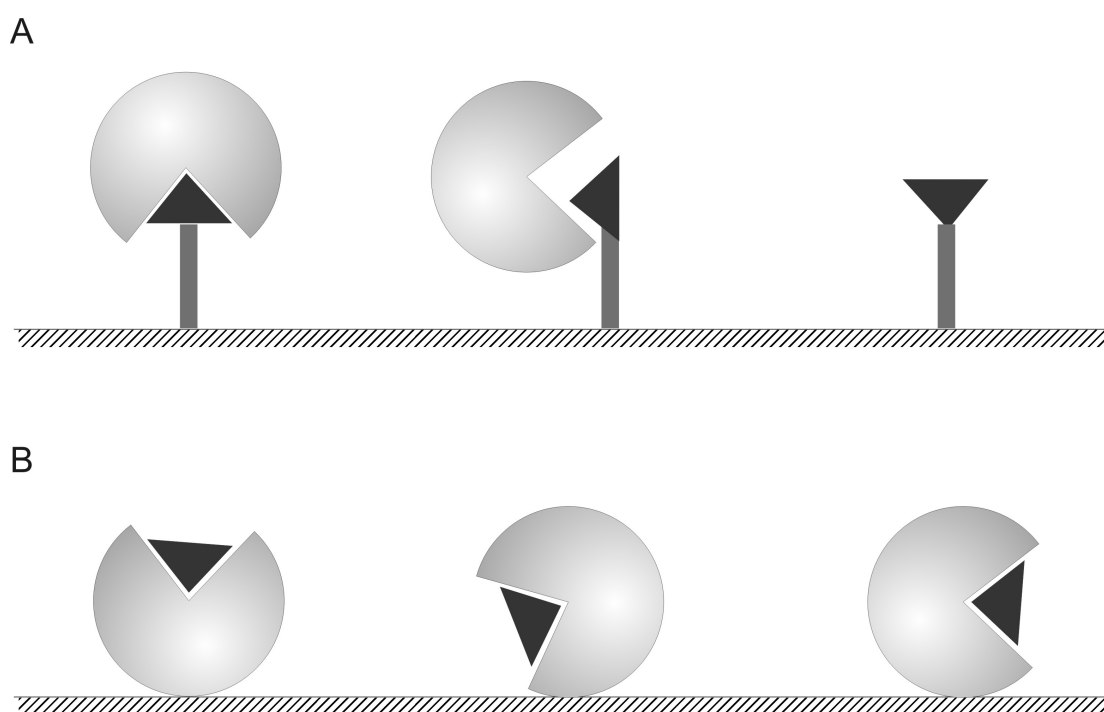




**Figure 13.** Commonly used spacer arms and immobilization procedures of ligands. (A) Ligands are directly coupled with matrix by reaction between Cyanogen bromide activated hydroxyl on matrix and amino group on ligand. (B) Ligands are coupled with spacer arms by reaction between N-hydroxysuccinimide activated carboxyl and amino group on ligand. (C) Ligands couple with spacer arms by reaction with epoxy group. (D) Coupling through condensation between a free amino and a free carboxyl group. (E) Coupling through bisulfide bond or additive reaction between thiol and double bond in ligand, such as  $N=N$  or  $C=N$ .

#### 6.1.4. Steric interference

For a small ligand, it should be paid attentions to the influences of steric interference even if a spacer arm has been used. For small ligands the amount of each functional group is rare. even just one. A bad choice that makes a wrong spatial orientation in coupling will likely cause a serious decrease in binding capacity or even complete failure. On the contrary, large ligands have several equivalent groups through which coupling takes place, so that a large proportion couplings leave sufficient space for binding with target molecules (Figure 14). Therefore in coupling a small ligand, it is important to choose a suitable functional group without introducing significant steric interference. The information of structure can be obtained from databases of X-ray crystal diffraction or NMR, or prediction by computational biology.



**Figure 14.** The influences of steric interference to small and large ligands. (A) For a small ligand, an inappropriate coupling orientation likely results in steric interference and inefficient adsorption. (B) This situation is less happened on large ligands.

## 6.2. Binding and elution

An ideal binding buffer should be optimized to ensure efficient interaction between target molecules and ligands and minimize the nonspecific interaction at same time. Since the ligand-protein interaction is a result of combination of electrostatic attraction, hydrophobic interaction and hydrogen bonds, the binding conditions can be optimized on these aspects.

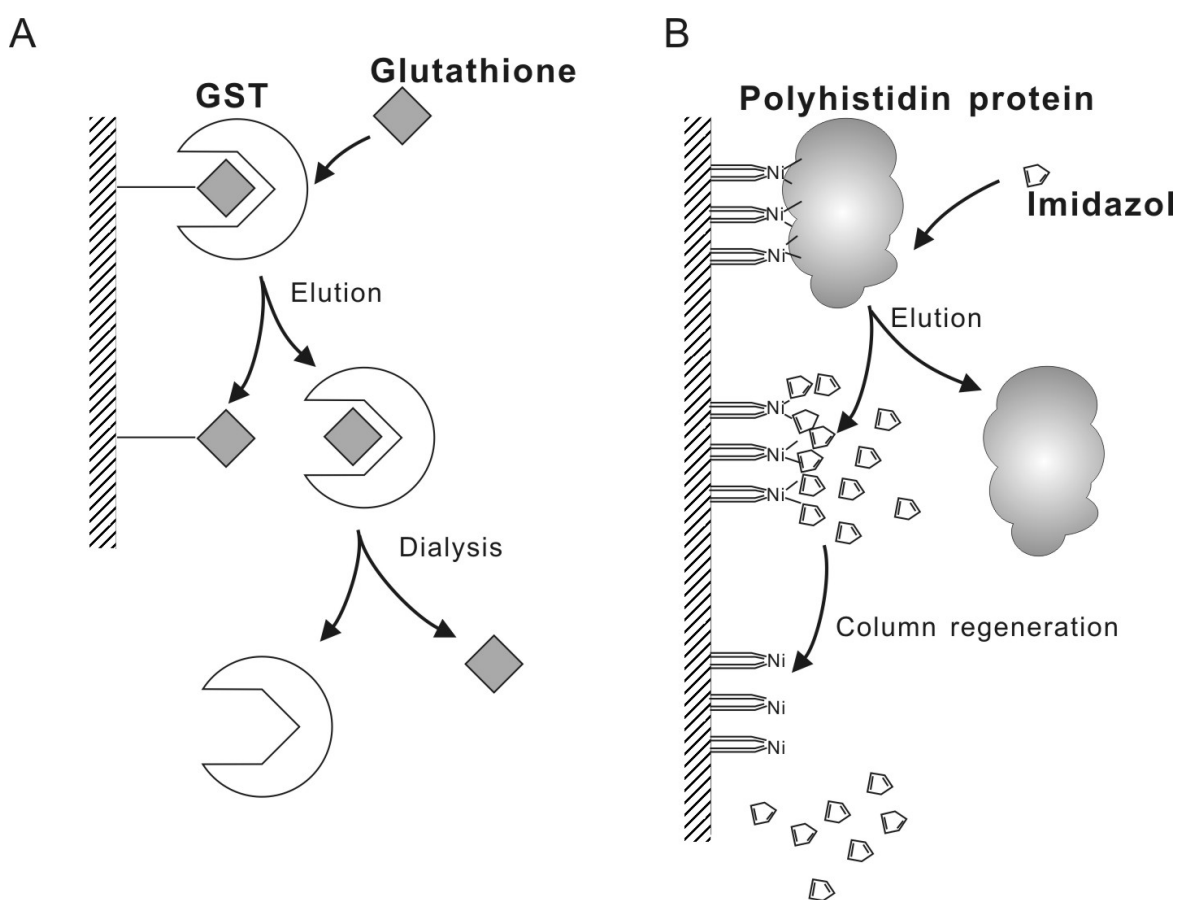
Adsorbed proteins could be eluted by modification of pH value, ionic strength, or polarity. pH value could be decrease to pH 2~3 to reduce the charge property of interaction surface between proteins. For example, immunoglobulin could be adsorbed by a protein A column and

eluted by a glycine buffer with pH 3.0. But the eluted sample should be neutralized as soon as possible to avoid being destroyed in extreme circumstance.

The ionic interaction also can be weakened by adding neutral salt, for example 1M NaCl is frequently used in practice.

A specific elution can be performed by adding competitors of either ligands or target proteins in elution buffer. An ideal competitor should have a moderate dissociation coefficient to the ligand or the target molecule, so that the competitor can elute target with high concentration but can be easily removed from column by wash or isolated from target protein by dialysis. Two classic applications is affinity chromatography of Glutathione S-transferase (GST) and polyhistidine [24] (Figure 15).

In binding process, flow rate should be control at a relative low degree to ensure an effective binding capacity.



**Figure 15.** Different elution mechanisms in GST affinity chromatography and metal chelate interaction chromatography (A) In GST purification, GST is captured by a medium with immobilized glutathione, and then dissociated by adding excess reduced glutathione. The excess glutathione is eluted together with target protein and removed by dialysis. (B) In nickel ion chelate interaction chromatography, protein with polyhistidine sequence is adsorbed by a medium with immobilized ionized nickel through a chelation between nickel ion and imidazolyl on polyhistidine sequence. The protein is eluted by adding high concentration of imidazol, a competitor of the imidazolyl on the protein. Finally, the small competitor is washed away from column by binding buffer.

### 6.3. Tag purification strategy

AC separates protein typically on the basis of interactions between ligands and local domains of target proteins. The interactions are not interfered by other domains in most case. Therefore, the tag purification strategy was invented to rapidly separate recombinant protein by fusion expression and co-separation [25].

First the target protein is expressed with a tag protein in fusion form. Then the target protein is purified using an affinity column that is specific to tag protein. After that if the tag needs to be removed, a restrictive protease is used to hydrolyze the fusion protein and the freed tag is finally be separated from target protein by running the same column once again.

The ideal tag protein should (1) have economical affinity chromatography media for convenient separation, (2) be very stable in bioactivity, and (3) have a good expressing property that is helpful to increase the expression of target protein. Commonly used tags are GST tag, FLAG tag, S tag, Strep tag, His tag, and so on.

### 6.4. Application

Affinity chromatography is a rapid and efficient chromatography technique. The high specific biorecognition give the technique an extremely high selectivity, by which a protein or a group of proteins could be separated from a crude sample in one step and reaches to a satisfying purify. However the excellent performance is based on the complex productive technology. Development of each noval medium needs a plenty of trials on finding suitable ligand and coupling the ligand on matrix properly. It is worth time and effort to develop a new specific affinity medium for high scale protein production, otherwise, the alternative method such as tag purification or other chromatography should be a better choice for small scale preparation in expiremental research.

## 7. Summary

This chapter introduces principles and applications of several basic chromatography techniques. Different techniques separate proteins depending on different properties including net surface charge, hydrophobicity, molecular size, and affinity interaction. Affinity chromatography has the highest selectivity and can purify target proteins in one step to >95% purify. But due to the difficulties on obtaining and immobilization of suitable ligand, this chromatography technique is not used as widely as other ones. HIC and RPC are both based upon hydrophobic interaction. PRC is widely used in analytic separation because of its high resolution, but less used on preparative separation of proteins since the high nonpolarity of the eluent likely causes irreversible inactivation of proteins. IEXC, HIC and SEC separate proteins in mild conditions and are suitable for large scale separation of active proteins. However, their resolutions are comparatively lower and hard to purify a protein from complex components by a single technique. An ideal purification could be achieved by combined application of several techniques.

## Author details

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