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AFFINITY LIQUID CHROMATOGRAPHIC SYSTEMS

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INTRODUCTION

Among all the chromatographic techniques, affinity chromatography, developed during the 1960's and 1970's, is one of the most powerful. Using this technique, it is possible to purify compounds ranging from small molecules to entities such as viruses and cells. In principle, affinity chromatography uses biospecific and reversible recognition between molecules. Affinity interactions can be classified into two broad categories: group specific and highly specific. Examples of the former are the binding of the triazine dyes to enzymes that contain nu-

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cleotide binding regions or concanavalin A with sugar moieties of glycoproteins. Examples of the latter are the binding of enzymes with inhibitors and substrate analogues or antibody and antigen interactions. Because of the high specificity of these interactions it is quite common to get a thousand-fold purification with close to 100% recovery in a single step.

Affinity chromatography has proven to be extremely useful to the biotechnology community because many biological samples are complex mixtures of large polymers. The complex mixtures are made up of proteins, carbohydrates, lipids, and nucleic acids. Affinity chromatography has sufficient resolution to separate closely related solutes because of the specific interactions with groups on these complex molecules. In most cases affinity chromatography is done in aqueous solutions and under mild conditions, so that the biological samples maintain their activity. Affinity ligands can bind dilute samples in large volumes so that elution into a small volume results in significant increases in concentration. The large number of publications shows the popularity and utility of affinity chromatography. This article will guide the reader to the general principles of affinity chromatography and provide an overview of the major forms of affinity chromatography. Extensive reviews and books written on affinity chromatography include the original references that contain the technical details and extensive applications of affinity chromatography.[1–7]

TECHNICAL PRINCIPLES

In affinity chromatography the ligand, which is capable of specific recognition and reversible binding of the compound of interest to be purified, is attached to the solid chromatographic matrix and packed into a column. The process of affinity purification consists of the following steps as illustrated in Figure 1: equilibration of the column with the loading buffer; loading the column with the sample mixture; washing off the unbound material; elution and recovery of the compound of interest; and regeneration of the chromatographic support to the original condition. If an affinity chromatography medium is not commercially available for your particular target compound, an affinity chromatography medium and a purification protocol can often be developed.

Instrumentation

Affinity liquid chromatography requires the basic components of a chromatography system. Figure 2 shows the components of a typical liquid chromatography system. The selection of the components depends upon the particular

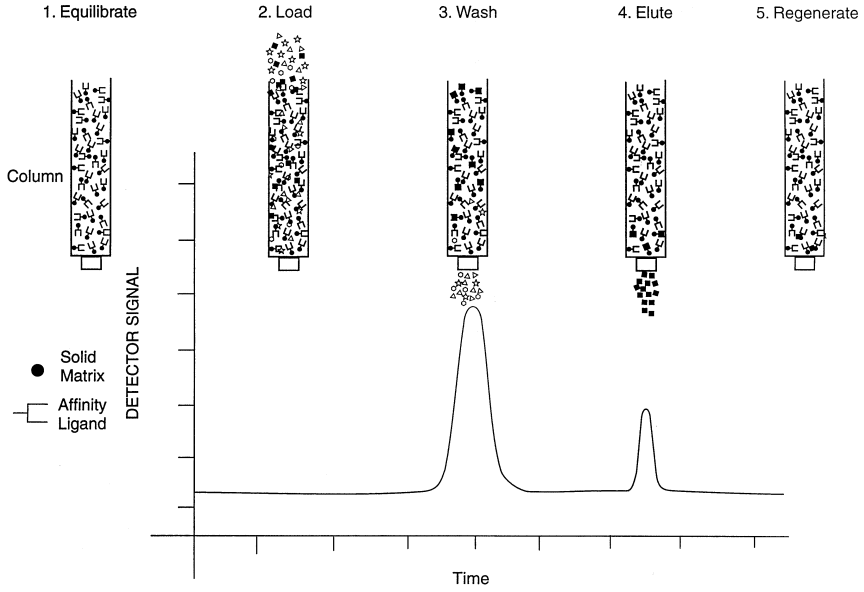


Figure 1. An illustration of the main steps in affinity chromatography.

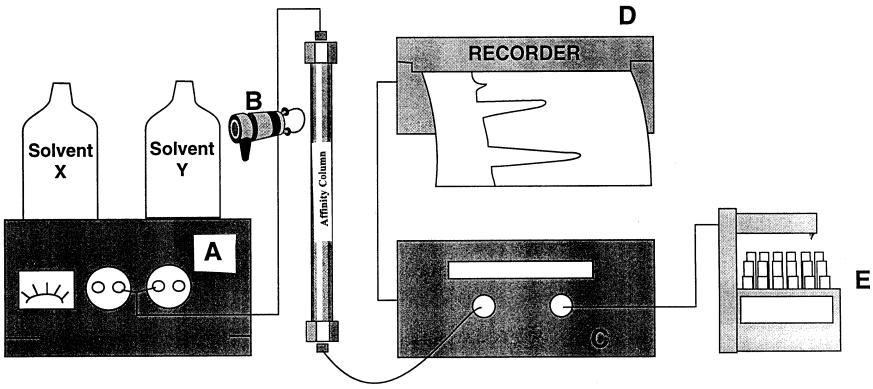


Figure 2. Components of an affinity chromatography system. A: pump. B: injector. C: detector. D: recorder. E: fraction collector.

application. Affinity chromatography can be done very simply using gravity flow in a column packed with large diameter packing materials and fractions collected manually. The fractions can then be analyzed for the specific compound of interest using other techniques. Affinity chromatography can also be done using a commercially available liquid chromatography system. We will cover the important features of each component to guide the selection for a particular application.

Pumps

The simplest arrangement is to use hydrostatic pressure to establish buffer flow, but this is limited to low pressures and the flow rate will vary with the resistance of the column. Compressed gases can be used with a pressurized solvent vessel, but these systems also suffer from the variations in flow due to any changes in column resistance. There are also safety concerns when using pressurized vessels, and if the gas dissolves in the solvent, degassing can occur in the detector resulting in a noisy baseline. Mechanical pumps are the most convenient way to control the flow. The most popular types of mechanical pumps for liquid chromatography include peristaltic pumps, syringe pumps, and piston pumps.

Peristaltic pumps are simple in construction and popular for low pressure applications. Peristaltic pumps have a circular head containing a variable number of rollers that compress a section of fixed tubing. The space in the tubing trapped between two rollers contains a section of fluid. When the pump head rotates the rollers trap a new section of fluid that is drawn into the pump head and a section of fluid is released at the outlet. The result is that the tubing remains stationary and fluid is moved through the pump in sections (pulses). Since the tubing is alternatively compressed and released, elastic types of tubing have the longest lifetime and the most reliable results. Peristaltic pumps are available in a variety of sizes from analytical applications (microliters per hour) to large industrial pumps (thousands of liters per hour). An advantage of peristaltic pumps is that the fluid does not contact any moving parts of the pump. This feature allows the use of corrosive fluids and fluids containing particulates. Contaminated fluid paths can be easily replaced by changing the tubing. Peristaltic pumps are relatively gentle to shear-sensitive substances compared to other pump types, such as piston pumps. The speed of rotation, the number of rollers, the tubing diameter, and the elastic properties of the tubing determine the flow rate of peristaltic pumps. Disadvantages of peristaltic pumps include their limitations for low-pressure applications, variations in flow rate due to differences in column resistance, and pulses in the flow. Pumps with greater numbers of rollers have pulses of lower magnitude. The pulses in the flow can be reduced by the use of commercial pulse dampeners or by the use of multi-channel pumps arranged so that where pulses tend to cancel each other out. A coil of elastic tubing before the injector will also function as a simple pulse dampener.

Syringe pumps are syringes with motor driven pistons. These pumps deliver very steady flows without pulses. One design of syringe pump uses two syringes with one syringe filling while the other is pumping. A valve switches flow from one syringe to the other at the end of the cycle to maintain a constant flow. Solvent compressibility of the solution inside the syringe can lead to flow inaccuracies at higher pressures with some solvents. The volume of solution in the syringe can also make solvent changes inconvenient.

Piston pumps use inlet and outlet check valves and are suitable for high pressure applications. The pump heads contain small volumes that can be compressed to high pressures (up to approximately 40 MPa). The flow rates of these pumps are determined by the stroke volume and piston velocity. One configuration is to have two pump heads with one head filling, while the other head is pumping. A single pump head with a rapid fill stroke can be used and decreases the number of moving parts in the pump. Piston pumps must be used with pulse dampeners. Because of the check valves and small diameter tubing used, the solvent and samples must be free of particulates. This is most easily accomplished by filtration through a filter (0.45 micron or less) or by centrifugation. Filtration also prevents the column inlet frits from becoming plugged. The disadvantages of piston pumps include their complexity (large number of moving parts), expense, and susceptibility of stainless steel to corrosion. High-pressure pumps made with corrosion-resistant materials are available for higher costs.

Injectors

Injectors function to introduce the sample to the inlet of the column. Large sample volumes are sometimes used in affinity chromatography because it is possible to concentrate large volumes of dilute samples. Analytical applications use smaller volumes. A septum injector is used with a needle and syringe. Different size syringes can be used to change the sample volume injected. The septum injector is limited to low pressure applications.

A popular design of injector uses a valve system with a rotary seal to switch the solvent paths between a loop and a bypass. A sample loop is filled with a syringe when the column buffer flow is diverted from the sample loop. The rotary seal is turned to inject the contents of the loop into the fluid path that carries it to the top of the column. The loop has a fixed volume but can be changed to different sizes. The loop can be filled entirely or partially with the sample. These injectors are available in either stainless steel for high pressure applications or plastic for lower pressures. An injector for preparative systems could consist of valve systems that switch the fluid stream to a pump that injects the sample onto the column and then back to the buffer.

Detectors

A commonly used detector for affinity liquid chromatography is an ultraviolet-visible wavelength absorbance detector. These detectors offer the best combination of sensitivity, flexibility, and value. The sensitivity will depend upon the molar absorptivity of the particular component that is being measured. The absorbance detector should have a wavelength range of 200 nm to 800 nm, which will cover the λ_{max} (wavelength of maximal absorption) for most compounds. Many proteins and peptides that contain aromatic amino acids (phenylalanine, tyrosine and tryptophan) are detected with highest sensitivity at 280 nm. Proteins and peptides, which lack aromatic amino acids, can be readily detected at 210 to 220 nm due the absorption of the peptide bond. It should be noted, however, that many buffer components used in affinity chromatography absorb in the low UV and are not suitable for use at these wavelengths due to high background noise. An absorbance spectrum should be run to determine the wavelength for optimal absorbance, if it is not known. Photodiode array UV-visible detectors are capable of continuous or periodic scanning of a wide wavelength range during chromatographic elution. These detectors are useful for samples that have mixtures with compound that absorb at different wavelengths and the spectrum data helps to identify peaks.

Other detectors should be used when the compound of interest does not absorb in the UV-visible range or when structural information is needed. Other detectors used for liquid chromatography include fluorimetric, refractive index, conductivity, electrochemical, and mass spectrum. Flurometric detectors are available for compounds that are fluorescent or can be tagged with fluorescent moieties. Refractive index, light scattering, and conductivity detectors measure a bulk property of the solvent can be used for compounds that do not absorb in the UV-visible range. Less commonly used detectors include radioactivity, infrared, optical activity, and viscometry. These detectors are selective for specific properties of certain samples.

Mass spectrometric (MS) detectors are becoming popular with liquid chromatography now that major instrumentation companies have developed tabletop models. These detectors can be used to determining the structure and sequence of peptides and proteins as the peaks elute. MS detectors are very useful to help identify unknown peaks in complex samples. It is necessary to have an interface to combine the liquid chromatography solvent stream to the mass spectrum. The buffers and solvents used for the liquid chromatography run should be compatible with the MS detector.

Recorders and Collection of Data

Strip chart recorders offer a low cost method to record data from a single or a few channels. The data collected with a strip chart recorder cannot be retrieved

and displayed at different scales. Computers allow the easy storage, retrieval, and manipulation of data on chromatographic runs. Data such as pump performance (pressure and flowrate), solvent pH, solvent conductivity can be stored and later reviewed for documentation and analysis. Such data is valuable when troubleshooting chromatography runs. Software is available for the comparison of multiple chromatography runs and the integration of peaks.

Fraction Collectors

A number of configurations are possible for fraction collectors. Either the tubes are moved beneath a fixed dispensing head or the dispensing head moves to a fixed array of tubes (or containers). The important considerations for fraction collectors are the tube size (determines range of fraction volumes), the number of tubes (total capacity of fractions), collection modes available, and the amount of automation possible. The collection modes include collecting by time interval, counting drops, or collection by peaks. Collection by drops assures a constant volume of each fraction, which is advantageous when the flow rate may vary due to changing conditions. Collection by peaks requires that the fraction collector be in communication with a detector. A threshold can be set that will trigger the beginning or end of a fraction. The threshold must be set so that only real peaks and not noise trigger fraction collection. A fraction collector for a preparative application could consist of a valve system controlled by a computer to "cut" the peak of interest into a collection vessel and then divert the other components to flow to a waste container.

DEVELOPMENT OF AN AFFINITY CHROMATOGRAPHY MEDIUM

The development of an affinity chromatography separation medium consists of the following four main steps:

1. Choosing a chromatographic support.
2. Activation of the support and ligand attachment.
3. Blocking of the remaining active groups.
4. Development of a purification protocol.

Step 1. Choosing a Chromatographic Support

Chromatographic media are available in a variety of chemical compositions and forms. We will discuss some general principles to guide the selection of a support for a specific application. An ideal affinity support should possess good me-

chanical and chemical stability, give reproducible results, be stable in aqueous and organic solvents, and it should minimize the retention of any component of the sample in a nonspecific manner. Such supports must also contain functional groups for facile activation and ligand attachment.[5,6,8]

To achieve the high purity levels desired in affinity separations, it is important to avoid nonspecific binding of other sample components on the chromatographic matrix. Such nonspecific interactions are often hydrophobic or ionic in nature. Hydrophobic interactions can occur between the nonpolar groups in the sample and the support matrix or the spacer arm (see below) used for the attachment of the ligand. Ionic interactions are also possible between the charged groups in the sample and any ionic groups present on the solid support, in the spacer arm, or any that are introduced by the immobilization process. While ionic interactions can be suppressed by using high ionic strength buffers (0.2 mol/L to 1.0 mol/L), these conditions can also enhance the hydrophobic interactions. In order to avoid coelution of the nonspecifically bound components along with the compound of interest, elution conditions which specifically desorb the bound compound of interest should be used. Such conditions include the use of soluble forms of the attached ligand, substrates, or inhibitors or substrate analogues.

In the early development of affinity chromatography, agarose was the most popular solid support. The advantages of agarose include high hydrophilicity, porous structure, low incidence of nonspecific interactions, and stability to aqueous mobile phases. However, it is not stable to organic solvents and high temperatures. Other disadvantages of agarose include susceptibility to microbial degradation, low binding capacity, and limited flow rates. To overcome these disadvantages, other synthetic and natural gel matrices have been developed (Table I).

Many of these solid supports are soft gels and can be used only in low pressure chromatography. Often affinity chromatography procedures involve macromolecules, such as proteins and polynucleotides, either as ligands or components of a complex mixture to be purified. In either case, these macromolecules have to diffuse into the pores of the chromatographic matrix to achieve good binding capacities and separation. For these reasons, the support should have pore diameters $\geq$300 Å. To achieve high binding capacities, the gels should also have a high density of the ligand immobilized in a functional form.

High Performance Liquid Affinity Chromatography makes use of either rigid silica or controlled-pore glass beads.[9,10] Silica supports can tolerate pressures over 40 MPa. Other advantages of silica supports are facile activation and availability in a range of particle sizes, surface areas, and pore diameters. The surface chemistry of silica has been extensively studied. A common method of activation is epoxy chemistry, and amino propyl silica gels can also be used for ligand attachment. Table I illustrates some of the variety of supports available from various suppliers.

Table I. Particulate Support Materials for Affinity Chromatography

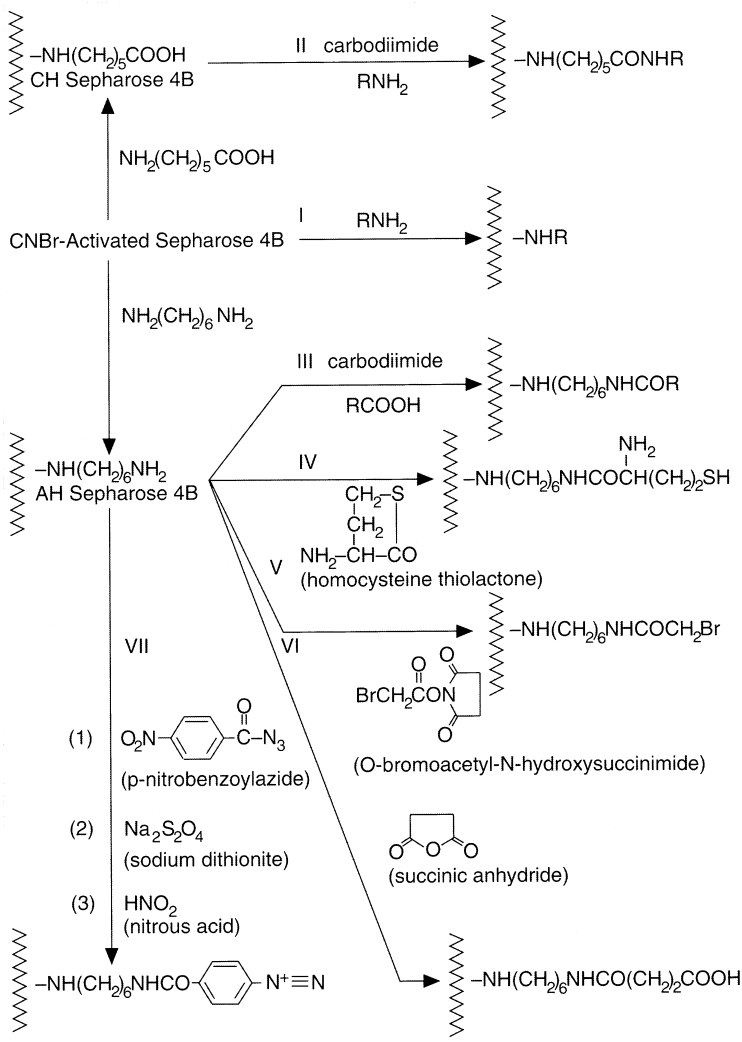
Trade name	Supplier	Support material
Low to medium pressure		
Trisacryl®	IBF, France	Totally synthetic
Ultrogel®	IBF, France	Polyacrylamide/agarose
Sepharose®	Pharmacia LKB, Sweden	Agarose
Sephadex®	Pharmacia LKB, Sweden	Crosslinked dextran
Sephacryl®	Pharmacia LKB, Sweden	Polyacrylamide/dextran
Macrosorb™	Sterling Organic, UK	Various organic powders
Eupergit® C	Roehm Pharma, FRG	Polyacrylamide
Affi-gel®	Biorad, USA	Crosslinked polyacrylamide
Matrex Cellufine™	Amicon, USA	Crosslinked cellulose
CPG	Pierce, USA	Controlled pore glass
Bakerbond Wide-Pore™ (Prepscale™)	J. T. Baker Inc., USA	Polymer-clad silica
Synthetic		
Dynospheres®	Dyno Particles, Norway	Synthetic polymer
Separon® H 1000	Tessek, Czechoslovakia	Synthetic polymer
TSK PW®	Tosoh Inc., Japan	Polymer
Fractogel®, TSK/Toyopearl®	E. Merck, FRG	Vinyl polymer
High pressure		
Hypersil WP300™	Shandon, UK	Silica
Lichrospher®	E. Merck, FRG	Silica
Ultraspere™	Beckman, USA	Silica
Spheron™	Waters, USA	Silica
Superose®	Pharmacia LKB, Sweden	Crosslinked agarose
Zorbax®	E. I. Dupont, USA	Silica

Work from reference 8, used by permission.

Step 2. Activation of the Support and Ligand Attachment

Hydroxyl or other functional groups (such as amides on polyacrylamide gels) that are present on many of the chromatographic matrices can be used for activation and subsequent ligand attachment. Cyanogen bromide activation is a popular method, but other, more efficient and convenient methods have been devel-

Table II. Reactions That Can Be Used to Couple Ligands to Sepharose and Other Affinity Supports



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oped. Table II shows some of the reactions that have been used to couple ligands to chromatography supports. The choice of immobilization chemistry is determined by the available groups on the ligand and the support material as well as the reaction conditions that can be used tolerated by a particular ligand. For instance,

a protein would require milder conditions in the coupling step compared to a small organic molecule.

In attaching the ligand to the surface of a solid matrix, one should also consider the impact of steric effects on affinity binding and separation. If a macromolecule (such as a protein or DNA) is attached very near to the surface of the matrix to be used as a ligand, then steric hindrance may prevent the binding of large or even small molecules to this ligand. Similarly, if a small molecule ligand (such as an amino acid or sugar) is bound very near the surface of the matrix, then a macromolecule can be inhibited from binding to it because of steric hindrance. For these reasons it is customary to use a spacer arm between the matrix and the attached ligand.

Spacer arms can be of different lengths and chemistries.[6] The most important aspect of the spacer arm is that it should not contribute to nonspecific interactions with any component of the sample. Table III illustrates some of the spacer arms that are used in affinity chromatography. Two routes are possible for the incorporation of the spacer arm. First, the spacer arm can attached to the matrix followed by the attachment of the ligand to the spacer. Second, the spacer can be incorporated into the ligand and then attached to the gel matrix. The most commonly used spacer arms contain alkyl chains, if the side chains are too long hydrophobic interactions with sample components can result.

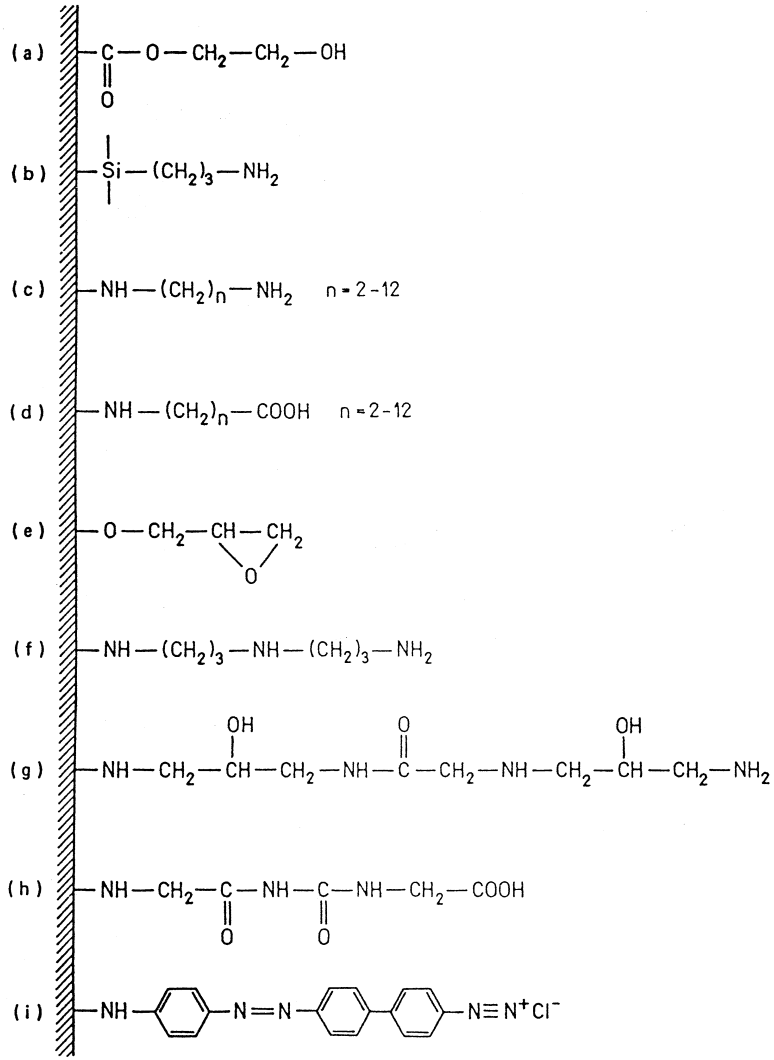
As discussed above, attachment of the ligand to the matrix should allow optimal interaction between the ligand and the sample. A good example is immunoaffinity chromatography. The use of a general activation and bonding chemistry results in a variety of possible orientations of the immunoglobulin on the gel surface (Figure 3). Some of these orientations lead to sterically hindered or inactive antigen binding sites. However, in a site-directed immobilization procedure, the antigen binding site is free to interact and leads to much higher specific binding capacity.[11,12]

Step 3. Blocking of the Remaining Reactive Groups

Once the matrix is activated and the ligand attached, it is important to deactivate or block the remaining active sites. Otherwise, these sites may react with the components of the sample and lead to surface heterogeneity. Depending on the activation chemistry used, different blocking reagents are available for deactivation. For example, cyanogen bromide activated gels can be deactivated with 2-aminoethanol, ethylamine, or glucosamine. The blocking groups should also have low nonspecific binding.

It should be noted that many activated supports for ligand attachment are commercially available. Leaching of the ligand or the blocking group is an important consideration and should be measured and controlled by careful selection

Table III. Structures of Some Spacers Used in Affinity Chromatography



The details of the different structures resulting from different spacer groups and activation chemistry are contained in the source of this figure, reference 6, used by permission.

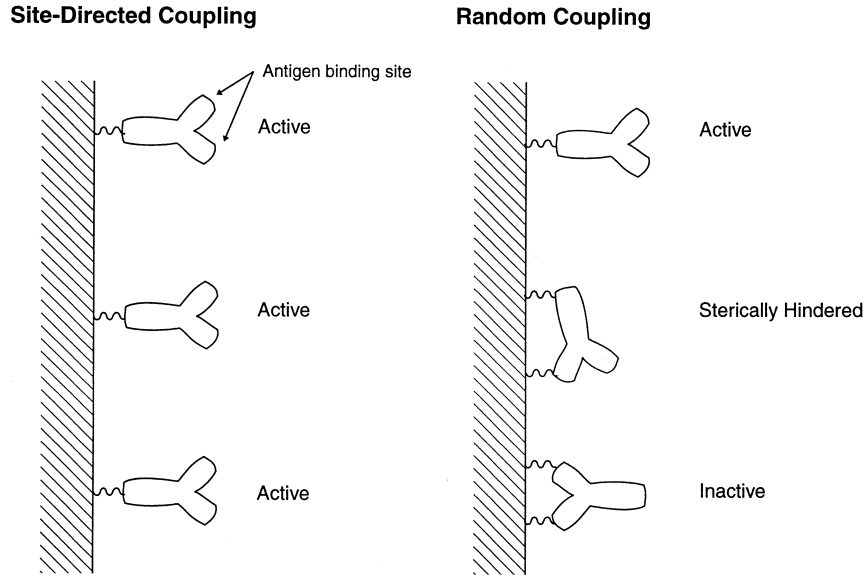


Figure 3. Immunoaffinity chromatography, effect of site-directed and random attachment on antigen binding.

of the operating and storage conditions. This is especially important for the purification of products used for therapeutic uses.

Step 4. Development of a Purification Protocol

There are two important steps in the purification protocol: developing the optimal loading conditions and developing the optimal elution conditions. The column should be thoroughly equilibrated with the loading buffer before sample application. The loading conditions are chosen such that they maximize the strength and specificity of binding of the target molecule to the affinity ligand. This depends on the properties of the loading buffer, such as ionic strength, pH, and the components of the buffer. These parameters should be carefully examined and optimized. The sample is dissolved in or changed into the loading buffer before applying to the column. For tightly bound compounds, the sample volume may not be critical. However, weakly bound compounds should be applied in small volumes (no more than 5% of the column bed volume). Low flow rates should be used in order to allow sufficient time for the component to bind to the ligand. After loading, the column should be washed with the starting buffer in order to remove the unbound components.

The elution conditions in affinity chromatography are of two types: selective and nonselective. Selective conditions can be used where group-specific interactions are involved or when the binding constants are low (K_d between 10^{-5} mol/L to 10^{-3} mol/L). In such cases the elution buffer contains compounds that compete for the affinity ligand on the column. Examples are: the use of substrate or substrate analogues in the elution of enzymes, the use of histidine or imidazole in metal affinity chromatography, and the use of a free sugar to elute proteins bound to a lectin affinity column. Nonselective elution conditions are generally employed for highly specific (tightly bound) affinity interactions. Such elution conditions include a change in pH or ionic strength of the elution buffer and a change in temperature. Nonselective conditions can lead to the coelution of nonspecifically bound compounds.

EXAMPLES OF DIFFERENT FORMS OF AFFINITY CHROMATOGRAPHY

Affinity chromatography is a diverse subject and many types of affinity interactions can be used for separations using liquid chromatography. Table IV shows the major forms of affinity chromatography that are in common use. We will briefly highlight each type and discuss the applications. Applications for each type of affinity chromatography are discussed in each section.

Biosorption Affinity Chromatography

Biosorption affinity chromatography uses the principles of molecular recognition that biological molecules have developed. There are many examples of molecular recognition, such as enzymes binding to cofactors, substrates, and effectors that regulate their activity. Molecular recognition is fundamental to almost all biological processes and can be utilized for affinity chromatography.[1,13] Naturally occurring compounds as well as synthetic compounds such as transition-state analogues (for enzyme reactions) can be used. A few of the many ligands used to for biosorption include small molecules like amino acids, peptides, carbohydrates, nucleotides, cofactors, substrates, and inhibitors. Examples of large complex molecules include proteins, oligosaccharides, and nucleic acids.

Immunoaffinity chromatography uses antibodies as ligands. Antibodies can be developed that recognize small differences in conformation and modifications of single amino acids (i.e. post-translation modifications) in large, complex proteins. Many antibodies bind very tightly to their antigens and this can a problem during the elution step. The release of bound targets may require harsh conditions such as low pH or the addition of protein-denaturing agents. When using monoclonal antibodies it is usually possible to select for antibodies that are weaker

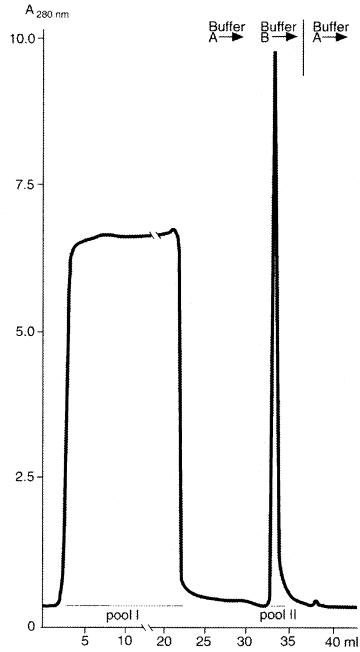
Table IV. Major Forms of Affinity Chromatography

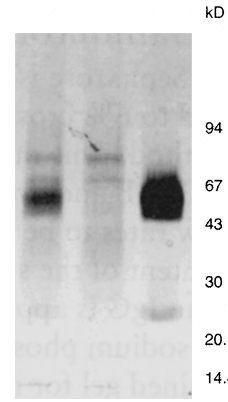
Type	Typical Ligands	Applications (Targets)
Biosorption	proteins, peptides, amino acids, nucleic acids, receptors, carbohydrates, nucleotides	proteins, peptides, nucleic acids, cells, viruses, cellular organelles, carbohydrates, glycoconjugates, oligonucleotides
Dye Ligand	Cibacron Blue F3G-A	nucleotide-binding proteins, blood proteins, other proteins
Immobilized Metal Ion	iminodiacetic acid chelated with Zn^{+2} or Cu^{+2}	proteins, peptides
Hydrophobic Interaction	ether, isopropyl, phenyl groups	proteins, peptides
Covalent Chromatography	activated thiol group	proteins, peptides, mercurated nucleic acids
Charge Transfer Adsorption	π-electron donors or acceptors	amino acids, peptides, nucleotides, oligonucleotides
Thiophilic Adsorption	sulfone group in close proximity to a thioether	immunoglobulins, other proteins

binders. The use of weaker-binding antibodies allows milder elution conditions to be used, which increases the life of the affinity column and preserves the biological activity of the proteins being purified. The role of binders and ligands can be easily reversed using affinity chromatography. For instance, antibodies that are specific for an antigen can be enriched from antibody mixtures by immobilizing the antigen and using it as the ligand in an affinity column. The nonspecific antibodies are washed off the column and an elution step will yield antibodies that bind specifically to the antigen.

Protein A and protein G are proteins derived from bacteria that specifically bind to the Fc region of many immunoglobulins. Proteins A and G exhibit significant differences in their affinity for a particular subclass of immunoglobulins and differences are seen depending upon the animal species.[3] Figure 4 shows an example of protein G used to separate immunglobulin IgG from a crude mixture. Immunoglobins are generally bound to immobilized protein A or G at a pH near neutrality and are eluted under acidic conditions.

Sample:	20 ml rat hybridoma cell culture fluid
Column:	HiTrap Protein G 1 ml
Flow rate:	0.5 ml/min
Buffer A:	20 mM sodium-phosphate pH 7.0
B:	0.1 M glycine-HCl pH 2.7
Chromato-graphic procedure:	1 ml buffer A. 20 ml sample, 8 ml buffer A 6 ml buffer B, 5 ml buffer A. The eluted fractions were neutralized with 1 M Tris
Electro-phoresis:	SDS-PAGE PhastSystem PhastGel Gradient 10–15 1 µl sample, silver stained





Lane 1: Rat hybridoma cell culture fluid reduced, diluted 1:10
Lane 2: Pool I, reduced, diluted 1:10
Lane 3: Pool II, purified monoclonal rat IgG, reduced, diluted 1:5

Figure 4. Purification of monoclonal rat IgG using HiTrap Protein G. (Work from Pharmacia LKB Biotechnology, Uppsala, Sweden). Property of Pharmacia Biotech, Inc., used by permission.

Lectins are a group of proteins that bind specifically to carbohydrates or groups of carbohydrates. Immobilized lectins are used to purify carbohydrates (including oligosaccharides), proteins that have carbohydrates attached to their surface (glycoproteins), cells, organelles, and other glycoconjugates. Lectins with a variety of specificities are available for use as ligands for affinity chromatography. The mixtures to be separated using lectin affinity columns are generally loaded in a buffer at neutral pH containing some salt to reduce nonspecific binding. The column is washed, and then the bound glycoconjugates or carbohydrates

are usually eluted using a carbohydrate that competes with binding to the lectin.[3,14]

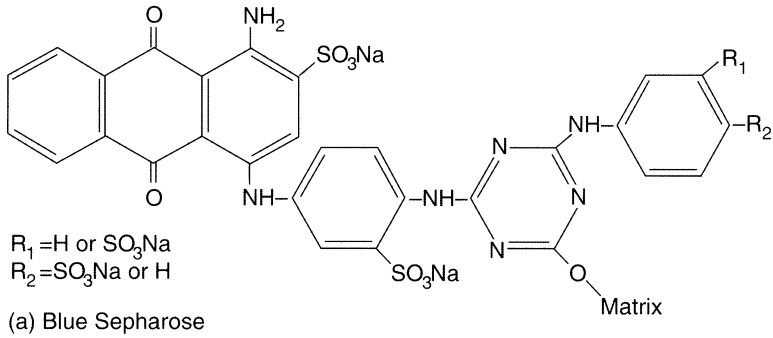
Nucleic acids can be used as affinity ligands for the purification of other nucleic acids and for proteins that associate with nucleic acids. Purification of nucleic acids using immobilized nucleic acids depends upon the specificity of base pairing between the ligand and the target sequence. Single-stranded nucleic acid fragments or synthetic oligonucleotides are immobilized and then a mixture of nucleic acids is added. The temperature, ionic strength, and pH are variables that need to be carefully controlled to insure fidelity of base pairing between the ligand and the target nucleic acid sequence. The unbound nucleic acids are washed off the column and generally the ionic strength is decreased or the temperature is increased to elute the specific nucleic acid sequences. A commonly used nucleic acid affinity chromatography step is to purify messenger RNA (mRNA).[15,16] The ligands employed are either oligo d(T), containing generally 12 to 18 thymine bases or poly (U), containing approximately 100 bases. Most eukaryotic mRNA molecules contain poly-adenine tracts on their 3' ends that will base pair to the immobilized d(T) or poly (U) ligands.

A variety of proteins that are responsible for the modification of DNA (i.e. restriction endonucleases), the regulation of the transcription, the repair, and the replication of DNA have been purified using sequence-specific nucleic acid affinity chromatography.[15, 17–19] Proteins that bind single-stranded and double-stranded nucleic acids can also be purified using the appropriate ligands. Many of the proteins that modify, regulate, repair, and replicate nucleic acids are present at low concentrations in cells and are extremely difficult to purify using standard protein purification methods. Affinity chromatography using nucleic acid ligands has proven to be essential for the enrichment and isolation of these relatively rare proteins. Reducing non-specific binding of proteins to affinity ligands becomes increasingly important when isolating proteins that are found at low abundance.

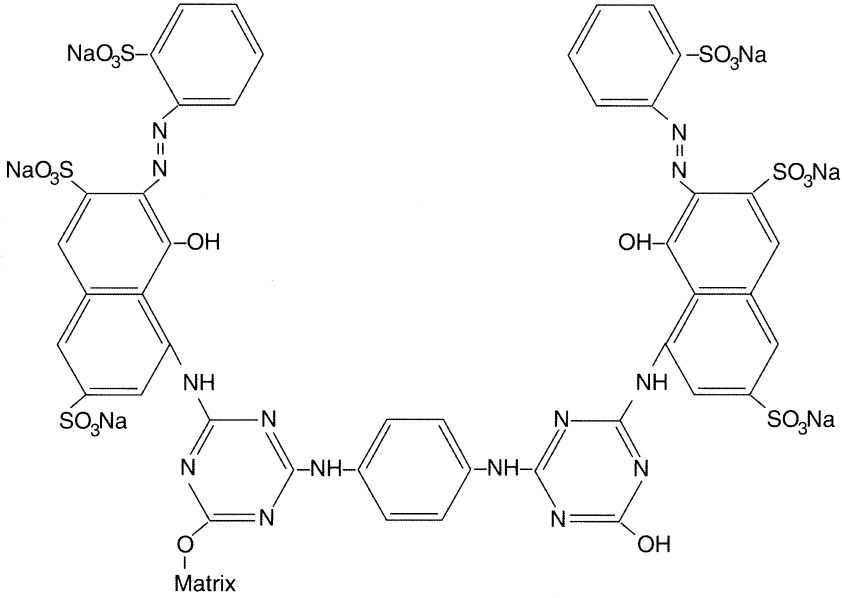
Dye-Ligand Chromatography

The traizine reactive dyes are a class of affinity ligands with diverse chemical structures that have proven useful for protein purifications. The triazine reactive dyes are made in large-scale for the dying of fabrics, but have been shown to bind to specific groups of proteins. The reactive dyes are easily immobilized to a variety of substrates using the reactive chloride(s) on the heterocyclic triazine ring.[20] A widely used dye in this class is Cibacron Blue F3G-A. Cibacron Blue F3G-A has been used as an affinity ligand for the purification of a wide variety of proteins. Dye ligand affinity chromatography has been used for proteins containing nucleotide-binding sites such as kinases and dehyrogenases, serum albumin, lipoproteins, and blood coagulation proteins.[3,21] The complex aromatic ring struc-

ture of the dyes offers the possibility of hydrophobic interactions, charge-transfer interactions, and cation-exchange mechanisms. Figure 5 shows the structure of Cibacron Blue F3G-A and Procion Red attached to a gel chromatography medium. The binding of the dye to kinases and dehydrogenases (those classes of



(a) Blue Sepharose



(b) Red Sepharose

Figure 5. Partial Structure of (a) Blue Sepharose Fast Flow, Blue Sepharose High Performance and Blue Sepharose CL-6B, and (b) Red Sepharose CL-6B. Property of Pharmacia Biotech, Inc., used by permission.

proteins that use the nucleotide cofactors, NAD^+, $NADP^+$, and ATP) can be reversed by the addition of low concentrations of these cofactors suggesting that the binding is specific at the cofactor binding site. Other proteins require high concentrations of cofactors or salts to cause their release from the dye, indicating that the immobilized dyes can also function as cation exchangers.

Other members of the reactive dye groups have been used with success to purify a variety of different proteins.[20] Analogues of Cibacron Blue F3G-A have been made that have different apparent affinity constants for horse liver dehydrogenase.[21] This opens the possibility of engineering specific dye affinity ligands for the purification of specific proteins.

Immobilized Metal Ion Affinity Chromatography

Immobilized Metal Ion Affinity Chromatography (IMAC) was first utilized for the separation of serum proteins[22], but is now being used for an increasing number of different proteins and peptides. This type of affinity chromatography is based on the ability of transition metal ions to form specific coordination complexes with histidine and cysteine on the surface of these proteins. The metal ions are immobilized by chelation to the chromatography media. In the original report for the purification of proteins, the metal ions (Zn^{+2} and Cu^{+2}) were immobilized to agarose beads using iminodiacetic acid via a spacer arm.[22] At pH's around neutrality the interactions are specific for histidine and cysteine, but at alkaline pH's, interactions with amino groups can occur resulting in less specific binding. It is important to load proteins in buffers of sufficient ionic strength to suppress any ionic interactions between the protein and the affinity complex.

Besides the divalent transition elements, Zn^{+2} and Cu^{+2} , other metal ions, including Co^{+2}, Ni^{+2}, Ca^{+2}, and Fe^{+3}, have been used. [23, 24] Elution of bound proteins can be accomplished either using changes in pH or the addition of histidine, imidazole, or some other compound that competes for the binding of the metal ions on the column.[23, 24]

Hydrophobic Interaction Chromatography

Hydrophobic interaction chromatography (HIC) is based on the association of hydrophobic (nonpolar) groups on the surface of proteins with weakly hydrophobic ligands immobilized on chromatography medium. HIC is carried out in aqueous solution and this distinguishes it from reversed phase chromatography, which uses organic solvents to elute proteins bound to strongly hydrophobic stationary phases.

Salts are added to the loading buffer to promote the binding of proteins to HIC medium. The influence of salts on the binding of proteins to HIC media is most

conveniently considered in terms of the salt's position in the Hofmeister series, which ranks anions and cations as lyotropic (salting out) or chaotropic (salting in) ions. Salts that are lyotropes will increase water structure and decrease protein solubility, resulting in salting out of proteins. Lyotropic salts such as ammonium sulfate and potassium phosphate will increase the retention of proteins on HIC. The choice of salt is an important factor in determining specificity of the separation.[25,26] The sample is loaded onto the column in a buffer containing a sufficiently high concentration of salt in order to effect binding of the protein of interest. It is important to keep the concentration of salt below the concentration at which the protein will precipitation. Unbound proteins are washed off the column and then the concentration of salt in the buffer is decreased in steps, or continuously in the form of a gradient, to achieve a selective elution of the protein of interest. Different salts will determine the specificity and the strength of interaction with the affinity matrix. The type of hydrophobic ligand attached to the resin is an important variable that will influence the strength of binding and consequently the salt concentration necessary to bind proteins. Commonly used ligand groups include ether, isopropyl, and phenyl that differ in the strength of protein binding.[27] Other important variables that should be controlled to obtain reproducible results include pH, temperature, and additives that influence protein stability and structure.[25, 26]

Covalent Chromatography

Covalent Chromatography (CC) is a form of chemisorption. CC is used for proteins and peptides that have available thiol groups (cysteine amino acids) to form covalent bonds with a thiol-containing ligand, resulting in a disulfide. CC can also be used to separate mercurated polynucleotides.[3] The affinity ligand coupled to the solid matrix must be in an "activated" form, meaning that the thiol group has a good leaving group that will readily be displaced by the thiol group in the protein or peptide. After the protein/peptide solution is loaded onto the CC matrix the unbound proteins are washed off and bound proteins are generally eluted by solutions of thiols that have greater reducing strength (i.e. cysteine, reduced glutathione, and dithiothreitol). Some proteins have a reduced thiol group available, while other proteins have thiol groups that are not accessible or are already in the form of disulfides. Disulfide bonds in proteins can be reduced prior to applying the sample and proteins can be partially or fully denatured to allow thiol groups to bind to the activated thiol ligand.[28]

Charge Transfer Adsorption

Charge transfer adsorption chromatography is based on the ability of organic molecules to form complexes, which are stabilized by the sharing of elec-

trons, typically the π-electrons of aromatic rings.[29] Compounds that are either electron donators or electron acceptors have been used as ligands for charge transfer adsorption. Most of the applications for charge transfer adsorption have been designed to separate peptides, nucleotides, and other low molecular weight molecules.[6] The adsorption of solutes to charge transfer ligands can also be due to another mechanism such as hydrophobic interactions between aromatic groups. The use of non-ionizable ligands can be used to prevent ionic interactions between the solute and the ligand.

Thiophilic Adsorption

The affinity ligands for thiophilic adsorption chromatography consist of a sulfone group in the general structure —SO_2—CH_2—CH_2—X—R, where Xis either sulfur or nitrogen.[23] Adsorption of proteins to these hydrophilic ligands is promoted by salt. T-gel was formed by the reaction of β-mercaptoethanol with divinyl sulfone-activated agarose and contains the ligand with the structure, —O—CH_2—CH_2—SO_2—CH_2—CH_2—S—CH_2—CH_2OH. The T-gel specifically adsorbs immunoglobulins in the presence of salt.[30] It is believed that the thiophilic ligands possibly act as electron donors (thioether) and as electron acceptors (sulfone).[23] The adsorption of other proteins to T-gel can be promoted by increased concentrations of salt such as ammonium sulfate in the binding buffer.

CONTINUING RESEARCH

Advances in molecular biology and the ability to manipulate gene structure have led to the routine modification of proteins by changing specific amino acids (site-directed mutation), deleting regions (domains) of proteins, and adding new domains. This technology has been utilized to identify and add domains responsible for ligand binding (affinity tags) to proteins that had not previously had such properties. Examples of adding affinity tags to proteins include histidine amino acids to increase interaction with IMAC, carbohydrate binding domains for lectin affinity chromatography, the calmodulin binding peptide for purification on a calmodulin-affinity columns, and a signal peptide that results in biotinylation for purification using avidin affinity columns. It is possible to remove affinity tags by means of chemical or enzymatic cleavage after the protein has been purified. Cloning systems are commercially available for expression in a variety of systems including *E. coli*, yeast, and baculovirus that will add affinity tags and in some cases cleavage sites (for removal of affinity tags after purification) to recombinant proteins.[31] A continuing area of research is the development of new affinity tags that minimally influence protein structure and function and are easily removed.

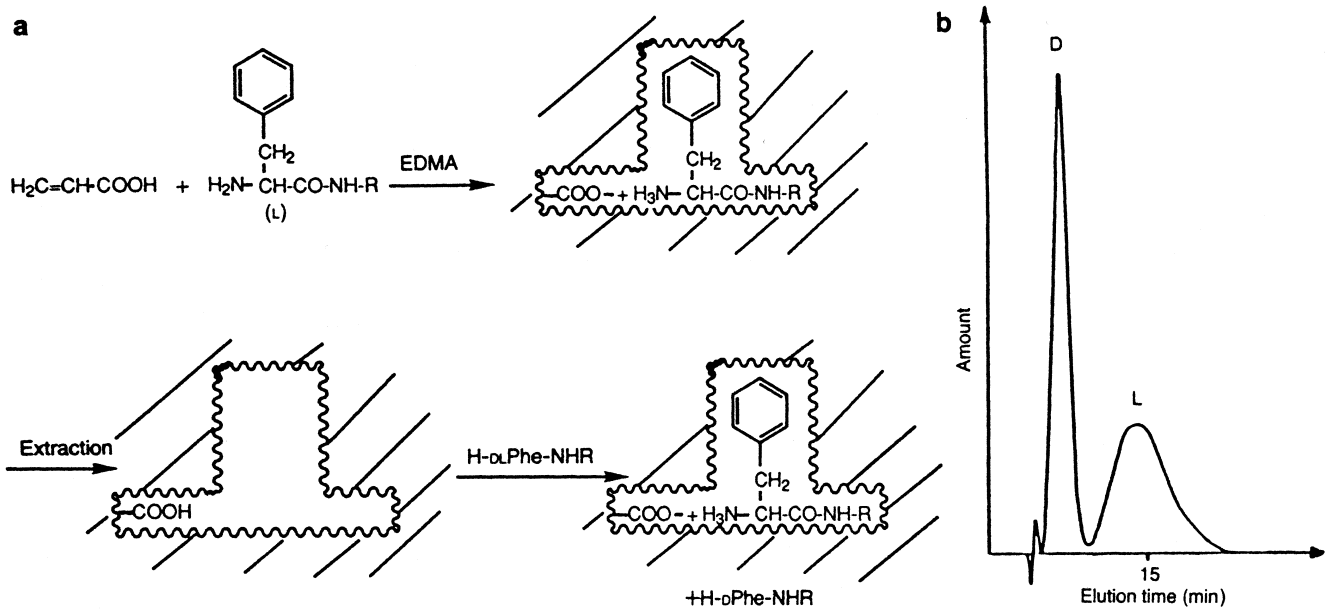


Figure 6. Molecular imprinting using non-covalent interactions (a) The ionic and other interactions between an L-phenylalanine anilid ($R=C_6H_5$) and acrylic acid determine the size, shape and character of the 'print site'. After crosslinking with ethylene glycol dimethylacrylate (EDMA) and extraction of the print molecule, the polymer is selective for L-phenylalanine anilid. (b) The chromatographic racemic resolution of DL-phenylalanine anilid on the polymer. Figure from reference[36], used with permission.

Phage display is a recently developed technology that uses a biological system for selection, replication, and amplification. Peptides or proteins can be expressed as fusion proteins on the surface of a filamentous virus (a phage) that infects *E. coli.* Randomness can be introduced into the peptide or protein expressed on the surface by making gene constructs in the viral genome that result in different amino acids being expressed at one or a number of locations in the fusion protein. Extremely large numbers of phage can be constructed, replicated, selected, and amplified in a process termed "biopanning".[32,33] The random display of amino acids on the surface of the phage constitutes a library of diversity that can be utilized to select for new affinity ligands. The variable regions of antibodies can also be expressed in a phage library and selected without loss of specificity or affinity.[34] These techniques allow the selection of affinity ligands for a variety of targets. The selection step in the biopanning procedure can be tailored so affinity ligands are selected on the basis of elution under mild conditions, strength of binding, and differential binding to different targets.

The field of combinatorial chemistry exploits molecular diversity in biological and chemical systems. Diverse forms of combinatorial chemistry are being used to isolate affinity ligands for a variety of targets. Combinatorial libraries are being developed using a variety of materials including nucleic acids, oligosaccharides, and small organic molecules. Screening these libraries and identifying affinity ligands from these libraries are areas of intense interest and activity for the development of new affinity ligands that can be applied to affinity chromatography.

Another promising area of research for selective recognition is termed molecular imprinting.[35–38] This is especially suitable for the purification of small molecules. With this technique, a polymeric matrix is synthesized in the presence of the compound (the print molecule) to be purified. This leads to the formation of cavities, which resemble the shape of the molecule in the polymer matrix. After the polymerization, the print molecule is removed by washing the matrix. The polymer gel matrix subsequently recognizes and binds only the print molecule that can then be eluted under appropriate conditions (Figure 6).

REFERENCES

1. C. Jones, A. Patel, S. Griffin, J. Martin, P. Young, K. O'Donnell, C. Silverman, T. Porter, and I.M. Chaiken, J. Chromatogr. A., *707*, 3 (1995).
2. R.K. Scopes, "Separation by Adsorption, Affinity Techniques in Protein Purification, Principles and Practice. Principles of Affinity Chromatography," Third Edition. Springer Verlag, New York, 1994.
3. Pharmacia, Laboratory Separation Division, "Affinity Chromatography, Principles & Methods" Phamacia: Lund, Sweden, 1993.

4. J. Carlsson, J.-C. Janson, and M. Sparrman, "Protein Purification, Principles, High resolution Methods and Applications," J.-C. Janson and L. Ryden, L., ed, VCH Publishers, Weinheim, 1989, p. 276.
5. E.V. Groman and M. Wilchek, Trends Biotechnol., *5*, 220 (1987).
6. P. Mohr, P and K. Pommerening, "Affinity Chromatography, Practical and Theoretical Aspects," Marcel Dekker, Inc., New York, 1985.
7. I.M. Chaiken, M. Wilchek and I. Parikh, I., ed. "Affinity Chromatography and Biological Recognition"**,** Academic Press, Orlando, 1983.
8. R.N. Narayanan and L.J. Crane, Trends Biotechnol.*, 8*, 12 (1990).
9. H.H. Weetall, Adv, Mol. Cell Biol.*, 15A*, 161 (1996).
10. N. Cooke, LC-GC, *5*, 8 (1987).
11. D.J. O'Shannessy and W.L.Hoffman, Biotechnol. Appl. Biochem., *9*, 488 (1987).
12. A. Murayama, K.Shimada, and T. Yamamoto, Immunochem., *15*, 523 (1978).
13. W. H. Scouten, "Affinity Chromatography Bioselective Adsorption on Inert Matrices"*,* John Wiley & Sons, New York, 1981.
14. I. West and O. Goldring, Mol. Biotechnol., *2***,** 147 (1994).
15. H.W. Jarrett, J. Chromatogr., *618*, 315 (1993).
16. N.K. Tanner, Methods Enzymol., *180*, 25 (1989).
17. J. Wittmeyer and T. Formosa, Methods Enzymol., *262***,** 415 (1995).
18. C. Pozidis, G. Vlatakis, and V. Bouriotis, J. Chromatogr., *630***,** 151 (1993).
19. J.T. Kadonaga, Methods Enzymol., *208*, 10 (1991).
20. Y.D. Clonis, T. Atkinson, S.J. Burton, and C.R. Lowe, ed. "Reactive Dyes in Protein and Enzyme Technology," Stockton Press, New York, 1987.
21. C.R. Lowe, S.J. Burton, N.P. Burton, W.K. Alderton, J.M. Pitts, and J.A. Thomas, Trends Biotechnol., *10*, 442 (1992).
22. Porath, J. Carlsson, I. Olsson, and G. Belfrage, Nature, *258*, 589 (1975).
23. J. Porath, Biotechnol. Prog., *3*, 14 (1987).
24. L. Kågedal, in "Protein Purification, Principles, High Resolution Methods and Applications", J.-C. Janson and L. Rydén , ed., VCH Publishers, Weinheim, 1989, p. 227.
25. P. Gagnon and E. Grund, BioPharm.*, 8*, 54 (1996).
26. K.-O. Eriksson, in "Protein Purification, Principles, High Resolution Methods and Applications," J-C Janson and L. Rydén , ed., VCH Publishers, Weinheim, 1989 p. 207.
27. P. Gagnon, E. Grund, and T. Lindbäck, BioPharm. 1995, *8*, (1995).
28. L. Rydén and J. Carlsson, in "Protein Purification, Principles, High Resolution Methods and Applications," J.-C. Janson and L. Rydén , ed., VCH Publishers, Weinheim, 1989, p. 252.
29. Porath and K.D. Caldwell, J. Chromat. 133, 180 (1977).
30. J. Porath, F. Masisano, and M. Belew, FEBS Lett., *185*, 306 (1985).

31. K.J. Morrow. Genet. Eng. News., *Mar.15*, 12 (1997).
32. S.F. Parmley and G.P. Smith, Gene, *73*, 305(1988).
33. B.K. Kay, J. Winter, and J.McCafferty, "Phage Display of Peptides and Proteins, A Laboratory Manual," Academic Press, San Diego, 1996.
34. J. McCafferty, A.D. Griffiths, G. Winter, and D.J. Chiswell, Nature, *348*, 522 (1990).
35. C. Alexander, C.R., Smith, E.N. Vulfson, and M.J.Whitcombe, M.J. in " Separations for Biotechnology, 3," D. L. Pyle, ed., SCI, the Royal Society of Chemistry.1994, p. 23.
36. B. Ekberg and K. Mosbach, Trends Biotechnol., *7*, 92 (1989).
37. L. Andersson, B. Sellergren, and K. Mosbach, K. Tetrahedron Lett. *25*, 5211 (1984).
38. K. Mosbach and O. Ramström, Biotechnol. *14*, 163 (1996).