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A REVIEW ON "AFFINITY CHROMATOGRAPHY

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ABSTRACT

Affinity Chromatography is a type of separation technique which is widely used to analyze the pharmaceutical substances and biological substances. The main principle involved here is based upon affinity. The separation is based on highly specific macromolecular binding interaction between the biomolecule and substance. The interaction is based upon the analyte of interest. The biomolecule interaction takes place between antigen and antibody, enzyme and substrate, ligand and receptor. This chromatographic method is used to purify the molecules in a complex mixture by specific interactions with the ligand. It is used in the separation of pharmaceutical, therapeutically, clinical and biological samples. Affinity chromatography has advantage for its high specificity and selectivity and resolution of separation compared to other chromatographic methods.

KEYWORDS: Affinity chromatography, matrix, ligand, spacer arm.

INTRODUCTION

Affinity chromatography is a type of liquid chromatography in which a biologically related agent is used in a column as a stationary phase to purify or analyze the components of a sample. The ability of this method to selectively bind and purify its target compounds is based on the specific and reversible interactions that are present in many biological systems, such as the binding of a hormone to a receptor or an antibody to its antigen.[1]

Affinity chromatography (also called affinity purification) makes use of specific binding interactions between molecules. A particular ligand is chemically immobilized or "coupled"

to a solid support so that when a complex mixture is passed over the column, those molecules having specific binding affinity to the ligand become bound. After other sample components are washed away, the bound molecule is stripped from the support, resulting in its purification from the original sample. The basic idea has been widely exploited as a powerful tool for the separation and purification of a wide variety of biological macromolecules. Its effectiveness for purification rests on the selectivity of interaction, and thus of adsorption, of a biological macromolecule on an affinity adsorbent which is prepared by the covalent immobilization of a specific ligand on solid polymeric matrix.[2]

To develop a method based on affinity chromatography, one of the pair of interacting species is first immobilized to a solid support, such as agarose beads or silica particles. The immobilized agent, called the affinity ligand, acts as the stationary phase for the affinity column. The other interacting compound is then injected onto the affinity column or applied in the presence of an application buffer, which allows the desired target to bind to the immobilized ligand. After non retained sample components have been washed from the column, the retained target analyte is typically released in the presence of an elution buffer. If the retained compound has only weak or moderate binding to the immobilized ligand, it is also possible to use the application buffer to elute this target under isocratic conditions; this approach is known as Weak-affinity chromatography (WAC). As the target elutes, it is collected for further use or analyzed by an on-line or off-line detector. The column is regenerated by reapplying the application buffer before the next sample injection.[3]

Affinity purification involves 3 main steps

i. Incubation of a crude sample with the affinity support to allow the target molecule in the sample to bind to the immobilized ligand.
ii. Washing away non-bound sample components from the support.
iii. Elution (dissociation and recovery) of the target molecule from the immobilized ligand by altering the buffer conditions so that the binding interaction no longer occurs.[3]

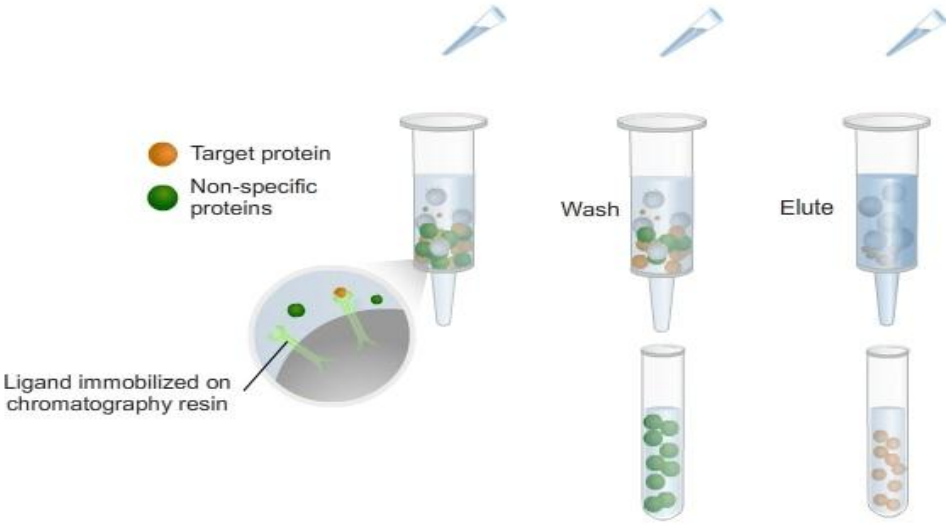

Figure 1: Process of Affinity Purification.

Components of Affinity Chromatography

The success of any affinity separation depends largely on the selection of the matrix, ligand, or binding agent that is immobilized in the column. Many of the ligands used in affinity chromatography are obtained from a biological source; examples of these ligands are antibodies, serum proteins, and lectins. Other binding agents and ligands that are useful in affinity chromatography are boronic acid, metal chelates, and triazine dyes, which are synthetic agents or inorganic molecules.[4]

Matrix

A support or matrix in affinity purification is any material to which a biospecific ligand is covalently attached. Typically, the material to be used as an affinity matrix is insoluble in the system in which the target molecule is found. Usually, but not always, the insoluble matrix is a solid. It consisted of porous support materials such as agarose, polymethacrylate, polyacrylamide, cellulose, and silica. All of these support materials are commercially available and come in a range of particle and pore sizes. Nonporous support materials consist of nonporous beads with diameters of 1- 3 µm. These supports allow for fast purifications, but suffer from low surface areas when compared to traditional porous supports.[5]

Agarose Bead Adsorbents

Of the beaded agarose derivatives commercially available, Sepharose 4B is the most useful for affinity chromatography. It is more porous than the 6B derivative, and has considerably greater capacity than the 2B gels. Chemical compounds containing primary aliphatic or

aromatic amines can be coupled directly to agarose beads after activation of the latter with cyanogen bromide at alkaline pH. The chemical nature of the intermediate formed by cyanogen halide treatment of polysaccharide derivatives is not known, but the products formed upon coupling with amino compounds appear to be principally derivatives of amino carbonic acid and isourea. Notably, both of these postulated linkage groups retain the basicity of the amino group of the coupled ligand. It must be borne in mind, therefore, that even if the charge on the ligand amino group is known to contribute to the binding interaction, the resultant agarose ligand gel may nevertheless demonstrate considerable binding effectiveness. Beaded agarose gels, unlike the cross-linked dextrans, cannot be dried or frozen, since they will shrink severely and essentially irreversibly. Similarly, they will not tolerate many organic solvents. Dimethyl formamide (50%, v:v) and ethylene glycol (50%, v:v) do not adversely affect the structure of these beads. These solvents are quite useful in situations where the compound to be coupled is relatively insoluble in water (e.g., steroids, thyroxine, tryptophan derivatives) since the coupling step can be carried out in these solvents. Similarly, the final, coupled derivative can be washed with these solvents to remove strongly adsorbed or relatively water-insoluble material.[6]

Spacer Arm

In affinity chromatography, the chain of carbon and/or other atoms that positions a functional group away from the solid matrix to which it is covalently bound and makes it more available to a ligand and less restricted by steric hindrance by the matrix.

The length of these spacer arms is critical. Too short or too long arms may lead to failure of binding or even non-specific binding. In general, the spacer arms are used when coupling molecules less than 1000 Da.[7]

Properties of ideal spacer arm

1. It should be long enough (at least 3 atoms) to keep the substance at an appropriate distance.
2. It should be inactive not to cause a non –specific binding.
3. It should have bi functional group for the reaction with both support and the sample.[7]

Ligand

It is a molecule that binds reversibly to a specific target molecule or group of target molecules. Antibodies have several advantages including their high specificity and relatively

large binding constants. Antibodies or immunoglobulins are a type of glycoprotein produced when a body's immune system responds it has been estimated that antibodies can be produced for millions or even billions of different foreign agents. Antibodies which are produced by separate cell lines are referred to as polyclonal antibodies. Monoclonal antibodies are produced when a single antibody producing cell is combined with a carcinoma cell to create a hybridoma which can be grown in a cell culture. Monoclonal antibodies are often more desirable than polyclonal antibodies in affinity chromatography due to their lack of variability which allows for the creation of a more uniform affinity support.[8]

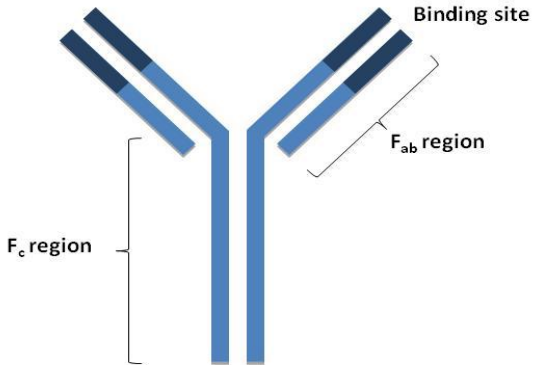

Figure 2: Typical structure of an antibody.

DNA can also be used as an affinity ligand. It can be used to purify DNA-binding proteins, DNA repair proteins, primases, helicases, polymerases, and restriction enzymes.

Peptide affinity chromatography is another method which can be used for purifying biomolecules.[9]

The amino acids in the Fc region generally have the same sequence, whereas the amino acids in the Fab region have variable amino acid sequences which allows for the specificity of the binding interaction against a wide range of antigens.

Table 1: Biological Interactions Used in Affinity Chromatography.

Sr. No	Types of Ligand	Target Molecule
1	Substrate analogue	Enzymes
2	Antibody	Antigen
3	Lectin	Polysaccharide
4	Nucleic acid	Complementary base sequence
5	Hormone	Receptor
6	Avidin	Biotin/Biotin-conjugated molecule
7	Calmodulin	Calmodulin binding partner
8	Glutathione	GST fusion protein

9	Proteins A and G	Immunoglobulins
10	Metal ions	Poly-histidine fusion protein

Attachment of Ligand To Matrix

Several procedures have been developed for the covalent attachment of the ligand to the stationary phase. All procedures for gel modification proceed in two separate chemical steps:

1) Activation of the functional groups on the matrix and
2) Joining of the ligand to the functional group on the matrix.

A wide variety of activated gels is now commercially available. The most widely used are described in the following:

Cyanogen Bromide-Activated Agarose

- This gel is especially versatile because all ligands containing primary amino groups are easily attached to the agarose. Since the gel is extremely reactive, very gentle conditions may be used to couple the ligand.

6-Aminohexanoic Acid (Ch)-Agarose and 1,6-Diaminohexane(Ah)-Agarose

- These activated gels overcome the steric interference problems by positioning a six carbon spacer arm between the ligand and the matrix.
- Ligands with free primary amino groups can be covalently attached to CH-agarose, whereas ligands with free carboxyl groups can be coupled to AH-agarose.

Carbonyldimidazole (Cdi)-Activated Support or Matrix

- Reaction with CDI produces gels that contain uncharged N-alkylcarbamate groups.

Epoxy-Activated Agarose

- This gel provides for the attachment of ligands containing hydroxyl, thiol, or amino groups.

Group Specific Adsorbents

- Group specific adsorbents contain ligands that have affinity for a class of biochemically related substances.
- For example cibacron blue-agarose is an adsorbent which would react with enzymes that have nucleotide cofactors (DNA polymerase, kinase and serum albumin).[10-11]

Immobilized Ligand

- The ligand can be selected only after the nature of the macromolecule to be isolated is known.
- When a hormone receptor protein is to be purified by affinity chromatography, the hormone itself is an ideal candidate for the ligand.
- For antibody isolation, an antigen can be used as ligand.
- If an enzyme is to be purified, a substrate analogue, inhibitor, cofactor or effector may be used as the immobilized ligand.[12]

Procedure

Step 1: Attach ligand to column matrix, where the substrate cannot reach the matrix hence ligand is used.

Step 2: Load protein mixture into the column, where the protein solution flows downward due to gravity and attracted to the gel.

Step 3: Proteins bind to the ligands, where proteins of interest are bounded to the gel.

Step 4: Wash column to remove unwanted materials.

Step 5: Wash thoroughly the proteins that bind loosely.

Step 6: Elute the proteins that are tightly bind to the ligands and collect purified protein of interest.[13-14]

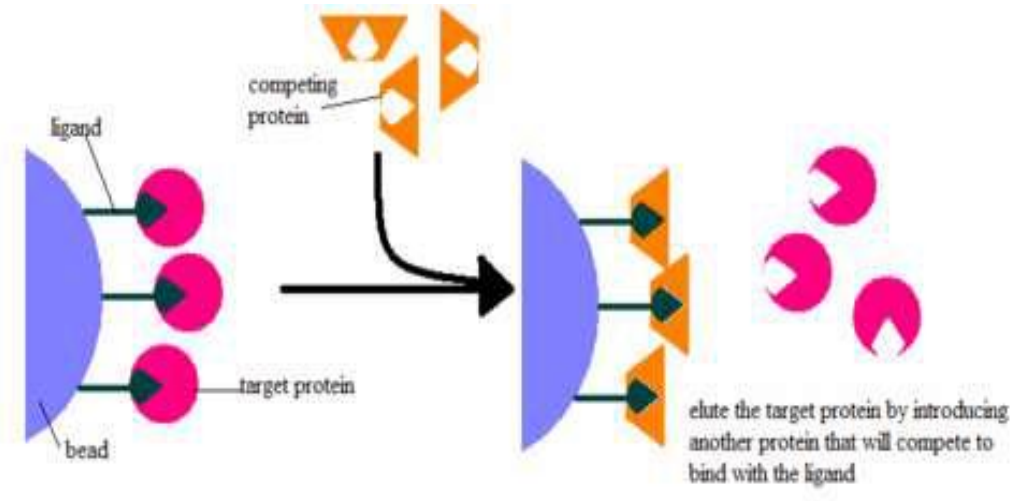

Figure 3: Procedure of Affinity Chromatography.

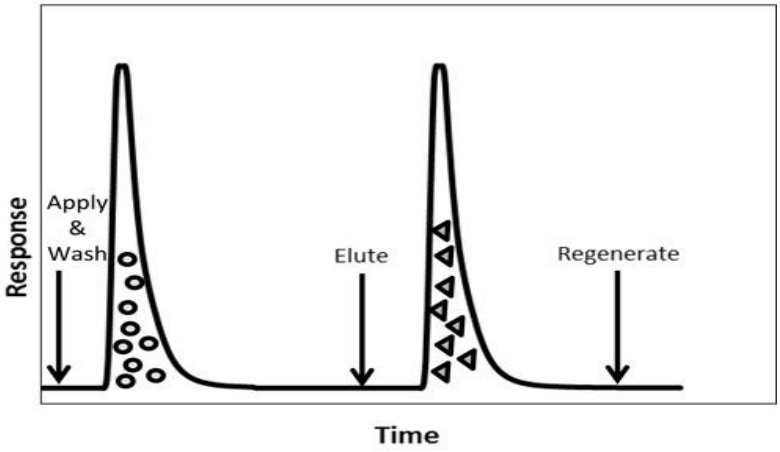

Figure 4: Time vs Response Chromatogram of Affinity Chromatography.

Applications of Affinity Chromatography

- Helps to purify and concentrate a substance from a mixture into a buffering solution.[15]
- Used to reduce the amount of a substance in a mixture.
- Used to purify and concentrate an enzyme solution.
- Used in Genetic Engineering for nucleic acid purification.
- Production of Vaccines which involves antibody purification from blood serum.[16]
- And Basic Metabolic Research helps in protein or enzyme purification from cell free extracts.
- Affinity chromatography is widely used in the pharmaceutical industry to purify and extract molecules of interest from complex mixtures.[17]
- These molecules tend to be enzymes, proteins or amino acids, but other biological species can be selectively retained.[18]
- Once isolated, these biological species can be selectively amplified to produce larger quantities, although at large concentrations.[19]
- In therapeutic and clinical applications, Hyper-lipidemia were the sample is made to pass through column containing antibody & plasma LDL so, it can easily be separated out by eluting with glycine hydrochloride buffer (pH 3).[20]
- It is used in Pregnancy test.
- It is used in Allergy test.
- It is used to study Immuno assay of the substance.
- It is used in Kinetic studies of the compound.
- It is used in Qualitative measurement of substrate.

- Affinity chromatography also plays an important role in other aspects of bioprocess and bioprocess development. For instance, purification of non-antibody biologics for quality attribute analysis in process development has been achieved using immune affinity chromatography.[21]
- Also, analytical affinity chromatography with Protein A can be used to determine antibody titres during process development and for other antibody quantification purposes.[22]

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