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DYE-LIGAND CHROMATOGRAPHY: REVOLUTIONIZING SEPARATION SCIENCE

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ABSTRACT

Dye-ligand chromatography is a powerful technique used in analytical chemistry to separate and analyse complex mixtures. This method involves the use of two different ligands, each with a distinct chemical affinity towards specific molecules in the mixture. By combining these ligands in a single chromatographic column, scientists can achieve enhanced separation and detection capabilities, leading to a more comprehensive understanding of the sample under investigation. The principle behind dye ligand chromatography is based on the concept of selectivity. Different ligands have different affinities for specific types of molecules, allowing for the separation of the mixture into its individual components. This selectivity is essential in achieving accurate and reliable results in analytical chemistry.

KEYWORDS: Complex mixtures, dye ligand, chemical affinity, selectivity.

INTRODUCTION

Chromatography designates the **generic name** collectively assigned to host **divergent separation techniques** that have been duly recognized right from early 1900s till date.

The term '**Chromatography'** emerged as the fusion of *two* Greek words: Chroma means **color,** and Graphein means **'to write'.**

Chromatography is defined as a physical technique of separation wherein the components required to be separated between the two phases, one of which being 'stationary phase' and the other 'mobile phase' that moves in a definite direction.

There are various chromatography methods have been developed among them Dye-ligand affinity chromatography is a type of affinity chromatography. Affinity chromatography is a highly specialized form of adsorption chromatography in which a specific ligand is immobilized chemically into an insoluble matrix to adsorb reversibly a single molecular species from a mixture of solutes. This technique is originally developed for the purification of enzymes and also extended to nucleotides. This involves the immobilisation of appropriate ligands in such a way that the enzyme is still capable of re-engaging to that immobilised form of the ligands in such a way that the enzyme is still capable of re-engaging to that immobilised form of the ligand.

In the vast field of separation science, dye ligand chromatography has emerged as a powerful technique that enables the separation and purification of complex mixtures with exceptional efficiency and precision. This cutting-edge chromatographic method encompasses the use of unique dye ligand ligands, which possess the ability to specifically bind to analytes of interest. By leveraging this remarkable selectivity, scientists are able to isolate and separate target compounds from highly intricate matrices, providing invaluable insights into the world of chemical analysis.

CLASSIFICATION OF AFFINITY CHROMATOGRAPHY

This is mainly classified into two classes

1. Bio-selective: The affinity is based on biologically relevant binding groups involving specific ligands, lectins, nucleotide cofactors, immunobiotin labelled proteins, rarely employed cofactors, receptor proteins and immunosorbents.

2. Chemiselective: The affinity is based on chemically defined interactions including hydrophobic chromatography, ion exchange chromatography, conventional chromatography, etc.

DYE-LIGAND AFFINITY CHROMATOGRAPHY

Dye-ligand chromatography has developed into an important method for large-scale purification of proteins. The utility of the reactive dyes as affinity ligands results from their unique chemistry, which confers both the ability to interact with a large number of proteins as well

as easy immobilization on typical adsorbent matrices. Reactive dyes can bind proteins either by specific interactions at the protein's active site or by a range of non-specific interactions. Divalent metals participate in yet another type of protein-reactive dye interactions which involve the formation of a ternary complex. All of these types of interactions have been exploited in schemes for protein purification. Many factors contribute to the successful operation of a dye-ligand chromatography process. These include adsorbent properties, such as matrix type and ligand concentration, the buffer conditions employed in the adsorption and elution stages, and contacting parameters like flowrate and column geometry. Dye-ligand chromatography has been demonstrated to be suitable for large-scale protein purification due to their high selectivity, stability, and economy. Also, the issue of dye leakage and process validation of large-scale dye-ligand chromatography has been discussed. Reactive dyes have also been applied in high performance liquid affinity chromatographic techniques for protein purification, as well as non-chromatographic techniques including affinity partition, affinity membrane separations, affinity cross-flow filtration, and affinity precipitation.

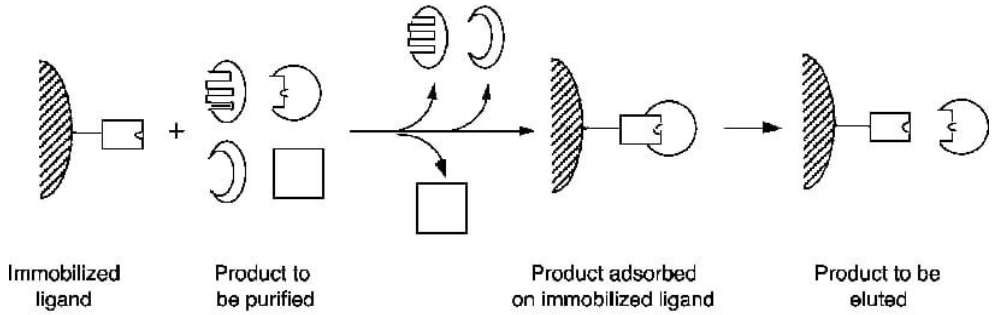
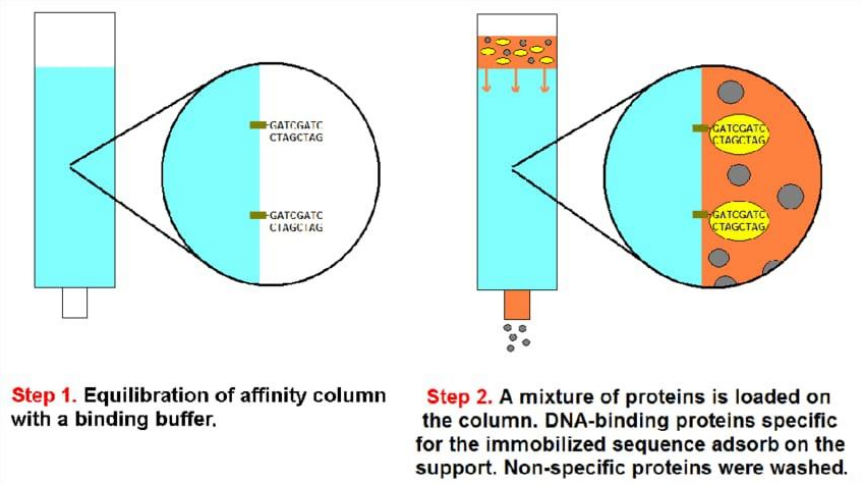


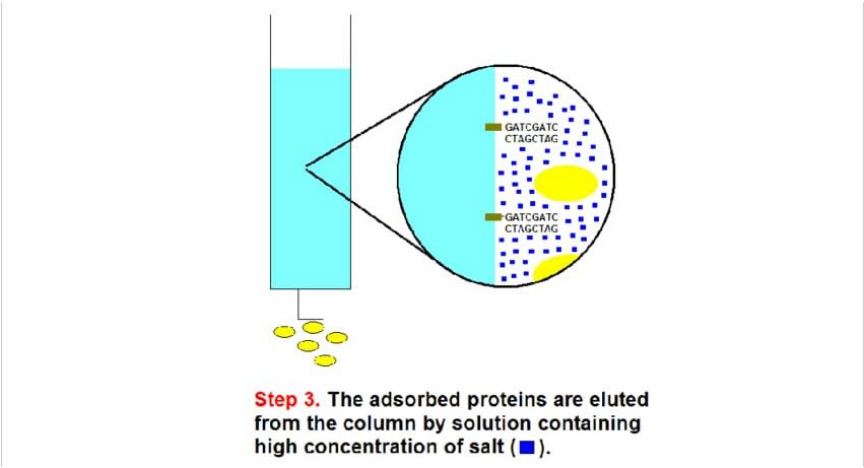
Fig. 1. Principle of affinity chromatography.

In the vast field of separation science, dye-ligand chromatography has emerged as powerful technique that enables the separation and purification of complex mixtures with exceptional efficiency and precision. This cutting-edge chromatographic method encompasses the use of unique dye-ligand ligands, leveraging this remarkable selectivity, scientists are able to isolate and separate target compounds from highly intricate matrices, providing invaluable insights into the world of chemical analysis.

The science behind dye-ligand chromatography lies in the development and synthesis of ingenious dye-ligand ligands. These ligands are characterized by their dual affinity for target compounds, often bearing both a specific functional group that confers selectivity and an additional chromatographic functionality that aids in the separation process. The synthesis of such dye-ligands requires a meticulous approach, with a multi-step process involving the design, functionalization, and immobilization of these ligands onto suitable stationary phases.

Once the dye-ligand ligands are firmly anchored onto the stationary phase, the chromatography column is ready for sample analysis. The separation process commences with the injection of the sample onto the column, where it interacts with the dye-ligand ligands. The analytes of interest form reversible complexes with the ligands, leaving behind undesirable compounds that do not possess the necessary affinity. This selectivity allows for the specific separation of target molecules, leading to enhanced resolution and purity.





Selection of matrix

Selection of an appropriate matrix depends on the type of separation desired. It shows the considerable effect on the stability of complex formed between ligand and affine molecule.

Support matrix should have the following properties

1. Adequate particle size and shape: Increased particle size reduces flow resistance and they become clogged. Irregularly shaped particles leads to unequal path lengths for substance to be separated and subsequently broadening of bond. Spherical shape is best sited.
2. It should be stable and resistant against micro-organisms.
3. It should be inert to prevent the affine from interacting non-specifically with the matrix itself.
4. It should possess functional groups to which ligand can be attacked easily by vanity of chemical reactions.
5. It should allow the free diffusing of the biomolecules to the binding sites.
6. It should be economical.

Ligand is a type of matrices which is polyacrylamide. The ligand to be immobilised can be an inhibitor, w-enzyme, substrate analogue (or) any other bimolecular with an affinity for a specific site such as active site, allosteric site, membrane binding site, etc., on the protein to be purified. Such biomolecules are usually small although macromolecules have been successfully employed. The ligand must have sufficient affinity for the enzyme being purified to retain on the ligand matrix completely.

Dye-ligands may function as analogues of their respective enzymes substrates, enabling affinities and interactions. In mammalian cell culture, a dye-ligand chromatography method is described for removing proteins that have been overexpressed. In order to quickly choose binding and elution conditions, the approach begins with batch binding. Gradient elution is then used to increase the final packed bed chromatography method's selectivity. An accompanying sample chromatogram and instructions for purifying a protein from mammalian cell culture on Cibacron blue are provided. In a protein purification procedure for chromatography, synthetic dyes can function as substitute ligands for proteins. Compounds are separated from mixtures using chromatography. In order to extract certain proteins from a mixture, affinity chromatography is routinely employed. Here, the targeted proteins are captured when the sample is run through the chromatography column using ligands that interact with the targeted proteins.

A ligand in Affinity chromatography should possess the following properties

1. It should specifically form a reversible complex with the substance to be purified.
2. The compound should possess a chemically modifiable group, so that the covalent linkage to the matrix can occur.
3. It should form a stable complex on binding to the substance to be purified.
4. It should be able to withstand harsh conditions during the chromatographic process.
5. It should be easy to dissociate the complex by a simple change in the medium without adversely affecting the compound to be purified.
6. It should be consistently available and economical.

Ligand classification

Ligands are generally classified as non-specific (or) group specific.

They are further divided into lower and higher molecular weight ligands.

- Ligands with mono-specificity have a high affectivity for the purification of a particular substance. It has a disadvantage that is a special ligand–matrix combination is required for every substance to be purified and also monoclinic ligands bind more strongly and require harsh elements than group specific ligands.

Example: Steroid hormones, vitamins and certain enzyme inhibitors.

- A ligand with group specificity called general ligands, which has one of the great values, allows a variety of substances to be purified with single affine matrix. Example: Coenzymes such as NAD, NADP, FMN and FAD used in dehydrogenases enzymes separation. The above two are the bio-specific ligands. They are also non bio-specific ligands.

They are as follows

1. Dyes as ligands which are used in dye-ligand chromatography:

Examples:
(a) Cibacron blue F 3G-A is very suitable group-specific ligand for the enzymes requiring adenylyl containing co-factors.
(b) Dextral-blue sepharose is used for purification and isolation of albumin.

2. Metal chelates as ligands (metal chelate AC).
Examples:
(a) Zn+2 is carbonyl methyl amino sepharose 6B used to purify human fibroblast interferon.
(b) Fe+3 bis-carboxy methyl amino sephadex G25 used to purify tyrosine containing peptides.

3. Charge transfer chromatography: Change transfer materials such as acriflavine, acridine yellow, pentachlorophenol and malachite green are used in AC.
Example: Acriflavine enables to separate nucleotides, oligo nucleotides, single chain and double chain nucleic acids.

4. Hydrophobic ligands
Example: Octyl-sepharose-CL 4b is used to purify staphylococci lipase and phenylsepharose-CL 4b is used to purify heparin.

5. Organo-mercurial and disulphide-containing ligands.
Examples: (I) 2-Phridyldisulfide-hydroxyl-pyopyl-ether-agarose:
‰ To separate copper thiamine from human foetal liver.
‰ To separate disulphide anion reductive from beef liver.

(II) Agarose glutathione-2-pyridyl-disulphide:
Examples: Globulin phospholipids from human serum. Chains of haemoglobin from bovine haemoglobin.

Spacers
‰ Although a ligand may be directly attached to an activated matrix, an intervening organic group usually serves as a method of attachment (or) as spaces (or) to separate the ligand form the matrix.

‰ A spacer is important. For example, when a series of eight agarose–insulin complexes are used for the purification of insulin receptor protein, the derivative with the longest spacer arm is most effective.

‰ The spacer arm positions the ligand at a certain distance from the matrix in order to reduce stearic interference from the matrix. There are three principle possibilities for introducing spacers. They are as follows:
1. One end of the spacer is first bond to the matrix and then the biological ligand is bound to the other end of the spaces using various methods for matrix fixations.
2. The spacer can be first bound to the ligand as a subsistent and then the other end of the spaces can be fixed to the matrix.
3. Commonly used spacer arms are aliphatic, linear hydrocarbons C two functional groups one at each end of the chains. Example: Of spacers: hetero ethylene domains and b-amino hexanes.

Elution
‰ In the elution step, it is attempted to weaken the interactions between the ligand-matrix and ligand analytic molecule to achieve the desired from the column.

‰ Different elution methods are as follows:
(a) Specific methods: Affinity elution with substrates, cofactors, inhibitors and other specific soluble compounds.
(b) Non-specific methods: Changing of pit, ionic strength, temperature, polarity (or) by using reagents such as deforming elements, urea, guanidine, detergents, etc.
(c) Special methods: Electrophoresis, cleavage of matrix-ligand bond, buffer effects, various elution variants such stepwise (or) gradient elution. In many cases, a change in pH leads to description, however, it is limited because of chemical stability of the substance, the matrix and the covalent linkage between matrix and ligand.

Example: In the case of proteases and inhibitors, adsorption occurs at pH 8.1 and elution at pH 3.1.

‰ Increase in the ionic strength either stepwise (or) in a gradient fashion is one of the gentle method of non-specific elution. NaCl / KCl mostly in concentration between 0.1 and 0.2M is used. The case of high chaotropic salts, SCN^-/CCl_3 CN^- can be used particularly in immune affinity chromatography for plasma protein isolation.

In some cases, decrease of polarity by addition of ethanol/dioxan with human lymphoid interferon up to 50% ethylene glycol is used for desorption.

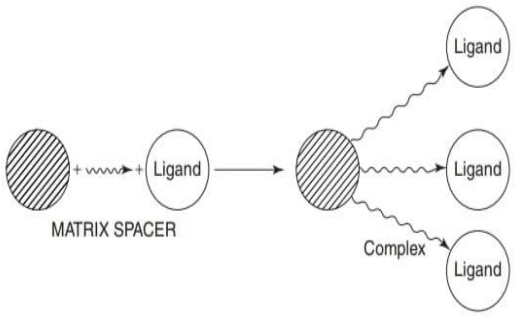
‰ A special method to elute biomacromolecules is electrophoresis. It is suited for desorption of antibiotics and hormone-binding proteins. v Another special method is selective cleavage of bond between the matrix and the ligand. This method is mainly used for high affinity complex when normal elution leads to penetration. Example: Oestradiol-binding proteins.

‰ The elution of the compound is monitored by several methods such as spectrophotometric methods, chemical detection methods and electrophoresis.

GENERAL PROCEDURE FOR THE DEVELOPMENT OF AFFINITY CHROMATOGRAPHY

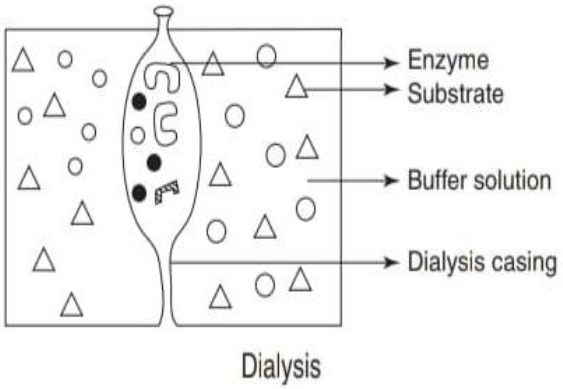
This involves several steps. They are as follows:

(a) A necessary chromatography packing (or) bio-selective adsorbent is synthesised and the bioligand is immobilised.



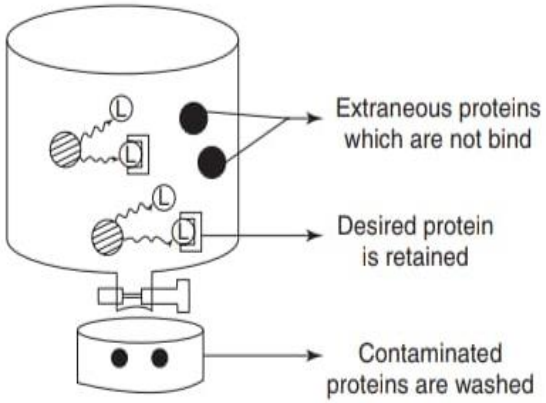
Attachment of the ligand to the matrix through spacer

(b) A crude extract is prepared and freed from endogenous substrate which is achieved by dialysis (or) enzyme precipitation.



Dialysis

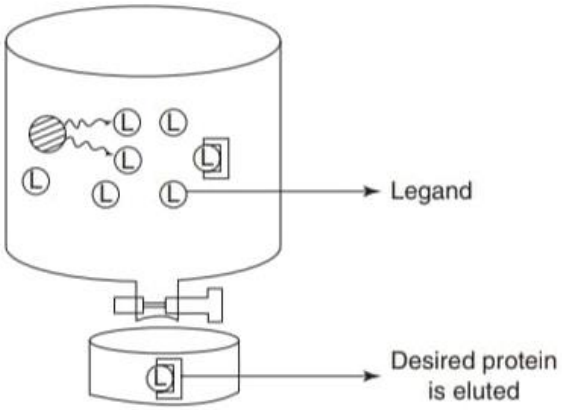
(c) The substrate free extract is applied to the chromatographic packing of immobilised bioligand.



Extraction of the desired proteins

(d) The contaminated proteins which have no affinity for the column are removed by washing.

(e) The protein to be purified remains bounded to the column owing to its affinity for the immobilised ligand. The desired protein is eluted possibly with other soluble ligand.



Separation of mixture into individual compounds

ADVANTAGES OF DYE-LIGAND AFFINITY CHROMATOGRAPHY

- Ability to handle complex mixtures
- Making it an invaluable tool in fields such as pharmaceuticals, biotechnology, and environmental analysis.
- In the pharmaceutical industry, dye-ligand chromatography plays a crucial role in drug discovery and development, enabling the isolation and purification of potential drug candidates from impure samples. By effectively removing impurities, dye-ligand chromatography ensures the safety and efficacy of pharmaceutical products.
- Selective/specific adsorption increases the purity by 100 folds.
- The number of purification steps is reduced.
- The species is concentrated.
- The species is stabilised on binding.
- This process is very rapid.

- This method is purely bowed on biological specificity not on physicochemical properties.
- Suitable for large-scale purification.

FACTORS AFFECTING DYE-LIGAND AFFINITY CHROMATOGRAPHIC SEPARATION

This depends on the following factors

- The ligand.
- The preparation of chromatographic packing.
- An inert matrix.
- The methods of attacking the ligand to matrix.
- Eluting the molecule from the bio-selective adsorbent.

APPLICATIONS OF DYE-LIGAND AFFINITY CHROMATOGRAPHY

- In biotechnology, dye-ligand chromatography has become indispensable for the purification of bioactive molecules, such as proteins and enzymes. Due to the high selectivity and specificity of dye-ligand ligands, scientists can isolate and purify these complex biomolecules with unprecedented accuracy. This is of paramount importance, as the purity of bioactive compounds directly affects their performance and functionality in various applications, including therapeutic treatments and diagnostics.
- Environmental analysis is another sphere where dye-ligand chromatography proves its worth. The ever-increasing concern over the presence of contaminants in the environment demands robust separation techniques to monitor and control such pollutants effectively. Dye-ligand chromatography, with its ability to selectively separate target analytes, offers a promising solution for the accurate determination of contaminants in environmental samples, aiding in environmental conservation and safeguarding public health.
- Dyes were originally introduced as cheap alternatives to costly nucleotide ligands for enzyme purification. Given the advantages of dyes over biological ligands, their broad protein-binding spectrum, and also the possibility of improving their selectivity for targeted proteins via molecular modelling, it is no surprise that these colourful tools are finding increasing application in various affinity-based bio-separation techniques. The literature abounds with examples where dye ligands have been employed to purify individual proteins and enzymes: albumin, antibodies, blood-clotting factors, plasminogen activator, proteolytic activities, growth factors, interferons, cellulolytic and lipolytic enzymes, collagenases, restriction endonucleases, and numerous nucleotide-binding proteins and enzymes, to name some examples. It is no exaggeration to claim that for almost every protein purification problem, a dye ligand can be found to help towards its solution.
- Beyond its applications, dye-ligand chromatography continues to advance with new innovations and improvements. Research efforts are focused on developing novel ligands with enhanced selectivity and stability, as well as optimizing the chromatographic systems for higher resolution and better reproducibility. Additionally, the integration of advanced detection methods, such as mass spectrometry, presents exciting opportunities for further expanding the capabilities of dye-ligand chromatography.

CONCLUSION

Dye-ligand chromatography has revolutionized the world of separation science by enabling the precise and efficient separation of complex mixtures. By harnessing the power of dye-ligand ligands, this technique offers remarkable selectivity, leading to enhanced purity, resolution, and accuracy. Its applications in pharmaceuticals, biotechnology, and environmental analysis underscore its significance in various industries. As researchers continue to innovate and improve upon this method, dye-ligand chromatography promises to shape the future of chemical analysis and contribute to advancements in numerous scientific disciplines.

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