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Immunoaffinity Chromatography

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Introduction

Immunoaffinity chromatography is a general term that covers a range of techniques the use of which is now widespread. Often these are based upon the use of antibodies to a specific target molecule or macromolecule immobilized on some form of support (**Figure 1**). This is then used to separate or isolate the target molecule (or molecules of a similar structure) from a matrix in order to purify it for some subsequent purpose. Alternatively, immunoaffinity chromatography can be used to isolate antibodies by immobilizing the antigen, and indeed the first example of the use of the technique can be traced back to the pioneering work of Campbell *et al*. who, in 1951, immobilized bovine serum albumin to a derivatized cellulose in order to purify antibodies that had been raised to it (**Figure 2**). These immunologically-based methods include in addition immunoaffinity precipitation, immunoaffinity adsorption and immunoaffinity extraction. Indeed the use of the term 'chromatography' is perhaps something of a misnomer as the technique often corresponds more to the online extraction of the target molecule onto the sorbent. Following extraction, a wash step is used to remove unwanted material followed by the recovery of the desired molecule with a strong eluent. It could thus be argued that in many applications immunoaffinity chromatography is simply immunoaffinity extraction in a column format. The term 'immunoaffinity chromatography' is however, widely used and understood by its practitioners.

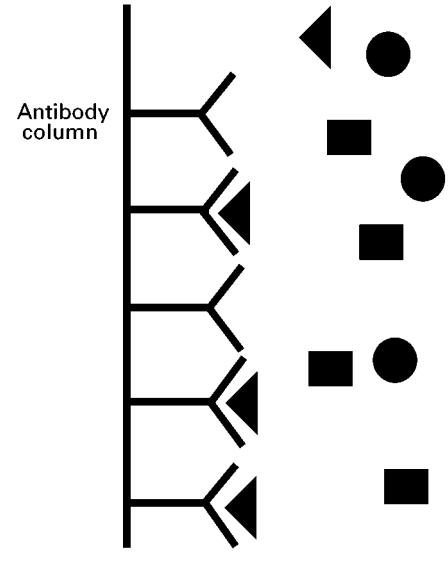

Figure 1 Stationary phase with antibodies bound, only the antigen to which the antibodies were raised is retained. Other molecules pass through with little or no retention.

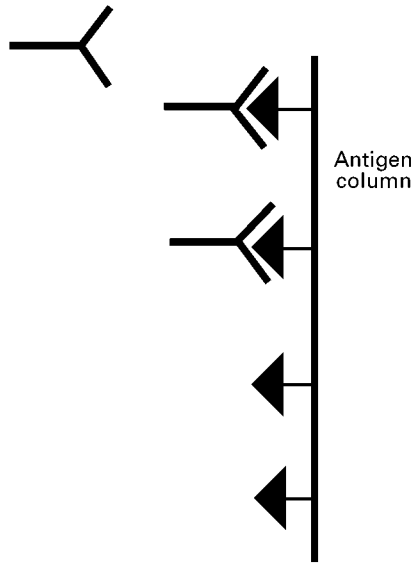

Figure 2 Stationary phase with antigen bound. Only antibodies to the antigen are retained.

Two, essentially different, types of applications of affinity chromatography can be distinguished. Thus there are those applications where isolation is with the intention of analysis (e.g. pesticides from water or drugs from blood plasma). Alternatively, immunoaffinity chromatography is used for preparative purposes and the latter is often used for the isolation of high-value proteins in the biotechnology industry.

Antibodies

The most important reagent in immunoaffinity chromatography is the antibody. These are produced by the immune system in response to foreign compounds. They are of large molecular mass (150 000–900 000). Most small molecular mass compounds such as drugs and pesticides will not provoke an immune response. The usual approach is to bind the analyte (or a structural analogue) to a carrier protein and to immunize the test species with this over a period of up to one year. Blood samples are taken and assessed for the presence of antibodies. One of the disadvantages of this type of work is that it is not certain that antibodies of suitable quality will be produced. If they are produced they are purified by techniques such as ion-exchange chromatography. Although much work is carried out on mice and rabbits, sheep are preferred as much greater volumes of antisera are produced. The antibodies produced contain a heterogeneous population of antibodies known as 'polyclonal'. These will only be produced for the lifetime of the animal. Monoclonal antibodies contain a homogeneous population and can be produced by the fusion of myeloma cells with the secreting cells of immunized animals to produce a hybridoma cell. In theory, these can be produced for an infinite length of time.

In the case of techniques where the antibody is immobilized to the support, both polyclonal and monoclonal antibodies can and have been used. However, with the development of methods for the production of monoclonal antibodies, these have been preferred because they offer advantages of reproducibility and a more defined specificity. In addition to the whole antibody, fragments can also be used, and these may confer advantages in terms of attaching them to the support (see below).

Immunoaffinity Supports

A considerable number of different materials have been used as supports for immunoaffinity chromatography. Traditionally, these have generally employed materials such as agarose or cellulose or synthetic polymers such acrylamide or polymethacrylate-based materials. These provide stationary phases that can be operated under gravity flow but are less suited to systems generating high pressures or flow rates because of limited stability. The main disadvantage of such phases is that they have slow mass transfer properties and thus have a relatively low performance.

Supports based on more rigid materials such as glass and silica, or certain organic polymers such as azalactone beads or polystyrene have been produced which, because of their higher mechanical stability and efficiency, enable higher back pressures and flow rates to be used which may be important in some applications. Because of the increased performance of these materials the term 'high performance immunoaffinity chromatography' (HPIAC) has been coined for methods based on the use of these materials.

There are also many methods for attaching the antibody to the support. A common method is simply to covalently attach the antibody directly to the support. One method of achieving this attachment is by reacting free amino groups on the antibody with supports that are activated with e.g. *N*,*N*′-carbonyl diimidazole, *N*-hydroxysuccinamide, or cyanogen bromide, etc., or to supports sporting reactive epoxide or aldehyde groups. Although technically undemanding and readily achieved, such methods of attaching the antibodies to the support bring with them the problem that the random orientation of the antibodies can interfere with their subsequent ability to interact with the antigen.

Antibodies (or fragments) can also be attached to the support via rather more selective means using, e.g.

free sulfhydryls produced in the production of Fab fragments or by coupling through the carbohydrate residues of the antibody rather than amino groups. A number of sulfhydryl-reactive supports are available, e.g. maleimide, divinylsulfone, etc., for the coupling of the Fab fragments. Coupling via the carbohydrate moiety of the antibody is facilitated by mild oxidation (periodate or enzymic) to yield aldehydes. Once formed, the aldehyde can then be reacted with amine or hydrazide-derivatized supports to bond the antibody. Such immobilized antibodies are believed to provide greater accessibility for the antigen to the antibody binding site and thus provide immunoaffinity supports with higher binding capacity relative to less selective methods. However, it should be noted that this is not always the case, and some workers have compared such 'site-directed' methods with 'random coupling' using monoclonal antibodies (Mabs). These experiments used murine Mabs to either Factor IX or protein C (human plasma proteins) which were immobilized at low density to agarose matrices. The results for this study showed that the site-directed hydrazide-coupled immunosorbents had lower binding capacity for Factor IX and higher capacity for protein C than the equivalent cyanogen bromide-coupled materials.

It has also been demonstrated that the masking of the Fab regions of the antibody with a synthetic antigen prior to covalent immobilization can result in improved immunosorbent efficiency. Thus masking of a murine Mab to protein C with water-soluble adducts of poly(2-methyloxazoline) polymers and a synthetic peptide epitope was performed followed by the immobilization of the antibody complex and then the removal of the Fab-masking antigen (FMA). The procedure resulted in significantly improved antigen binding and accessibility of the Fab domain for protein C, with the best results obtained using the largest FMA employed. Whilst this work was performed on a membrane support rather than beaded material, there seems no *a priori* reason why the approach should not work in immunoaffinity chromatography as well.

There are also a variety of indirect methods of noncovalently attaching the antibody to a support. Thus the aldehyde-containing antibodies generated above can also be reacted with biotin hydrazide, which can then be attached to a streptavidin support. Alternatively, the antibody can be adsorbed onto the bacterial proteins 'protein A' or 'protein G' attached to a support. These proteins will bind to the Fc (stem) region of the antibody reasonably strongly under physiological conditions but this can be reversed by changing the pH, etc. Whilst this does not produce particularly robust immunoaffinity supports, it can be useful in that the antibody can be replaced should the need arise, enabling the column to be regenerated. It should also be noted that protein A does not recognize all the subclasses of IgG, and has varying avidity for the IgGs of different species.

Retention and Elution in Immunoaffinity Chromatography

Retention

Retention of the compounds of interest, be they low-molecular-mass compounds or macromolecules is effected by the appropriate combination of buffer concentration and pH. Typically a pH of 7.0–8.0 would be used to promote binding to the antibody. It is also quite common to add a small percentage of sodium chloride to the buffer. Phosphate-buffered saline is the most common retention buffer quoted. In addition, in some cases, binding to the antibody depends on metal ions. For example, in the case of protein C, binding only occurs in the absence of calcium ions.

Elution

Having retained the compound or macromolecule of interest on the immobilized antibody, elution can be accomplished by a variety of methods. If the antibody is covalently bound to the support, relatively strong eluotropic conditions can be used including organic solvents such as ethanol, changes in buffer concentration and/or pH, or the use of chaotropic reagents. For speed and sharp elution profiles of the analyte/product, a rapid change in eluent composition from conditions promoting retention to those favouring elution can and are used, i.e. a simple step gradient. However, gradient elution can be used if less aggressive conditions need to be employed and the dilution of the target molecule, relative to step gradient elution, can be accommodated. A further method of elution that can be used where antibodies with relatively weak affinities are used is competitive displacement using another molecule that has an affinity for the antibody (this technique has been termed 'weak affinity chromatography'). In addition, as described below in greater detail, peptides (against which the antibodies were raised) that correspond to a particular region of the target proteins can be used to promote desorption.

Furthermore, as noted above, certain proteins binding to the antibody is metal ion dependent. Thus the protein C bound in the absence of calcium ions was eluted from the immunosorbent using a calcium chloride-containing buffer.

'Analytical' Applications of Immunoaffinity Chromatography

Immunoaffinity chromatography in essentially analytical applications can be considered under a number of headings. These include clinical analysis of endogenous macromolecules for the diagnosis and monitoring of disease, drug analysis in biological fluids (clinical, pharmaceutical and toxicological) and environmental monitoring (e.g. for pesticides in water, etc.). In such methods, a variety of analytical end points are possible ranging from the direct detection of the analyte following elution from the immunoaffinity column. Alternatively, many systems have been developed where the immunoaffinity column is placed in series with an analytical chromatography column and appropriate detector. Whatever the ultimate configuration of the system, the matrix containing the analytes passes through the immunoaffinity column which selectively extracts it allowing contaminants to pass through unretained. The analyte is then eluted from the immunoaffinity support for quantification.

Macromolecules

There are a considerable number of immunoaffinity chromatography-based methods in the literature for clinical analysis. Analytes include anti-idiotypic antibodies, fibrinogen, granulocyte colony-stimulating factor (GCSF), immunoglobulin G and E antibodies, transferrin and interferon, etc. These methods have been demonstrated to compare well with other techniques such as for example, electrophoresis or immunoassay. In such assays the columns seem to be stable up to several hundred sample applications.

Drugs

Immunoaffinity has been used for the measurement of several drugs and endogenous compounds, including anabolic steroids, betamethasone, bufuralol, clenbuterol, corticosteroids, dexamethasone, fluoroquinones, leukotrienes, LSD, morphine, *S*-phenylmercapturic acid, salbutamol, sulphathiazole, tetracyclines, and the thromboxanes TxB1 and TxB2. Matrices include blood, plasma, urine, faeces, liver, milk and honey. In the case of immunoaffinity extraction, this has been used offline (as a form of solid-phase extraction) and in HPLC column-switching mode, with the immunoaffinity column as the first column. In some instances, the immunoaffinity column was used directly on diluted urine or plasma as the only sample preparation; in others, it was used in combination with other sample pretreatment steps such as liquid–liquid extraction or protein precipitation. Even with these most challenging samples, immunoaffinity chromatography was able to produce clean chromatographic traces.

Environmental Samples

A number of pesticides and other trace organics of environmental interest have also been determined by methods incorporating immunoaffinity chromatography. Examples include aflatoxins, algal toxins, atrazine and triazines generally, carbendazim, chlortoluron, isoproturon and other phenylurea herbicides, mycotoxins, ochratoxin A, polyaromatic hydrocarbons, and TCDD. Matrices have included nuts, milk, shellfish, water, cereals, coffee, beer, wheat, sludge, sediment, tissue, soil, potatoes, carrots, peas, serum, and fruit juice. One of the most popular examples of the use of immunoaffinity chromatography is for the determination of pesticides in water. The antibodies bind the analyte very tightly so large volumes of water can be passed through an immunoaffinity column to facilitate trace enrichment and clean-up in one step.

Immunoaffinity chromatography has also been proposed as a simple inexpensive device for monitoring chlortoluron in water. Antibodies were bonded to a column and water passed through. Reagents to give a colour stain, the length of which would give a semiquantitative measure of pesticide concentration provided a simple rapid test.

Protein Purification by Immunoaffinity Chromatography

The purification of high-value products from complex biological matrices such as fermentation broths or extracts still represents a considerable challenge. In such preparative applications there is clearly a requirement to deal with large quantities of biological matrices (either fermentation broths or e.g. plasma after varying degrees of preliminary clean-up) compared to the analytical examples cited above. The use of larger columns also means that larger quantities of antibody are required in order to prepare sufficient immunosorbent resulting in considerable expense, which can effectively limit the range of applications. A further consequence of the high cost of the columns is the need to protect them from contamination or mechanical damage which might shorten the lifetime of the column. In addition, where the purified proteins from immunoaffinity chromatography are designed for use in the clinic it is important to ensure that the antibody does not contaminate the product due to leakage from the sorbent as it may itself produce an immune response in the patient. The cost of

the antibody has led to a considerable amount of work aimed at optimizing the capacity of the sorbents, as well as the optimization of flow rates, pressure limits and mechanical stability. It seems to be generally accepted that the typical operational life of such a column is about fifty uses without significant loss of purification capacity, depending upon matrix and process conditions.

An illustrative example of the use of immunoaffinity chromatography for the purification of a protein is provided by studies on protein C, a vitamin K-dependent glycoprotein. This protein has a molecular weight of 62 000 Da, comprising a light chain of 21 000 Da and a heavy chain of 41 000 Da and contains some 23% as carbohydrate. Protein C has potent anticoagulant properties and it is envisaged that there may be potential for its use therapeutically for patients with protein C deficiency, abnormal clotting problems or in victims of heart attacks, etc. In this study a murine monoclonal antibody (Mab 8861) to human protein C was bonded to a variety of support materials and their comparative performance assessed. Columns prepared with these materials were equilibrated with an adsorption/wash buffer (consisting of 0.02 mol L^{-1} sodium citrate, 0.08 mol L^{-1} sodium chloride) at a pH of 6.0 (chosen to increase the stability of the load material). The sample containing protein C was then loaded on to the column which was then left for 30 min to ensure sufficient time for the protein to interact with the antibody. The column was then washed for a further 30–40 min (4–5 column volumes) after which the elution solvent (0.1 mol L^{-1} sodium carbonate, 0.15 mol L^{-1} sodium chloride pH 10) was applied to the column to recover the protein C. The eluent containing the protein C was then immediately taken to pH 7.5 with HCl. However, as described above the binding of protein C can also be dependent on the absence of calcium ions, and in an alternative protocol, using the murine antibody 7D7B10-Mab to human protein C (bound to either agarose or cellulose) this property was exploited. Thus feedstock from the recombinant protein (pre-centrifuged transgenic pig milk whey containing 50 mmol L^{-1} EDTA) was diluted with buffer (1.0 mol L^{-1} sodium chloride, 0.05 mol L^{-1} Tris, 0.025 mol L^{-1} EDTA at pH 7.0) (3 parts buffer to 1 part whey). After centrifugation, the sample was applied to the immunosorbent, and the columns then washed with 18 volumes of buffer. Elution was accomplished with the same buffer with the addition of 25 mmol L^{-1} calcium chloride. Following elution the columns were then regenerated with successive washes of 4 mol L^{-1} sodium chloride, 2 mol L^{-1} sodium thiocyanate and then the application buffer (with 5 mmol L^{-1} EDTA).

Obviously one problem that afflicts all immunoaffinity-based methods is that an antibody to the target protein must first be raised, which may be difficult if the target protein is not available in sufficient quantity or purity. It has now been demonstrated that, for example, peptides to the C-terminal regions of chimeric α-amylase, recombinant CD2 and the insulin β-chain can be used to obtain antibodies. These rabbit antibodies had sufficient affinities to the target proteins to be suitable for use on immunosorbents. The peptide to which the antibody has been raised can then be used as a mobile-phase additive in the eluent in order to displace the target protein from the antibody. Thus, when processed fermentation broths containing the target protein mentioned above were applied to the column they could be recovered either using non-specific eluents (e.g. 2.5 mol L^{-1} sodium thiocyanate, 5 mmol L^{-1} calcium chloride, pH 5.0 for the amylase), or by eluents containing the appropriate peptide in a sodium acetate (50 mmol L^{-1})–calcium chloride (5 mmol L^{-1}) buffer at pH 5.0. For the amylase example, concentrations of the peptide in the eluent of 0.153 or 0.48 mg mL^{-1} produced similar elution profiles, with recoveries of 50–60% of the adsorbed protein (similar to the recoveries with the nonspecific eluent). To regenerate the immunoaffinity column the peptide was then eluted with 0.1 mol L^{-1} HCl. This process of protein purification is interesting as it avoids the use of chaotropic reagents, and also resulted in high-purity products. Post-elution from the column, the proteins and eluting peptides could be separated by ultrafiltration or gel permeation chromatography.

As these examples show, immunoaffinity chromatography for protein purification is a well established and effective method for obtaining high-value proteins, with a continuing high level of innovation.

Conclusions

Immunoaffinity chromatography is a widely used, and useful, family of techniques for the isolation or analysis of both macromolecules and low-molecular-mass compounds for either preparative or analytical purposes. Continuing advances in the production of antibodies and immunoaffinity phases by the use of improved supports and coupling chemistries will result in higher capacities and longer-lived materials. These advances will undoubtedly result in the increased use of immunoaffinity methods in analytical chemistry and in biotechnological applications.

See also: **I/Affinity Separation. II/Chromatography:** Polymer Separation by Size Exclusion Chromatography;

Membrane Separations: Ultrafiltration. **III/ Immunoaffinity Extraction. Pesticides:** Extraction from Water. **Appendix 1/Essential Guides for Isolation/Purification of Enzymes and Proteins. Essential Guides for Isolation/ Purification of Immunoglobulins. Appendix 2/Essential Guides to Method Development in Affinity Chromatography.**

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Imprint Polymers

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Introduction

Molecular imprinting is now recognized as one of the most rapid and powerful methods for creating tailor-made synthetic receptors with strong, yet selective, affinities for a diverse selection of analytes. The imprinting of small organic compounds, metal ions and peptides is well developed and almost routine, and the imprinting of much larger analytes, such as proteins and cells, has also now been demonstrated. The impressive molecular recognition characteristics of molecularly imprinted materials, allied to their highly robust physical nature, makes them ideally suited for numerous applications in affinity separation. This article will outline the general principles behind molecular imprinting and the generic approaches to the preparation of imprinted materials. Particular emphasis will be placed on their role as affinity materials in separation science.

The Imprinting Principle

Molecular imprinting has been demonstrated in silica and in synthetic organic polymers, but it is organic polymers that have found the most favour and indeed probably have the most to offer to the affinity separation area. The rest of this article will therefore deal exclusively with molecular imprinting in the latter medium.

The technique of molecular imprinting in organic polymers is a polymerization process in which a rigid, and insoluble, macroporous polymer network is formed around an analyte (template) of interest (**Figure 1**). In a typical imprinting experiment the analyte is initially allowed to form, in solution, an assembly with one or more functional monomers, which interact with the analyte via either covalent or non-covalent bonds. Once the assembly has been generated, copolymerization with an excess of cross-linking monomer (usually >50 mol%) is initiated, and the insoluble polymeric product phase separates from solution as the polymerization proceeds. The analyte functions as a template during the