Immobilized Boronates/Lectins

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Lectins and boronates have affinity for very similar biological compounds. They are both useful for separating glycoproteins, glycolipids, and other glycated compounds as well as for the separation of sugars and, in the case of boronates, other compounds containing appropriate 1,2 or 1,3 diols. There is, however, a fundamental difference between lectins and boronates. Lectins bind to glycated molecules because of a natural biological interaction between the lectin and the sugar moiety of the glycated compound. Lectins have biological purposes and functions related to this sugar–lectin interaction. On the other hand, boronates have no biological function but serve as a compound which, by chemi-selection, binds to 1,2 or 1,3 vicinal, co-planer, diols. Since many sugars contain such diols, boronates are very useful functioning as a 'chemical lection'. However, lectins have a much higher degree of binding specificity, as would be expected from the fact that biological interactions depend on multiple binding sites and intricate interactions with complex stereochemistry. Thus, any given lectin may bind to a specific sugar; or in other cases, may bind to an array of sugars. Thus, there exists a broad repertoire of lectins that are capable of binding to various types of glycated biomolecules. Fortunately, a sufficient number of lectins are available commercially to provide essentially the entire array of binding sites and, certainly, that array which is needed for biological separations of glycated biomolecules.

The corollary to this is that while boronates have a fairly low degree of specificity, they have a much higher stability than lectins. Thus an immobilized boronate, such as a boronate chromatography matrix, can be stored for years, whereas a lectin may have limited storage and operational stability.

Finally, in lectin affinity chromatography it should be understood that lectins are purified by affinity chromatography on a variety of immobilized sugars. Such 'reverse' lectin chromatography is beyond the scope of this discussion but does provide a methodology for identifying and isolating lectins that have a unique specificity for a particular rare combination of one or more sugar molecules.

Applications

None of these can be considered *the* application for lectin or boronated chromatography. By far the largest application of both is in the separation of glycated haemoglobin from non-glycolated haemoglobin for diagnosis of diabetes (see below).

Other diagnostic applications for both lectins and boronates have been proposed. In particular, lectins have been used to bind proteins associated with specific forms of cancer. These have the potential to be developed into useful diagnostic procedures.

In addition, both lectins and boronates have the potential to be used in large-scale purification of various glycated proteins and other glycated biomolecules for commercial purposes. Since most extracelluar mammalian proteins are glycated, and since many of these have useful commercial value, boronate chromatography is likely to develop into a significant tool for commercial purification.

Basic research also utilizes affinity chromatography based on lectins and boronates. Lectins are far more commonly utilized when the goal is to identify different glycated isoforms of particular proteins or to determine which oligosaccharides are present on the surface of the cells and organelles. Utilizing successive chromatography with a bank of lectins of various specificities, coupled with glycases with distinct and varied specificities, the entire sequential composition of oligosaccharides can be readily determined.

Other uses, of course, exist for lectin and boronate chromatography; for example, the purification of specific organelles or cells utilizing the sugar moieties of glycated proteins forming the membrane surface of the organelle and/or cell.

Problems or Pitfalls

There are numerous difficulties with any type of chromatography. Perhaps the foremost is the expectation from a method containing the word 'specific' that one will obtain a highly purified material in a single chromatographic step. Occasionally this can be true, but far more commonly either lectin or boronate chromatography is just one of several steps needed to obtain a reasonably pure product. The true advantage of these two chromatographic methods, however, is that they complement standard chromatographic methods such as ion exchange, gel permeation and hydrophobic chromatography.

Among the other problems associated with boronate and affinity chromatography is that the binding of molecules to a particular lectin or boronate can only be determined by trial and error. This calls for a fairly large pilot study which employs various lectins and/or boronate derivatives bound to various matrices and at several ligand concentrations. Without this information, it is difficult to optimize the separation; nevertheless, simple separations are sometimes done by taking a commercial immobilized lectin off the shelf and attempting a full chromatographic separation. In most cases, this is done with a concanavilin A stationary phase, since Con A is relatively inexpensive, fairly stable, and has a broad specificity for a number of saccharide moieties. Thus it has a high probability of effecting a reasonable separation of glycated molecules from nonglycated molecules or from molecules which are glycated in significantly different ways. Con A has a primary binding to glucose or mannose moieties which are, fortunately, very common terminal sugars in glycated proteins.

An additional problem found in affinity chromatography is nonspecific binding. Both the lectin itself and the alkyl portion of the boronate can be the source of nonspecific binding.

One final problem with lectin chromatography is the high toxicity of some lectins. The reader should be warned that some lectins are highly toxic and must be handled with extreme caution, although others have little or no toxicity.

Applications of Boronate Chromatography

The interaction of boronate with low molecular weight compounds has been known since the mid-nineteenth century when it was first observed by Biot. Since then this interaction has been employed for the separation of many biologically important vicinal diols, particularly monosaccharides, polysaccharides and various glycated macromolecules. The earliest applications involved the separations of various sugars. Boronate forms a charged complex with a sugar diol and, occasionally, a bridge compound in which two sugar molecules are bridged by a single boronate. These complexes can be separated from one another and from unchanged molecules by ion exchange chromatography. Boronate in elution media converts normally neutral sugar molecules into an ionic component in which the charge eventually is dependent upon the structure and stability of the boronate–sugar complex.

A variation of this is boronate affinity chromatography, in which a boronic acid is coupled by means of a spacer organic molecule to an appropriate matrix. The most widely used is *m*-aminophenylboronic acid, which has been coupled with agarose, polyacrylamide gel and beaded cellulose, among other matrices. In this case, the sugar will bind to the boronate matrix and can be eluded using appropriate elution conditions. Binding is generally aided by an alkaline pH at a fairly high ionic strength, since the resulting complex between the bound sugar and the immobilized boronate is negatively charged and high ionic strength stabilizes the charged spacer. Elution can be readily effected using mildly acidic or neutral pH and a low ionic strength eluent.

Separation of sugars on boronate affinity matrices has been little utilized and is of only minor importance. On the other hand, the separation of many biological macromolecules containing sugars is of extreme significance. Nucleosides, nucleotides, catecholamines and tRNA are among the more important compounds which have been separated by this method.

The most important separations that have been performed on boronated affinity chromatography are those of glycated proteins, including glycated enzymes. Most proteins exported from cells are glycated prior to their secretion. This glycation has been observed to be of significance in determining the lifetime of, for example, a serum protein, as glycases remove the sugar residues until a nearly sugar-free molecule is recognized by liver cells and removed from the blood stream by phagocytosis.

Many proteins which are naturally glycated in mammalian tissue are produced in recombinant microorganisms in a nonglycated form. While many of these have utility in a research laboratory, there is considerable reluctance to utilize human proteins without normal glycosylation as therapeutic materials; therefore, many of these mammalian proteins are now produced by recombinant systems in organisms which glycosylate the proteins in a way that mimics their natural glycated state. For this reason, determination of the oligosaccharide structure of glycosylated residues is very important. As will be seen in the following section, this is done chiefly using lectin chromatography. Boronate chromatography, on the other hand, provides a general way in which glycated proteins can be purified and separated from nonglycated proteins and often other glycoproteins. The interactions between the matrix and the glycated proteins need to be minimized, while the chemi-selective adsorption of the sugar diols to form diesters with the boronate needs to be maximized. To do this, the chromatography must be done under conditions in which the pH is reasonably high, usually above the p*K* of the boronate, and, at the same time, the ionic

strength must be high enough to suppress the ion exchange properties of the naturally charged boronate residues. At the same time, too high ionic strength will increase hydrophobic interactions between the protein to be purified and the matrix on which the boronate residue is bound or the organic molecule which bears the boronate, for example the phenyl portion of the *m*-aminophenylboronic acid. To accomplish both of these goals simultaneously, normally a cation such as magnesium is employed. Magnesium is effective at suppressing the boronate charge at relatively low concentrations, which do not significantly promote hydrophobic binding of the protein to the matrix. Many glycated proteins and enzymes can be separated from their nonglycated counterparts in a single chromatographic step, which, while not always producing a homogeneous protein, effects considerable purification and selection.

By far the most important boronate separation is the separation of glycated haemoglobin from its nonglycated counterpart. Glycated haemoglobin is created in a very different fashion from the biological glycation of secreted proteins. Haemoglobin produced within the red blood cell is not glycosylated; however, in a nonenzymatic process, amine residues of the haemoglobin react with glucose and form a transient Schiff's base. This Schiff's base then undergoes a rearrangement (the Amadori rearrangement) to produce a stable fructosamine derivative of the haemoglobin molecule. The rate at which this reaction occurs and thus the percentage of haemoglobin which is glycated, is totally dependent upon the concentration of glucose in the blood stream. Since the haemoglobin molecule has a half-life of approximately 60 days in the blood stream, the percentage of glycated haemoglobin provides a good measure of the long-term average blood glucose concentration. By separating the glycated haemoglobin from nonglycated haemoglobin, the physician has a diagnostic tool for the diabetic management of patients. Diabetic patients notoriously mis-report their exercise and adherence to dietary prescriptions of the physician. By taking a small sample of blood and separating the diabetic, or glycated, haemoglobin from nondiabetic, or nonglycated, haemoglobin, a reasonable measure of the history of the patient's serum glucose levels can be achieved. This is a very widely used test of great significance in the control of this important disease. Proteins, other than haemoglobin, could be utilized for this assay, since other serum proteins such as serum albumin undergo nonenzymatic glycosylation. Glycated haemoglobin is the most readily measured in a reliable fashion and has, to date, provided the physician with the best diagnostic methodology.

In addition to the diabetic analysis, boronate chromatography has the potential to form the basis of other significant diagnostic assays, such as assays for catecholamines, as well as for various differences in protein composition and nucleoside concentrations in various tumours. This potential has not yet been fully realized.

Lectin Chromatography

Immobilized lectins are true complements to boronate chromatographic materials. Lectins do not have the stability that boronates possess, but they possess a considerably higher and broader range of specificity. While there are several immobilized commercial boronate materials available for boronate affinity chromatography, there are a much greater number of different types of immobilized lectins. For example, one commercial firm offers 19 different lectins immobilized by several different methods, chiefly to agarose. The same firm offers approximately 60 purified lectins in a nonimmobilized state, any one of which could be immobilized by a researcher for his or her own specific purposes. Since the specificity of lectins can vary from relatively narrow specificity to those which have very broad specificities, and since the specificities between lectins are so varied, they offer a very powerful tool for separating many glycoproteins. An idealized purification scheme utilizes boronate affinity chromatography in an initial purification step, which allows one to obtain first the glycated protein fraction as a whole, followed by lectin affinity chromatography to separate the glycated proteins.

Lectins are obtained from either plant or animal materials and have various biological functions. Originally, lectins were also termed phytohaemagglutinins because they were isolated from plant sources (phyto) and were used to classify blood cells by their agglutinizing property (haemagglutinins). The agglutinization occurs because lectins are multimeric proteins with multiple sugar binding sites which can crosslink red blood cells, thus aggregating them.

There are two basic approaches to lectin affinity chromatography. At the present time, the easiest approach, and the one most commonly used, is to survey the commercially available lectins, preferably those which are already immobilized, for their ability to bind the target protein. It is helpful if the terminal sugar of the glycated protein is already known, since that makes the choice of lectin much easier. On the other hand, it is also relatively simple to carry out a trial and error procedure to determine which immobilized lectin is best in the purification of a particular

protein. It is also possible to immobilize an appropriate lectin from among the many commercial lectins that are available if the terminal sugars of the glycoconjugate to be purified are known.

The second approach is to determine the terminal sugar of the glycoconjugate to be purified and then to purify, *de novo*, a new lectin, by immobilization of the desired terminal sugar to a matrix, such as agarose, by means of a spacer arm. This allows the screening of numerous potential lectin sources to find as many lectins as possible that will bind to the target sugar. This methodology was used for many years prior to the present significant commercial availability of purified lectins.

After the lectin has been chosen and obtained in an immobilized form, or is immobilized by one of many simple procedures, for example agarose or cyanogen-bromide-activated agarose, or Affi-gel 10, adsorption/desorption, method conditions need to be defined. The binding of glycoproteins to lectins is generally easier than their elution. The factors in binding are generally temperature and salt concentration, as well as the density of coverage of the lectin immobilized on the matrix. High densities of lectins are not desirable in most instances. Although high ligand density may yield a marginal increase in capacity, it significantly increases the difficulty of eluting the target protein. The use of a moderate salt concentration is often helpful during binding of the glycoprotein target to the immobilized ligand. In many instances, it is necessary to be certain that the required metal ions are included in the sample wash and elution buffers in order to prevent deforming and/or denaturation of the lectin. Once the protein binding conditions have been determined, elution conditions need to be investigated. If one wants to have the highest purity product and to be certain that binding the protein to the immobilized lectin is through the biologically significant, sugar–protein interaction, then elution should be done by a high concentration of the free sugar. This will bind competitively to the lectin, displacing the glycoconjugate and thus eluting it. The sugar concentration during elution must be high enough to compete effectively with the lectin for the glycoconjugate, particularly when the density of the immobilized lectin is high.

Nonspecific elution of the glycoprotein from the lectin is frequently used. Changes in pH, temperature and salt can affect elution by decreasing the affinity of the lectin for the glycoconjugate. If elution were either with free sugar or by changing binding conditions, for example pH, a good yield is not obtained. It is also possible to apply an eluent to the column and stop the flow, thus allowing equilibrium to be reached in the free solution. This may or may not be necessary, however, and frequently a simple clean elution can be obtained by a proper choice of conditions.

One of the more interesting applications of immobilized lectins is the determination of the structure of sugar residues bound to the glycoconjugate. For example, a protein will be applied to a specific immobilized lectin and found to bind to that lectin. If the lectin has a rather broad specificity, the investigator cannot be certain which sugar is the terminal sugar on the oligosaccharide moiety of the glycoprotein. However, by using various glycases to remove the terminal sugar, the investigator can determine which glycase yields a derivative that will no longer bind to the immobilized lectin used. The glycase specificity indicates the identity of the terminal sugar. This procedure can be done repetitively and from the results a resonable understanding of the structure of the oligosaccharide of the glycated protein can be determined.

Conclusion

Both boronate chromatography and lectin affinity chromatography have considerable potential for future use in biotechnology. Proteins with proper glycosylation are becoming more and more important because it is perceived to the desirable to have proper glycosylation for therapeutic purposes. In addition, researchers are becoming more and more aware that the structures of oligosaccharides bound to glycosylated proteins contain valuable information on the biological system from which the protein was isolated. It appears that both boronate and affinity chromatography will be valuable in basic research and in commercial protein purification and undoubtedly for other applications as yet unknown.

See also: **III/Immobilised Boronic Acids:** Extraction.

Further Reading

Adamek V, Liu X-C, Zhang YA, Adamkova K and Scouten WH (1992) New aliphatic boronate ligands for affinity chromatography. *Journal of Chromatography* 625: 91–99.

Beneš M, Štambergova A and Scouten WH (1993) In: Ngo T (ed.) Affinity chromatography with immobilized benzeneboronates. *Molecular Interactions in Bioseparations*, pp. 313–321. New York: Plenum Press.

Bergold A. and Scouten WH (1983) In: Scouten WH (ed.) Boronate chromatography. *Solid Phase Biochemistry*, pp. 149–188. New York: Wiley.

Freeze NH (1995) Lectin affinity chromatography. *Protocols in Protein Chemistry*, pp. 901–919. New York: Wiley.
Gerard C (1990) Purification of glycoproteins. In: Deutscher MP (ed.) *Guide to Protein Purification, Methods in Enzymology*, vol. 182. New York: Academic Press.
Liu X-C and Scouten WH (1994) New ligands for boronate affinity chromatography. *Journal of Chromatography A*, 687: 61–69.
Liu X-C and Scouten WH (1996) Studies on oriented and reversible immobilization of glycoprotein using novel boronate affinity gel. *Journal of Molecular Recognition*, 9: 462–467.
West I and Goldring O (1996) Lectin affinity chromatography. In: Doonan S (ed.) *Methods in Molecular Biology, Protein Purification Protocols*, vol. 59, pp. 177–185. New Jersey: Humana Press.

Immobilized Metal Ion Chromatography

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Introduction

Since its introduction by Porath in 1975, immobilized metal ion affinity chromatography (IMAC) has developed into a robust and versatile tool. The number of uses is large and includes the isolation of metal-binding compounds from sea water, separation of enantiomeric forms of amino acids, tetracycline removal from animal products and protein purification. This article will focus on its application to protein purification, where it relies on the ability of certain amino acid side chains to form coordinative interactions with immobilized metal ion chelate complexes. As a chromatographic method it falls somewhere between biospecific affinity chromatography and ion exchange chromatography. The evolution of the technique, current tools and some specific technical details are discussed.

Background

Knowledge of the interaction of metal ions with proteins and the potential utility of immobilized metal chelators began during 1940–50, although it was not until 1974 that the method was first used to isolate a metalloprotein. The general use of IMAC was initiated in 1975 with a *Nature* publication from Porath. A summary of key milestones in the history of IMAC is presented in **Table 1**.

In the late 1970s and 1980s there were numerous publications on the choice of metals and investigations on the precise nature of the interactions that take place with proteins. It was assumed that surface-exposed residues were coordinating with the immobilized metal ions. Studies using free amino acids, peptides, and eventually engineered recombinant proteins, revealed the importance of certain amino acids – in particular histidine. Additionally, depending on the metal and chelating ligand employed, the spatial arrangement of the amino acids within the peptide or protein was also found to influence binding. This led to studies using model peptides with a wide range of histidine-containing sequences and in 1988 the first use of six consecutive histidine-residues as a purification tag (6His tag). In parallel with this 1987 saw the introduction of a metal–chelate complex with a high degree of selectivity for adjacent histidine residues (Ni^{2+}–NTA). Proteins purified using the 6His tag have been found to retain biological activity and their structures have also been solved by both X-ray and NMR – illustrating that, in the absence of metal ions, the tag has no defined secondary structure. Despite the enormous utility of the 6His tag the use of metal chelating ligand/metal combinations still has a role to play in the isolation of nontagged proteins from a wide variety of sources. The literature contains many examples of using IMAC as a one-step process to isolate native proteins, e.g. α-lactalbumin from milk and factor IX from blood. In addition, immobilized Fe^{3+} has been successfully used to separate phosphoproteins and immobilized Ca^{2+} to purify calcium-binding proteins.

The potential exists for even wider application to the separation of protein mixtures, with new chelators being introduced (e.g. TACN, see below).

Table 1 Key dates in the history of immobilized metal ion affinity chromatography

1974	First use of immobilized chelators to isolate metalloproteins
1975	First description of general technique (IMAC) using IDA
1983	Introduction of high performance on silica based media
1986	Use of Fe^{3+} chelates to purify phosphoproteins
1987	Introduction of NTA
1988	Introduction of genetically engineered His tags
1992	Introduction of TREN
1998	Introduction of TACN