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Gel Filtration Chromatography*

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Summary

The technique of gel filtration chromatography is described, with a guide to relevant literature on methods, theory, and applications, especially the use of GFC in analysis of molecular interactions, both macromolecular and small molecule–macromolecule aggregation.

INTRODUCTION

Pores of molecular dimensions to separate molecules by size have been used for over a century, originating with the introduction of dialysis in 1861 (*1*). This technique, together with ultrafiltration under pressure or vacuum (*2*), is still of major practical importance.

It is, however, only within the last few decades that chromatographic materials which separate essentially by molecular size have been developed and widely used. Such materials have been known for 40–50 years in the form of zeolites and similar molecular sieves (*3*), and a sieving effect in ion exchange resins (*4*), starch gels (*5*), agar gels (*6*), and polyacrylamide gels (*7*) has occasionally been utilized in macromolecular separation. This molecular sieve effect in polyacrylamide gel is an important component of the high separation power of disc gel electrophoresis (*8*).

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In the above cases, the molecular sieve properties are secondary or incidental. In the last decade two major classes of materials for chromatography have been developed which rely primarily or exclusively on molecular size for separation. These are represented by the Sephadex series of cross-linked dextran gels (*9*) and highly cross-linked polystyrene gels (*10*).

These two classes of materials tend to be used by mutually exclusive groups and are generally called by different names. Thus the Sephadex-type materials are gels which swell in aqueous media and, under the term "gel filtration chromatography," have been extensively used in biochemistry; while the polystyrene gels are primarily used in organic solvents and, as "gel permeation chromatography," are employed in industrial and polymer chemistry.

The present conference is concerned almost entirely with gel permeation chromatography (GPC). It is hoped that in this context the present paper will provide an introduction for those familiar with GPC to the literature on "GFC," with particular reference to theories and applications that differ from those commonly used in GPC.

In essence this paper is an extension of a previous review by Altgelt and Moore (*10a*).

GFC MATERIALS

The materials used in GFC are characterized by their property of swelling in aqueous systems. There are two major groups of materials, the cross-linked polydextran gels (Sephadex), and cross-linked polyacrylamide (Bio-Gel P) gels (*11*, *12*).

Both materials come in a variety of types, varying in the molecular weight range over which fractionation occurs. For example, Sephadex is obtainable in types from G-10 (fractionation range 0–700) to G-200 (fractionation range 5,000–800,000). These MW fractionation ranges are specified for globular proteins; for dextrans the equivalent ranges are 0–700 and 1,000–200,000, respectively. Particle diameters of Sephadex (a bead polymer) vary from 20–300 μ, with a superfine grade (10–40 μ) available for thin layer chromatography (*13*). Bio-Gel is available in types from P-2 to P-300 (fractionation ranges 200–1,800 and 60,000–400,000, respectively).

For very high MW materials, such as nucleic acids or viruses, an agarose material, Sepharose, is supplied by Pharmacia (*14*).

As stated above, the Sephadex-type materials are designed for use

in aqueous systems, although alcohol–water mixtures, dimethylsulfoxide, formamide, and glycol may be used. Recently Pharmacia has developed the LH series of gels which swell in organic solvents. These gels, of which LH-20 is currently available, have been used for separations of such materials as steroids (*15*), petroleum hydrocarbons (*16*), and other organic molecules.

Although the primary mechanism of separation in GFC is by molecular sieve properties, it is important to recognize that under certain conditions (i.e., low ionic strength), adsorption, ion-exchange, or ion exclusion may occur. This is particularly noted in the retardation of aromatic compounds on Sephadex (*17*); this phenomenon has been used to fractionate amino acids (*18*). Other examples of applications of adsorption or ion-exchange in GFC include concanavalin purification by specific protein-carbohydrate interaction (*19*), fractionation of humic acid (*20*), and separation of nucleic acid components (*21*). It has even been possible to carry out a partial resolution of stereoisomers on Sephadex (*22*).

GFC TECHNIQUES

The usual techniques for gel preparation, column packing, and elution are summarized in relevant product literature (*11*, *12*), and in the work of Flodin (*23*). The swelling kinetics of Sephadex have been the subject of several reports (*24*, *25*), since this parameter is critical in gel preparation.

In general, simple open columns have been used, with typical diameters of 1–5 cm and lengths of 20–200 cm; closed columns with flow adaptors have been introduced by Pharmacia and several apparatus manufacturers. Automatic equipment has not been greatly utilized, partly because common applications of GFC, such as desalting, have not required such sophistication. However, closed systems with sample injection and provisions for recycling have been described (*26*), and are being used increasingly.

Sample application is one of the most critical steps in GFC, since a sharp boundary between sample and solvent is required. Several mechanical devices are available for this procedure (*11*), and applying the sample at a density above that of the solvent is a common technique; this is achieved by addition of neutral salt or sucrose (*27*). In this method, care must be exercised to keep sample viscosity low, or undesirable band spreading may result (*23*).

In general, for protein separation, desalting, or qualitative analysis of molecular binding, extreme accuracy is not required. It is possible, however, with meticulous attention to gel preparation, packing under reverse flow, slow elution rates, and small sample size to obtain elution volumes accurate to ~0.1% (B. F. Cameron, unpublished results, see Ref. *28*). For such accuracy, volume estimation by weight may be needed (*29*).

THEORETICAL ASPECTS

This topic will be only briefly covered, since most of the theories are common to GFC and GPC, and several reviews on theoretical aspects of GPC are available (*30*).

The process of GFC is usually looked upon as a process of steric exclusion with the gel pore geometry defined in various ways (*31*), or as a process of equilibrium partition in a three-dimensional gel fiber network (*32*). Careful study of column chromatographic and equilibrium partition of macromolecules by Ackers (*33*) suggested that at least for Sephadex gels G-150 and G-200 the chromatographic separation could not be explained by partition equilibrium. An expression was derived to characterize GFC as a process of restricted diffusion in cylindrical pores, a model essential similar to a capillary exclusion model proposed earlier (*34*). It is probably a reflection on adequacy of experiment rather than correctness of the theory that all these models fit experimental data equally well (*35*). A succinct summary of thermodynamic and hydrodynamic aspects of GFC has been published (*36*).

The thermodynamic parameters of swelling of Sephadex gels are a critical factor in a theoretical analysis of GFC, and have been the subject of a series of reports by Ogston and co-workers (*37*, *38*); the results on G-200 gel conflict with the conclusions of Ackers (*33*), and lend support to the equilibrium partition mechanism (*32*).

An alternate theory for molecular sieve partition has been suggested (*39*), based on a stochastic model of movement of a solute through the column (*40*). This model has not been applied to GFC, except to incorporate the formalism into an alternate expression (*41*) for general equations derived by Ackers for generalized pore geometry (*42*). However, elution profiles of both large and small molecules on Sephadex G-50 agree in shape with that proposed for the stochastic model by McQuarrie (*40*), and base widths are inversely proportional

to MW as predicted by Carmichael (*39*). (Unpublished experiments, B. F. Cameron.)

APPLICATIONS

The first applications of GFC were to desalting as a substitute for dialysis, i.e., separation of a macromolecule from low MW salts, or buffer exchange; and macromolecular fractionation, particularly on Sephadex types G-75 through G-200 (*23*, *43*). Such applications remain the most important uses.

Perhaps the application of GFC to biopolymers in aqueous systems, i.e., monodisperse materials, fixed the development of analytical methods of GFC. Conversely, in GPC, the problems involve nonaqueous systems and polydisperse polymer fractions, leading to an emphasis on problems of band spreading and data correction.

Thus, the problem of molecular weight determination in GFC, while an important use of the technique, has been relatively unimportant theoretically. In general, the elution volume at the peak of a monodisperse protein is correlated with an empirical function of molecular size, and the MW of the unknown is obtained from a calibration curve obtained on the same column with proteins of similar shape (i.e., globular or random coil) and known MW.

Several parameters of molecular size have been used in correlation to MW, those most often employed being a linear relation of V_e to logarithm of MW (*27*), or of the cube root of partition coefficient to the square root of MW (*44*). An alternate calibration is in terms of the relationship of elution volume to Stokes radius given by Ackers (*33*), although this is not as generally useful. It has been pointed out that any of these empirical approaches is useful as a method of estimating MW for one of a homologous series of macromolecules (*24*, *36*).

MOLECULAR ASSOCIATION

Very early in the development of GFC the technique was applied to detection and measurement of molecular association.

The simplest system is that of binding of a small molecule to a macromolecule, where both the macromolecule and the complex are excluded from the gel. For example, GFC has been extensively used to measure free and bound small molecules, such as calcium in serum (*45*). A sample is applied to the GFC column; concentration of the

bound small molecule is determined by analysis of the rapidly eluted macromolecular peak containing the complex, and concentration of the free species by difference. The method is equivalent to equilibrium dialysis of tightly bound species, and the two techniques have been shown to be quantitatively comparable (*46*).

This technique fails when the bound small molecule is freely dissociable from the macromolecule, since the complex will dissociate as it elutes and false low values of binding will be obtained. To assay such systems a variety of techniques have been used. Thus, binding may be determined as above in columns of varying length, and extrapolated to zero length (*47*), or the bound fraction may be determined by an equilibrium partition experiment rather than by chromatography (*48*, see Ref. *25*).

For the detection of such interaction, a GFC method was devised in which the macromolecule is equilibrated with a small molecule and chromatographed as a small sample on a column equilibrated with an equal concentration of the small molecule (*49*). If binding occurs, even weakly, the elution profile of the small molecule shows a positive peak at the elution volume of the macromolecule, and by conservation of mass, a corresponding trough at a greater volume. This method has been extended to quantitative estimation of equilibrium constants (*50*), although this is easier if analysis is carried out on the trailing boundary of a frontal analysis system (*51*), i.e., one in which sample volume is of the same order of magnitude as column volume such that a plateau region occurs.

The analysis of macromolecular association is the last area of major application for analytical GFC. Theoretical analyses of elution boundaries in rapidly interacting macromolecular species are based on the analysis of boundary forms in freely migrating systems (*52*). These concepts have been applied to the chromotographic case in several ways (*53–55*), all in essence relating the variation of elution volume or elution profile with concentration to the extent of interaction.

It is important to distinguish concentration dependence due to molecular interaction from that due to "physical" interaction; the latter is usually linear and positive, while the former exhibits curvature (*56*). Zonal (small sample) and frontal (large sample) techniques of measuring concentration dependence due to physical interaction have given discordant results, which have recently been reconciled by a consistent definition of concentration in zonal analysis (*57*).

In some cases it is necessary to use the GFC experiment to determine the nature of the system as well as the interaction constants. For a simple one-step polymerization reaction a method for analysis of stoichiometry has been described for zonal (*58*) or frontal (*29*) GFC. One useful feature of frontal analysis is that for reactions $nA \rightleftharpoons P$ with $n > 2$ the trailing boundary may be bimodal, and that the stoichiometric coefficient may be directly obtained from the profile (*59*). Such behavior has been described for chymotrypsin (*60*) and for soap micelles (*61*).

An alternative technique for analysis is to use elution data to define an apparent weight-average MW (*62*) and analyze the concentration dependence of this quantity by a general multinomial theory (*63*).

Finally, an equilibrium partition method may be used in which the partition coefficient is directly determined as a function of concentration (*25*, *64*).

For further details, especially in respect to analytical aspects of GFC, two recent reviews should be consulted (*65*, *66*).

A detailed study of enzyme association reactions by GFC has recently been carried out on rabbit phosphorylase (*67*). Phosphatase (*68*) has been studied at concentration levels equivalent to those used in kinetic studies; the ability of GFC to examine macromolecular association at these levels (pg/ml) is a unique advantage of the method. Many studies have been made of hemoglobin dissociation (*25*, *69*), illustrating the utility of GFC for macromolecular systems with a very low dissociation constant ($\sim 10^{-6} M$).

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