Chapter 2

Gel-Filtration Chromatography

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Abstract

Gel-filtration chromatography is a versatile method that permits the effective separation of biological molecules in high yield. This article describes the basis of the method, the selection of suitable operating conditions, and contrasts typical matrix types. Applications of the technique are described, with references to the scientific literature.

Key words Gel-filtration chromatography, Gel permeation, Gel exclusion, Size exclusion, Molecular sieve, Operating conditions, Separations, Molecular mass estimation, Size-exclusion reaction chromatography

1 Introduction

Gel-filtration chromatography is a form of partition chromatography used to separate molecules of different molecular sizes. This technique has also frequently been referred to by various other names, including gel-permeation, gel-exclusion, size-exclusion, and molecular-sieve chromatography. The basic principle of gel filtration is quite straightforward. Molecules are partitioned between a mobile phase and a stationary phase (comprising a porous matrix of defined porosity) as a function of their relative sizes. A column constructed of such a matrix, typically in bead form, will have two measurable liquid volumes, the external volume, consisting of the liquid between the beads, and the internal volume, consisting of the liquid within the beads. The external volume is usually referred to as the void volume (V_0), while the sum of the external and internal volumes is the total volume (V_t). Following sample application, molecules larger than the pores of the stationary phase matrix will be excluded from the internal volume within the beads and will, therefore, migrate quite rapidly through the column, emerging at V_0, while molecules both smaller than the matrix pores, as well as those intermediate in size, will equilibrate with both the external and internal liquid volumes, causing them to migrate much more

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slowly and emerge at a volume (V_e) greater than V_0. Molecules are, therefore, eluted in order of decreasing molecular size. The elution volume, V_e, of a particular molecule depends on the fraction of the stationary phase available to it for diffusion. This can be represented by the constant K_d or K_{av} (also referred to as the partition coefficient). Therefore:

$$V_e = V_0 + K_{av}\left(V_t - V_0\right)$$

Rearranging this equation gives:

$$K_{av} = \left(V_e - V_0\right) / \left(V_t - V_0\right)$$

In addition to molecular size or mass, the flow behavior of molecules through a gel-filtration column is also a function of their molecular shape or, to be more precise, hydrodynamic diameter. This is defined as the diameter of the spherical volume (hydrodynamic volume) created by a molecule as it rapidly tumbles in solution. When performing gel-filtration chromatography, one generally assumes that all of the molecules within a mixture have the same symmetrical shape, so that the order of elution will be one of decreasing molecular weight. Whereas this is an acceptable assumption in most cases, one must bear in mind that the operative molecule dimension during gel filtration is the hydrodynamic volume and, as such, an asymmetrical molecule will appear to elute with an abnormally high molecular weight compared with a symmetrical molecule of similar molecular mass. When separating out proteins, for example, the usual assumption is that all of the proteins in the mixture are globular. Asymmetrical proteins (fibrous proteins and certain glycoproteins), however, will elute with an anomalously high molecular weight compared with globular proteins of similar mass.

2 Selection of Operating Conditions

Various factors should be considered when designing a gel-filtration system. These include: (1) matrix choice, (2) sample size and concentration, (3) column parameters, (4) choice of eluent, (5) effect of flow rate, and (6) column cleaning and storage.

2.1 Matrix Choice

Under separation conditions, matrices should be inert with respect to the molecules being separated in order to avoid partial adsorption of the molecules to the matrix, not only retarding their migration through the column, but also resulting in "tailed" peaks (*see*, for example, [1]). The stability of the matrix to organic solvents, pH, and temperature is also an important consideration and these variables should be compatible with the properties of the molecules being separated.

When choosing a suitable matrix, one with a molecular mass fractionation range should be selected which will allow the molecule of interest to elute after V_0 and before V_t. The most suitable fractionation range, however, will not only be dictated by the molecular mass of the target molecule, but also by the composition of the sample being applied to the column. Therefore, the best separation of molecules within a sample having similar molecular masses is achieved using a matrix with a narrow fractionation range.

Gel-filtration matrices may be made from a wide variety of materials including dextrans (Sephadex™ series), agarose (Sepharose™ series), polyacrylamide (bio-Gel series), polyvinylethylcarbitol, polyvinylpyrrolidone, cellulose, silica-based materials, or from mixtures such as dextran-polyacrylamide (Sephacryl™ series) or dextran–agarose (Superdex™ series). Each of these materials has advantages and disadvantages. Dextran may be greatly improved as a support material by means of enhanced cross-linking and its "swelling" may be controlled by the surrounding ionic milieu. With agarose, this "swelling" is nearly independent of ionic strength and pH but high binding capacities may be obtained with synthesis of highly porous agarose particles. Polyacrylamide-based matrices have a swelling behavior similar to dextrans while cellulose has a hydrophilic surface (which is good for reducing nonspecific hydrophobic interactions). Although cellulose is relatively inexpensive, it requires extra cross-linking for stability. Acrylate-copolymer supports are stable across a wide range of pH values. Commonly used gel-filtration matrices consist of porous beads composed of cross-linked polyacrylamide, agarose, dextran (*see* Table *1*), or combinations of these, and are supplied either in suspended form or as dried powders.

Polystyrene-divinylbenzene matrices have hydrophobic surfaces which make them unsuitable for the separation of proteins. Silica matrices have good rigidity (making them suitable for high-pressure fast flow-rate systems) but have hydrophobic surfaces and are unstable at pH > 8. This has led to the development of coated polystyrene-divinylbenzene and silica bead matrices, which have hydrophilic surfaces.

There has been a drive to further reduce particle size in order to achieve faster speed and greater chromatographic resolution. Weaker polymeric resins may compress under pressure and flow, a drawback that limits the extent to which the particle size can be reduced for chromatographic applications. Porous hybrid organic/inorganic particles have been employed as the supports for size-exclusion chromatography [2]. More recently, bridged ethyl hybrid (BEH) particles, which have their surface modified with diol groups, have been developed to minimize nonspecific ionic interactions with proteins [3]. In addition, the high mechanical strength of BEH particles enables a reduction in particle size to 1.7 μm, providing significant improvements in chromatographic efficiency.

Table 1
Some media for gel filtration

Material	Fractionation range (globular proteins)	Advantages	Disadvantages
Dextran	Sephadex G-10: 0–700 Da Sephadex G-25: 1000–5000 Da Sephadex G-50: 1500–30,000 Da Sephadex G-100: 4000–150,000 Da Sephadex G-200: 5000–600,000 Da	Smaller fractionation ranges (G-10, G-25) are good for desalting	Expanded forms require low pressures/hydrostatic heads
Agarose	Sepharose 6B: 10,000–4,000,000 Da Sepharose 4B: 60,000–20,000,000 Da Sepharose CL-4B: 60,000–20,000,000 Da Sepharose CL-2B: 70,000–40,000,000 Da	Good for larger molecules. Cross-linked (CL) forms more robust	Must be kept wet and not allowed to dry out
Allyl dextran-bis-acrylamide	Sephacryl S-200 HR: 5000–250,000 Da Sephacryl S-300 HR: 10,000–1,500,000 Da Sephacryl S-400 HR: 20,000–8,000,000 Da	Not biodegradable, mechanically robust	Must be kept wet and not allowed to dry out

Da Daltons
This table lists some typical media used for gel filtration. It is not exhaustive; there are many others, including media for industrial use in special configurations. Users should consult manufacturers' technical information regarding suitability for particular requirements
Sephacryl®, Sephadex®, Sepharose®, and Superdex® are registered trademarks of GE Healthcare. Toyopearl resins from Tosoh Bioscience are made from polymethacrylate and are available with different fractionation ranges (Toyopearl® is a registered trademark of Tosoh Bioscience)

2.2 Overview Some of the More Commonly Used Matrices

2.2.1 Agarose-Based Support Media

Superdex is a series of size-exclusion chromatographic media consisting of a composite base matrix of dextran covalently attached to highly cross-linked agarose. Its relatively low nonspecific interaction generally permits high recovery of biological material, such as proteins. Higher flow/pressure tolerance, smaller bead size, and narrower particle size distribution results in increased resolution

and shorter run times for these media. They are also stable to repeated autoclaving and to exposure to abrasive chemicals and detergents (such as SDS, urea, and guanidine hydrochloride). These types of media are typically available both as small beads, which are optimal for micropreparative and analytical runs, as well as larger beads which are more suitable for larger scale preparative work.

The high rigidity of the Superdex media allows for viscous eluents to be run at relatively high flow rates. Nonspecific interactions are negligible when using buffers with ionic strengths in the range 0.15–1.5 M. For example, Superdex 200 Increase has a broad fractionation range that allows separation of a large variety of proteins (Mr 10,000–600,000 Da) with an optimized resolution for the antibody molecular weight range of Mr 100,000–300,000.

Agarose-based support matrices are alkali tolerant and, therefore, may be cleaned using sodium hydroxide (up to 1 M concentration). This type of cleaning is highly efficient, prolongs column life and minimizes the risk for carry-over between different runs.

2.2.2 Silica-Based Support Media

The support material in these media are polymers of silica. Silica gel, made from sodium silicate, is a granular, vitreous, porous mineral that can be processed into either granular or beaded form. As a desiccant, it has an average pore size of 2.4 nm and has a strong affinity for water molecules. As a chromatographic material, silica has the advantage that it has a larger pore volume and narrower pore size distribution. Hence, it often gives sharper peaks and better resolution in certain cases. However, larger pore size silica particles may be brittle and fragile, and subject to collapse at high pressures. In addition, only a limited range of pH values may be utilized with these media. Because silica dissolves quite readily above pH values of 7–8, it is not possible to exceed pH 7.5 in eluents used with silica columns. In general, silica has been found to give the best results for the separation of small proteins, ranging from 10,000 to 1,000,000 Da. Compared to other types of HPLC columns, silica-based SEC columns often claim to contain more pore volume per unit column volume, which would result in higher MW selectivity and better resolution.

2.2.3 Hydroxylated Meth-Acrylic Polymer Resins

Some SEC media are hydroxylated meth-acrylic polymer resins (e.g., TSKgel PW beads) and are made commercially in many different pore sizes and particle diameters (30 μm and smaller). They vary from the typical Toyopearl HW beads by having a higher degree of cross-linking (such cross-linking is often necessitated by the higher flow pressures required when using smaller particles for chromatographic separations). Since separation efficiency, peak width and resolution are all improved with this smaller particle size, these matrices are regarded as higher performing chromatography resins. TSKgel based resins may be used with high flow rates and are designed to withstand operating pressures up to 20 bar.

2.3 Sample Size and Concentration

Maximum resolution in gel-filtration chromatography depends on application of the sample in a small volume, typically 1–5 % of the total bed volume. For this reason, gel-filtration chromatography has an inherent low sample-handling capacity and, accordingly, should be performed late in a purification procedure when the numbers of different molecules in a sample are relatively low. The concentration of sample that can be applied to the column will be limited by the viscosity of the sample (which increases with sample concentration) relative to the eluent. A high viscosity will result in irregular sample migration through the column (with subsequent loss of resolution) and, in some instances, will reduce the column's flow rate. When separating proteins by gel filtration, the sample should not have a protein concentration in excess of 20 mg/mL.

2.4 Column Parameters

Maximum resolution in gel-filtration chromatography is obtained with long columns. The ratio of column diameter to length can range from 1:20 up to 1:100.

2.5 Choice of Eluent

As gel-filtration chromatography separates molecules only on the basis of their relative sizes, the technique is effectively independent of the type of eluent used. Elution conditions (pH, essential ions, cofactors, protease inhibitors etc.) should, therefore, be selected which will complement the requirements of the molecule of interest. However, the ionic strength of the eluent should be high enough to minimize protein-matrix and protein–protein associations by electrostatic or van der Waals interactions. The addition of 0.1 M NaCl or KCl to the eluent to avoid these interactions is quite common.

2.6 Effect of Flow Rate

Low flow rates offer maximum resolution during gel-filtration chromatography since flow rate and resolution are inversely related. The optimum flow rate for resolution of proteins is approximately 2 mL/cm^2/h, although much higher flow rates can be used, particularly with rigid matrices such as the Sephacryl HR range from GE Healthcare (30 mL/cm^2/h). Unfortunately, low flow rates mean longer separation times. Therefore, a compromise between desired resolution and speed must be decided upon.

2.7 Column Cleaning and Storage

Most gel-filtration matrices can be cleaned with 0.2 M sodium hydroxide or nonionic detergents. When left unused for long periods of time, matrices should be stored at 4 °C in the dark in the presence of an antimicrobial agent (e.g., 0.02–0.05 % w/v sodium azide or 20 % v/v ethanol).

3 Applications of Gel-Filtration Chromatography

One of the principal advantages of gel-filtration chromatography is that separation can be performed under conditions specifically designed to maintain the stability and activity of the molecule of

interest without compromising resolution. Absence of a molecule-matrix binding step also prevents unnecessary damage to fragile molecules, ensuring that gel-filtration separations generally give high recoveries of activity.

This technique, however, is not without its disadvantages. When separating proteins by gel-filtration chromatography, for example, proteolysis becomes an increasing problem, since the target protein frequently becomes the abundant substrate for proteases also present in the mixture, consequently reducing recovery of activity. Because of the large size of gel-filtration columns, large volumes of eluent are usually required for their operation, often creating excessive running costs. Gel filtration also has an inherent low resolution compared to other chromatographic techniques because none of the molecules are retained by the column and nonideal flow occurs around the beads. In addition, this technique has a low sample-handling capacity dictated by the need to optimize resolution.

Despite these disadvantages, gel-filtration chromatography still occupies a key position in the field of biomolecule separation because of its simplicity, reliability, versatility, and ease of scale-up. A brief overview of its main applications is given below.

3.1 Separation of Proteins and Peptides

Because of its unique mode of separation, gel-filtration chromatography has been used successfully in the purification of literally thousands of proteins and peptides from various sources. These range from therapeutic proteins and peptides, which together constitute a multibillion euro world-wide market, to enzymes and proteins for industrial applications; some examples are outlined below.

Recombinant human granulocyte colony stimulating factor (rhG-CSF) was refolded from inclusion bodies in high yield, with great suppression of aggregates formation, by urea-gradient size-exclusion chromatography on a Superdex 75 column [4]. A similar technique was used to purify human interferon-gamma, solubilized from inclusion bodies by 8 M urea, to a specific activity of 12,000,000 IU/mg with protein recovery of 67% [5]. Luteinizing hormone (LH) was purified 46-fold from crude pituitary extract by gel filtration on two Sephacryl S-200 columns. The method exploited differential binding of LH (in the crude extract) to blue dextran for the first chromatography step. Before the second step, addition of high salt released LH from the blue dextran, enabling effective purification [6]. Fusion ferritin (heavy-chain ferritin plus light-chain ferritin) has also been purified by urea-gradient gel filtration. In this case, fusion ferritin solubilized from inclusion bodies with 4 M urea was applied to the column. Refolding enhancers were included in the urea-diluent buffer subsequently applied to the column to produce properly folded fusion ferritin multimers [7].

A continuous rotating annular size-exclusion chromatography system permitted the purification of crude porcine lipase with productivity of approximately 3 mg lipase per mg gel per hour and an activity recovery of almost 99% [8].

Among food-use proteins, hen egg lysozyme has been successfully refolded using both acrylamide- and dextran-based gel columns (Sephacryl S-100 and Superdex 75, respectively) [9]. Gel filtration has also proven useful for the purification of the whey proteins alpha-lactalbumin and beta-lactoglobulin from aqueous two-phase systems [10].

Protein engineering techniques enable the design of self-assembling multimeric protein cages for applications in nanotechnology [11]. Grove et al. describe a gel-filtration method to examine the metal ion-mediated assembly of protein cages [12].

3.2 Size-Exclusion Reaction Chromatography: Protein PEGylation

Covalent attachment of PEG (polyethylene glycol; "PEGylation") to a protein can attenuate its antigenicity and/or extend its biological half-life or shelf life. Size-exclusion reaction chromatography (SERC) permits one to control the extent of a reaction (such as PEGylation) that alters molecular size and to separate reactants and products. In SERC, injection of reactants onto a size-exclusion chromatography column forms a moving reaction zone. Reactants and products partition differently within the mobile phase leading to different flow rates through the column. Thus, products are removed selectively from the reaction zone, shortening their residence time in the reaction zone and separating them into the downstream section of the column. In PEGylation, addition of PEG groups to the protein significantly increases molecular size, allowing the use of SERC to obtain a dominant final PEGylated protein size in high yield. The principle was successfully demonstrated using two model proteins, alpha-lactalbumin and beta-lactoglobulin [13].

3.3 Separation of Nucleic Acids and Nucleotides

Gel-filtration chromatography has for many years been used to separate various nucleic acid species such as DNA, RNA, and tRNA as well as their constituent bases, adenine, guanine, thymine, cytosine, and uracil. Linear phage lambda DNA and circular double stranded phage M13 DNA, for example, can be completely separated from chromosomal DNA and RNA by gel filtration on Sephacryl S-1000 Superfine [14]. Plasmid DNA can also be purified by gel filtration [15], although modern commercial kits often use a centrifugal spin column format for greater convenience. Limonta et al. [16] describe the novel use of two gel-filtration steps, one before and one after a reverse-phase operation, to purify plasmid DNA from a clarified alkaline *E. coli* cell lysate.

3.4 Virus Particles

Krober et al. [17] devised an open loop simulated moving bed (SMB) for the continuous size-exclusion chromatographic separation of influenza virus (derived from cell culture) from contaminating proteins. Overall productivity of the SMB process was estimated to be up to 3.8-fold greater than that of an optimized batch process.

Size-exclusion chromatography was used downstream of expanded bed adsorption chromatography to recover active recombinant hepatitis B core antigen (HbcAg) in 45% yield with a purification factor of 4.5 [18]. A Sephacryl S-1000 SF proved to be effective and economical in the purification of recombinant *Bombyx mori* nucleopolyhedrosis virus displaying human pro renin receptor [19]. Sephacryl S-1000 gel-filtration chromatography gave more effective purification of turkey coronavirus from infected turkey embryos than did use of a sucrose gradient [20].

3.5 Endotoxin Removal

The presence of bacterial endotoxin is unacceptable in injectable recombinant biologicals, since endotoxin in the bloodstream can induce a pyrogenic response. Good manufacturing practice (GMP) will effectively remove endotoxin, but preclinical biologics may be produced under non-GMP conditions. London et al. investigated various means of endotoxin removal from preparations of a recombinant human protein. Endotoxins typically form aggregates, which may be quite large. A Superdex 200 size-exclusion column (1.75 L bed volume) removed most of the "spiked" endotoxin from an applied sample of monomeric monoclonal antibody, which was obtained in good yield [21].

3.6 Absolute Size-Exclusion Chromatography (ASEC)

Absolute size-exclusion chromatography (ASEC) is a technique that couples a dynamic light scattering (DLS) instrument to a size-exclusion chromatography system for absolute size measurements of proteins and other macromolecules as they elute from the chromatographic system. Dynamic light scattering (DLS; also known as photon correlation spectroscopy or quasi-elastic light scattering) is a technique that uses light scattering patterns (usually from a laser source) to determine the size distribution profile of small particles in suspension, or of polymers (such as proteins) in solution [22]. DLS can also be used to probe the behavior of complex fluids such as concentrated polymer solutions.

The sizes of the macromolecules are measured as they elute into the flow cell of the DLS instrument from the size-exclusion column. It should be noted that the technique measures the hydrodynamic size of the molecules or particles and not their molecular weights. For proteins, a Mark–Houwink type of calculation can be used to estimate the molecular weight from the hydrodynamic size [23].

A big advantage of DLS coupled with SEC is the ability to obtain enhanced DLS resolution. Batch DLS is quick and simple to perform. Using SEC, the proteins and protein oligomers are separated, allowing oligomeric resolution. ASEC can also be used for aggregation studies: although the aggregate concentration may not be calculated, the size of the aggregate will be measured, being limited only by the maximum size eluting from the SEC columns. Limitations of ASEC include flow rate, concentration, and precision. Because a correlation function requires anywhere from 3 to 7 s to properly build, only a limited number of data points can be collected across the peak.

3.7 Molecular Mass Estimation

Gel-filtration chromatography is an excellent alternative to SDS-PAGE for the determination of relative molecular masses of proteins, since the elution volume of a globular protein is linearly related to the logarithm of its molecular weight [24]. One can prepare a calibration curve for a given column by individually applying and eluting at least five suitable standard proteins (in the correct fractionation range for the matrix) over the column, determining the elution volume for each protein standard, and plotting the logarithm of molecular weight versus V_e/V_0. When a protein of unknown molecular weight is applied to the same column and eluted under the same conditions, one can use the elution volume of the protein to determine its molecular weight from the calibration curve.

3.8 Group Separations

By selecting a matrix pore size which completely excludes all of the larger molecules in a sample from the internal bead volume, but which allows very small molecules to enter this volume easily, one can effect a group separation in a single, rapid gel-filtration step which would traditionally require dialysis for up to 24 h to achieve. Group separation can be used, for example, to effect buffer exchanges within samples, for desalting of labile samples prior to concentration and lyophilization, to remove phenol from nucleic acid preparations and to remove inhibitors from enzymes (*see*, for example, [25]).

3.9 Conclusion

Despite its disadvantages of sample dilution and the need for a low ratio of sample volume to column volume, gel filtration remains a popular separation method due to its versatility, the wide range of matrices commercially available and the mild conditions of operation. It is hoped that this article has given the reader some grasp of the technique's wide range of applications and how to choose appropriate conditions for its gainful use. Useful handbooks on both gel-filtration and other chromatography techniques are available through the GE Healthcare Lifesciences website (www.Gelifesciences.com).

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