

# Chapter 2

## Gel-Filtration Chromatography

Ciarán Ó'Fágáin, Philip M. Cummins, and Brendan F. O'Connor

### Abstract

Gel-filtration chromatography is a popular and versatile technique that permits the effective separation of proteins and other biological molecules in high yield. Here, the basis of the method is described and typical matrix types are contrasted. The selection of suitable operating conditions and applications of the method are also discussed.

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

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### 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 relatively simple. 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. They will, therefore, migrate quite rapidly through the

column, emerging at  $V_0$ , while molecules 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 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:

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

Rearranging this equation gives:

$$K_{av} = \frac{(V_e - V_0)}{(V_t - V_0)}$$

In addition to molecular size or mass, the flow behaviour 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 the molecules within a mixture have the same symmetrical shape, so that the order of elution will be one of decreasing molecular weight. Although 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 weight. When separating out proteins, for example, the usual assumption is that all the proteins in the mixture are globular proteins. Asymmetrical proteins (fibrous proteins and certain glycoproteins), however, will appear to elute with an abnormally high molecular weight compared with globular proteins of similar molecular weight.

### **1.1. 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.

#### **1.1.1. Matrix Choice**

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. The matrix should be compatible with the properties of the molecules being separated and its stability to organic solvents, pH, and temperature

**Table 1**  
**Some media for gel-filtration**

Material	Media <sup>a</sup> and fractionation range (globular proteins)	Advantages	Disadvantages
Dextran	Sephadex G-10 0–700 Da Sephadex G-25 1–5 kDa Sephadex G-50 1.5–30 kDa Sephadex G-100 4–150 kDa Sephadex G-200 5–600 kDa	Smaller fractionation ranges (G-10, G-25) are good for desalting	Expanded forms require low pressures/hydrostatic heads
Agarose	Sepharose 6B 10–4,000 kDa Sepharose 4B 60–20,000 kDa Sepharose CL-4B 60–20,000 kDa Sepharose CL-2B 70–40,000 kDa	Good for larger molecules. Cross-linked (CL) forms are more robust	Must be kept wet and not allowed to dry out
Allyl dextran-bis-acrylamide	Sephacryl S-200 HR 5–250 kDa Sephacryl S-300 HR 10–1,500 kDa Sephacryl S-400 HR 20–8,000 kDa	Not biodegradable, mechanically robust	Must be kept wet and not allowed to dry out

This table lists some typical media used for gel-filtration. It is not exhaustive, and there are many others, including media for industrial use in special configurations. Users should consult manufacturers' technical information regarding suitability for particular requirements

*Da* daltons, *kDa* kilodaltons

<sup>a</sup>Sephacryl<sup>®</sup>, Sephadex<sup>®</sup>, Sepharose<sup>®</sup>, and Superdex<sup>®</sup> are registered trademarks of GE Healthcare. Toyopearl resins from Tosoh Bioscience may also be used for size-exclusion chromatography; these are made from polymethacrylate and are available with different fractionation ranges (Toyopearl<sup>®</sup> is a registered trademark of Tosoh Bioscience)

is also an important consideration. 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 (e.g. see (1)).

When choosing a suitable matrix, one with a molecular mass fractionation range, which will allow the molecule of interest to elute after  $V_0$  and before  $V_t$ , should be selected. The most suitable fractionation range, however, will be dictated not only 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.

### 1.1.2. Sample Size and Concentration

Maximum resolution in gel-filtration chromatography depends on the application of 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 quite late in a purification procedure when the numbers of different molecules in a sample are relatively low. The concentration of a sample which can be applied to the column will be limited by the viscosity of the sample (which increases with the sample concentration) relative to that of 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 flow rate. When separating proteins by gel-filtration, the sample should not have a protein concentration in excess of 20 mg/mL.

#### 1.1.3. 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.

#### 1.1.4. 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.) which will complement the requirements of the molecule of interest should, therefore, be selected. However, the ionic strength of the eluent should be high enough to minimise protein-matrix and protein-protein associations by electrostatic or van der Waals interactions (e.g. see (1)). The addition of 0.1 M NaCl or KCl to the eluent to avoid these interactions is quite common.

#### 1.1.5. 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<sup>2</sup>/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<sup>2</sup>/h). Unfortunately, low flow rates mean longer separation times. Therefore, a compromise between desired resolution and speed must be decided upon.

#### 1.1.6. Column Cleaning and Storage

Most gel-filtration matrices can be cleaned with 0.2 M sodium hydroxide or non-ionic 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 (0.02–0.05% [w/v] sodium azide or 20% [v/v] ethanol).

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## 2. 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 separation technique, however, is not without its disadvantages. When separating proteins by gel-filtration chromatography, proteolysis, for example, becomes an increasing problem: the target protein frequently becomes an abundant substrate for proteases that may also be present in the mixture, leading to reduced 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 with other chromatographic techniques, because none of the molecules are retained by the column and non-ideal flow occurs around the beads. In addition, this technique has a low sample-handling capacity dictated by the need to optimise 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.

### **2.1. 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 worldwide market, to enzymes and proteins for the brewing, food-processing, and diagnostics industries; some examples of each type are cited 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 (2). A similar technique was used to purify human interferon- $\gamma$ , solubilised from inclusion bodies by 8 M urea, to a specific activity of 12,000,000 IU/mg with protein recovery of 67% (3). Luteinising hormone (LH) was purified 46-fold from a 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 (4). Fusion ferritin (heavy-chain ferritin plus light-chain ferritin) has also been purified by urea-gradient gel-filtration. In this case, fusion ferritin solubilised from inclusion bodies with 4 M urea was applied to the column. Refolding enhancers were included in the urea-diluent buffer that was subsequently applied to the column to produce properly folded fusion ferritin multimers (5).

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% (6). 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) (7). Gel-filtration has also proven useful for the purification of the whey proteins alpha-lactalbumin and beta-lactoglobulin from aqueous two-phase systems (8).

## **2.2. Separation of Other Biomolecules**

Carbohydrates represent a plentiful, but so far only scarcely exploited, reservoir of unique, multifunctional biopolymers which can be readily fractionated by gel-filtration chromatography on the basis of their relative sizes (e.g. see (9, 10)). Various problems, however, have limited the development of gel-filtration methods for oligosaccharides. First, many of the commercially available gel-filtration matrices are themselves carbohydrates (e.g. Sephadex, Sepharose, etc., manufactured by GE Healthcare), shedding milligram quantities of heterodisperse carbohydrate polymers into the mobile phase. Second, non-specific interactions with matrix materials are common, since sugars are essentially amphipathic with a hydrophobic ring structure and hydrophilic functional groups. Despite these problems, however, gel-filtration chromatography still remains an important option for the purification of complex oligosaccharides.

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 (11). Plasmid DNA can also be purified by gel-filtration (12), although modern commercial kits often use a centrifugal spin column format for greater convenience. One recent study describes 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 *Escherichia coli* cell lysate (13).

## **2.3. Separation of Cells and Virus Particles**

Cells of different sizes can be efficiently separated from one another using gel-filtration chromatography. Methods have been developed, for example, to separate both erythrocytes (14) and platelets (15) from blood (most workers now prefer density-gradient media such as Percoll™ or Ficoll™, trademarks of GE Healthcare, for tasks of this nature). 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 (16). 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 (17). Sephacryl S-1000 gel-filtration chromatography gave more effective purification of turkey coronavirus from infected turkey embryos than did use of a sucrose gradient (18).

#### **2.4. Group Separations**

By selecting a matrix pore-size which completely excludes all 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 lyophilisation, to remove phenol from nucleic acid preparations, and to remove inhibitors from enzymes (e.g. see (19)).

#### **2.5. 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 (20). 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.

#### **2.6. 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 (21).

### 3. 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. A useful handbook on gel-filtration is available from GE Healthcare's Life Sciences division through the following URL: [http://www6.gelifesciences.com/aptrix/upp00919.nsf/content/LD\\_153206006-R350](http://www6.gelifesciences.com/aptrix/upp00919.nsf/content/LD_153206006-R350).

Other useful chromatography handbooks may be accessed through links from URL: [http://www6.gelifesciences.com/aptrix/upp01077.nsf/Content/service\\_and\\_support~documents\\_and\\_downloads~handbooks](http://www6.gelifesciences.com/aptrix/upp01077.nsf/Content/service_and_support~documents_and_downloads~handbooks).

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