Gel Filtration Chromatography

8

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Abstract

Apart from finding multitudes of applications in chemical, medicinal, and pharmaceutical research, gel filtration chromatography (GFC) has also become a routine tool in almost every biomedical research laboratory especially protein biochemistry. With rapid advancement and implementation of recombinant DNA technology in basic research, the requirement of purifying bacterially expressed proteins for further characterization has increased manifold. Gel filtration chromatography is usually adopted in the last or polishing purification step to obtain highly purified proteins that are later used for biophysical and structural studies. The versatility and robustness of GFC lies in the fact that the protein molecules do not adhere to the column during separation unlike ion-exchange and affinity chromatography. This gives GFC a significant advantage as it allows proteins to be eluted in a buffer condition that is conducive toward their future applications or storage. Since GFC separates analytes as a function of their size or molecular weight, it is also used as an analytical tool to determine molecular weights and oligomeric properties of macromolecules. This chapter, which is a sequel to the previous two chapters on chromatographic techniques, describes the theory, instrumentation, and applications of gel filtration chromatography along with elaborate discussions on several important protocols and troubleshooting tips.

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8.1 Introduction

Gel filtration chromatography (GFC) or size exclusion chromatography (SEC) is a popular protein purification technique that separates the macromolecules based on differences in their hydrodynamic volume or size [1]. The general principle of gel filtration chromatography is fairly simple where the inert gel medium is a porous matrix comprising spherical beads with stable physicochemical properties. When a mixture of analytes with different size distribution is applied to the column, the molecules larger than the pore size are unable to enter into the beads. As a consequence, they pass through the interstitial spaces between beads and elute first. On the other hand, molecules smaller than the gel pore size get diffused into the pores and elute at later time points as a function of their molecular weight [2] (Fig. 8.1).

Although this chromatographic technique is widely used in recombinant protein purification in research laboratories, it is capable of scaling up the process at an industrial scale as well. Based on the type of solvents used in the mobile phase, and other subtle variations, it is also referred to as molecular exclusion, molecular sieve, gel-permeation and gel-exclusion chromatography [3].

One of the distinct features of GFC that separates it from the other two main chromatographic techniques (viz. ion–exchange and affinity chromatography) is that, there is no direct interaction between the protein molecules and the solvent, i.e., the mobile phase. This makes the method more flexible in terms of buffer selection as it has no adverse effect whatsoever on the column resolution. Since GFC is usually used in the last step of protein purification, this unique characteristic allows retention of the protein in a buffer that will be amenable to several structural and functional studies post purification. The other important determinants of column resolution other than the gel pore size are bed height, flow rate, sample volume, and the molecular weight of the protein [4, 5], which will be elaborated in the later sections of this chapter. As mentioned briefly in Chap. 5, the best resolution is usually achieved with an optimum flow rate (slow or medium), long and narrow columns, small pore-size gel and sample volumes (1–5% of the total column volume) [6].

Apart from separation of proteins based on their molecular weights, another important application of GFC is desalting [7]. Since GFC is generally used in the finishing step of protein purification, the loaded protein can be efficiently eluted in a buffer of interest. The salt component present in the loaded sample easily separates from the macromolecule due to their huge size difference. Therefore, sample volumes as much as 30% of the total bed volume can be safely loaded onto the column as chances of peak overlap is unlikely. Furthermore, since the separation

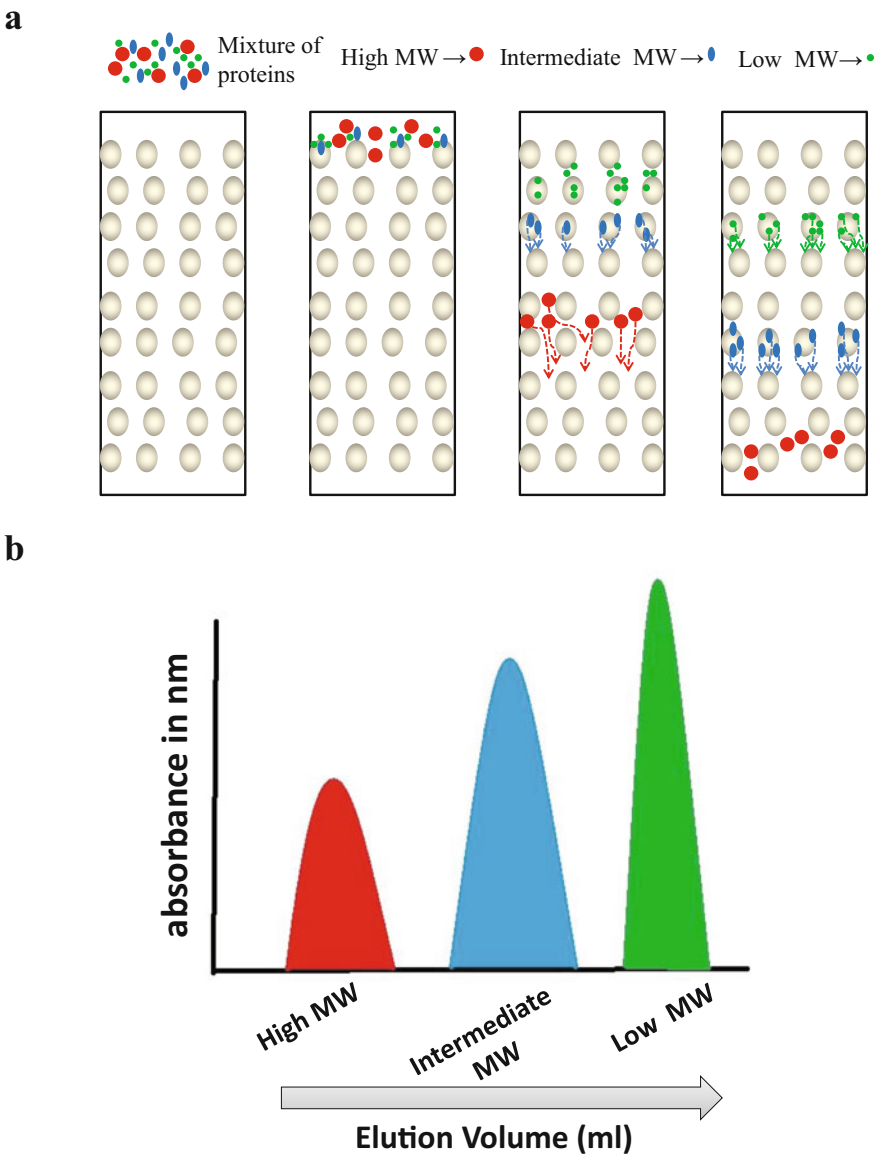

Fig. 8.1 Illustration of the separation principle in gel filtration chromatography. (**a**) The mixture of proteins (shown in different colors) applied to the column. Depending upon their size, protein molecules pass through the gel via different routes. High molecular weight proteins (shown in *red*) that are too large to enter the beads pass through the space between the beads and elute first. Small molecules (shown in *green*) enter the beads, travel slowly, and elute last. (**b**) Elution profile of proteins of different sizes as a function of elution buffer volume

technique is linearly dependent on the molecular weight of the macromolecule, this principle is used to determine the molecular weight and hence the oligomeric property of a protein of interest as described in later sections of the chapter [8].

This chapter, describes the principles of GFC along with its various applications in protein purification as well as studies pertaining to protein biochemistry. It also discusses several practical problems that are generally encountered and ways of circumventing them through efficient troubleshooting methods.

8.2 Instrumentation

Note: Gel filtration chromatography is commercially available as either a fast protein liquid chromatography (FPLC) or high performance liquid chromatography (HPLC) system. HPLC that is often used to characterize small chemical compounds works under high temperature and pressure, which makes it unsuitable for protein purification or molecular weight determination. On the other hand, FPLC system is optimized to purify large biomolecules [9]. Therefore, all protocols will refer to FPLC system in this chapter.

The typical instrumentation of Gel filtration chromatography system is as follows.

8.2.1 Pump

The pump pulls up the solvent from the reservoir and forces it to the column and subsequently to the detector. Operating pressure depends on composition of mobile phase, column dimensions, flow rate, and particle size.

8.2.2 Injector

Introduces the protein solution into the mobile phase.

An efficient injector should have the following properties:

1. Should be capable of introducing small as well as large volumes of samples (depending on applications).
2. Multiple injections of the sample solution should be possible.
3. It should not agitate the mobile phase of the column.

8.2.3 Column

Usually used for separation of protein of interest from its mixtures.

The size and dimensions of columns depend on the type of application.

1. *Analytical:* In analytical chromatography, the main objective is to characterize the sample so as to obtain its properties in a quantitative manner. This is mainly used in synthetic chemistry labs, pharmaceutical industry, etc. Recovery of the loaded sample is not of primary importance and hence the eluent is often removed as waste [10].
2. *Preparative*: This is mainly used for protein isolation and purification for further biochemical and biophysical characterization. Therefore, the eluents are collected in several fractions [11].

Typically, in GFC, analytical columns have 7.5–8 mm and preparative columns have 22–25 mm diameter. Column lengths range between 25 and 60 cm. In protein biochemistry labs, preparative columns are mainly used.

8.2.4 Detector

Regularly utilized detectors are UV-spectroscopy, fluorescence, mass-spectrometric, and electrochemical identifiers. In UV detectors, routinely used wavelengths are 214 or 220 nm (where the amide peptide bond has a strong absorbance) and 280 nm (to detect the presence of other chromophores primarily aromatic amino acids) [12].

8.2.5 Fraction Collector

An automated fraction collector with tube holders helps in collecting the sample eluted from the column.

8.2.6 Data Processing System

Provides data output from different fractions, stores data, and provides a graphical representation of the data. It also helps in column calibration.

A simple layout of the instrument setup is provided as shown in Fig. 8.2 below.

8.3 Principle of Macromolecular Separation Using Gel Filtration Chromatography

The gel filtration columns consist of beads that contain sieves cross-linked with agarose, dextran, polyacrylamide, or their combinations of a particular size, and are designed to suit specific separation tasks. The matrix that acts as a stationary phase consists of two measurable volumes, internal and external. The internal volume consists of liquid within the beads, and external volume, *aka* void volume (V_0), refers to liquid in the space between the beads. The sum of the external and internal volumes makes up the total volume (V_t) (Fig. 8.3) [3].

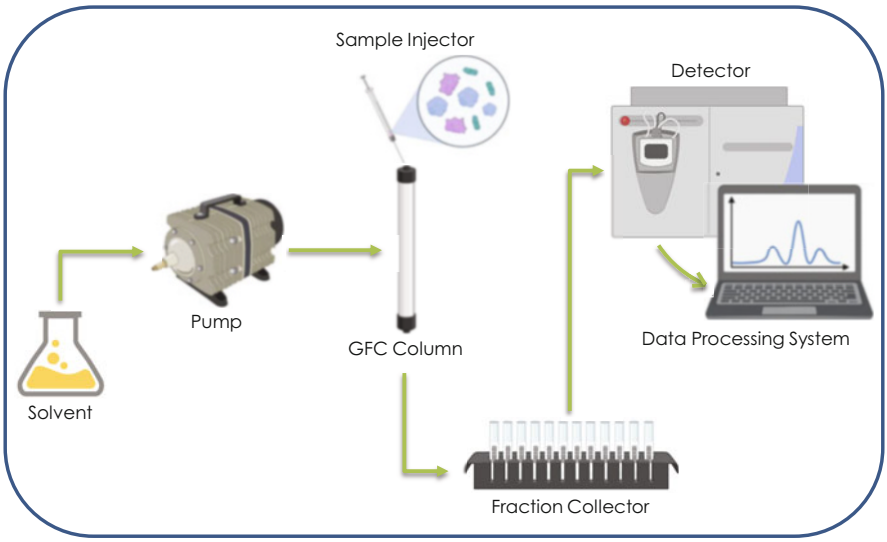

Fig. 8.2 A simplistic representation of gel filtration chromatography instrument setup. All the components of the schematic are labelled and are self-explanatory

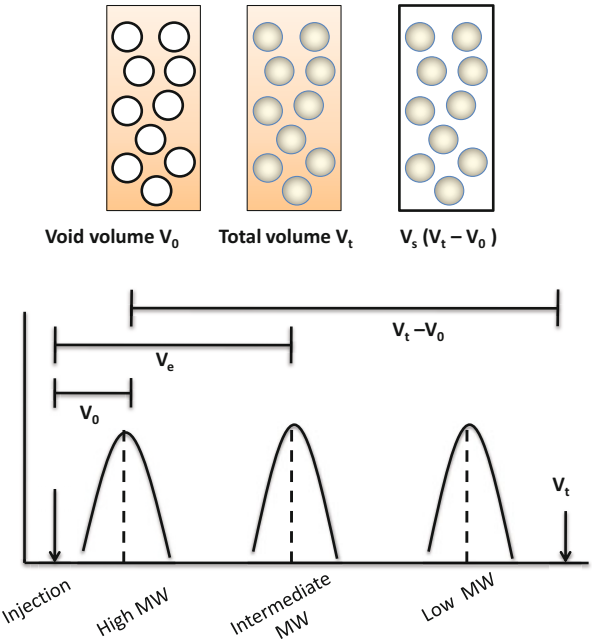

Fig. 8.3 Pictorial and graphical representation of molecular weight determination using gel filtration chromatography. V_t is the total volume of a gel bed, V_0 is the void volume, and V_s is space occupied by solvent inside the medium particles. The colored sections in the three rectangular boxes (representing a GFC column) in the upper panel represent the volume that has been considered, while the white section represents the part of the column volume that is not

When a mixture of proteins of different sizes is loaded onto the column, larger molecules migrate through the interstitial spaces between the stationary phase matrix pores and run down quite rapidly through the column eluting at V_0. Simultaneously, particles smaller than the matrix pores equilibrate with external and internal liquid volumes, causing them to migrate more slowly and eventually elute at a volume (V_e). The elution volume V_e, which is different for different proteins but greater than V_0, is linearly dependent on their size (Fig. 8.3). The elution volume (V_e) of a particular molecule depends on the fraction of the stationary phase available for diffusion. V_t refers to the total column volume. This principle can be mathematically represented by constant K_d or K_{av} (partition or distribution coefficient) as depicted in the following equations [3].

$$V_e = V_0 + K_{av}(V_t - V_0) \tag{8.1}$$

Rearranging the equation:

$$K_{av} = (V_e - V_0)/(V_t - V_0) \tag{8.2}$$

In GFC, K_{av} is represented as a function of its molecular size. When a molecule is adequately large (for example, blue dextran, MW ~ 2000 kDa) so as to bypass the mobile phase between the beads, then $K_{av} = 0$. On the contrary, for an extremely small molecule that enters the innermost mobile phase, then $K = 1$. For other proteins, the K_{av} value is intermediate and varies between 0 and 1. This shifting of the value of K_{av} between 0 and 1 is the fundamental basis for separation of macromolecules as a function of their molecular weight [3].

Other than size and mass, the hydrodynamic radius plays a vital role in determining gel filtration's flow rate. During separation in gel filtration, the general assumption is that molecules have symmetrical shapes with a similar hydrodynamic radius that fall in the resolution range of the matrix used. However, protein molecules that deviate from this assumption (i.e., asymmetric with larger hydrodynamic radius) will be eluted prior to the ones having same molecular weight but are symmetric with lower hydrodynamic radius. For example, fibrous proteins and some protein aggregates are asymmetric with higher hydrodynamic radius compared to their globular counterparts [3].

8.4 Choice of Matrix in Gel Filtration Chromatography

Matrix is a chemically inert but mechanically stable substance with a homogeneous porous structure and pore size. Gel filtration matrices are made from a wide variety of materials including agarose (Sepharose™ series), dextrans (Sephadex™ series), polyacrylamide (Bio-Gel series), polyvinylpyrrolidone, polyvinylethylcarbitol, silica-based materials, and cellulose. These materials have different pore size and particle size distribution that help in separation of molecules belonging to different ranges of molecular weights. Some of the commercially available matrices are a mixture of the above-mentioned substances such as dextran–agarose (Superdex™

Table 8.1 List of various commercially available media for gel filtration chromatography [3]

Sl. no.	Type of media	Commercial name	Molecular weight range (kDa)
1	Agarose	Bio-Gel A-0.5 Bio-Gel A-1.5 Bio-Gel A-5 Sepharose 6B Sepharose 4B	10–500 10–1500 10–5000 10–4000 60–20,000
2	Dextran	Sephadex G-50 Sephadex G-75 Sephadex G-100 Sephadex G-200	1.5–30 3–80 4–150 5–600
3	Polyacrylamide	Bio-Gel P-10 Bio-Gel P-30 Bio-Gel P-100 Bio-Gel P-150 BioGel P-200	1.5–20 2.5–40 5–100 15–150 30–200
4	Dextran-polyacrylamide gels	Sephacryl S-200 Sephacryl S-300 Sephacryl S-400	5–250 10–1500 20–8000

series), dextran-polyacrylamide (Sephacryl™ series), etc. as shown in Table 8.1. Each of them have distinct properties with certain *pros* and *cons*, and are suited for particular types of applications [3].

The type of column appropriate for a particular analyte is determined from "selectivity curve" that plots K_{av} (partition coefficient) versus $\log_{10}$ of MW for a set of standard molecules that is represented by the equations provided below.

$$K_{av} = (V_e - V_0)/(V_t - V_0) \tag{8.3}$$

$$K_{av} = \log\ (MW) + b \tag{8.4}$$

here "b" is the intercept on the Y-axis. The steeper the curve, the higher the resolution is reached [13]. The matrix is chosen for a particular analyte when its MW falls within the range of selectivity curve [13].

The variables such as buffers, organic solvents, pH, and temperature should be considered while choosing the matrix because they should be compatible with molecules or analytes being separated. The matrix should not adsorb the analytes during the separation process. For difficult separation problems, the resolution of the material will be critical and in these instances properties such as bead size, selectivity (given by the pore size distribution), and separation volume (available pore volume) become essential. Another factor to consider is the effect of the sorption properties (physicochemical property that includes both adsorption and absorption) of the matrix under running conditions. Although virtually no support can be expected to be completely free from sorption properties, the nature and degree of these properties vary with the nature of the matrix. Sometimes, these properties have been used to achieve increased separation of the sample component [14, 15].

8.5 Resolution of Gel Filtration Chromatography

Resolution can be defined as the extent of separation between peaks that correspond to MW of different analyte molecules.

Mathematically Resolution (R_s) can be expressed as:

$$R_s = \frac{Vr2 - Vr1}{\frac{W1+W2}{2}} \tag{8.5}$$

where V_{r1} and V_{r2} are the elution volumes of two consecutive peaks, whereas W_1 and W_2 are their peak widths, respectively [16] (Fig. 8.4).

There are different factors that determine resolution as described below. Therefore, by carefully balancing these parameters one can achieve good selectivity and sharp peaks in gel filtration chromatography.

8.5.1 Parameters Affecting Resolution

Several factors influence resolution. Pore size of the particles, particle size and distribution, column dimensions, medium packing density, flow rate, sample volume as well as viscosity of the sample and buffer. The medium selectivity is described by the selectivity curve (as mentioned above), in which a partition coefficient, K_{av}, is plotted against the logarithm of the molecular weight for a set of standard proteins.

The different parameters affecting resolution are discussed below.

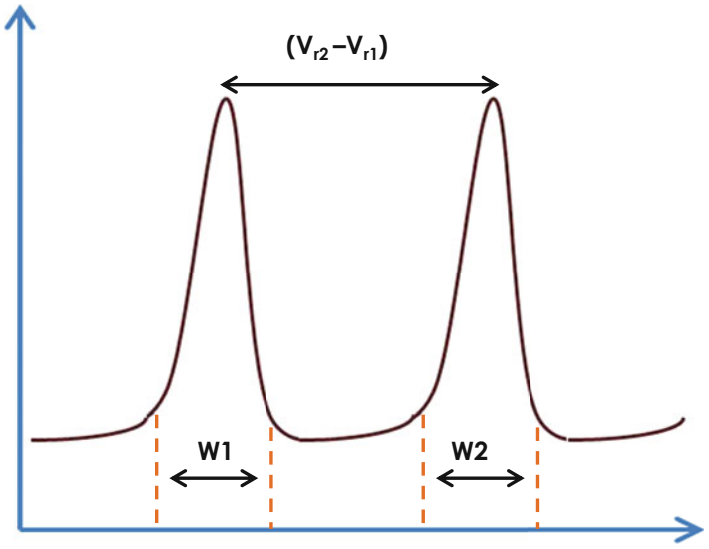

Fig. 8.4 Graphical representation of parameters affecting resolution (R_s) of gel filtration chromatography

8.5.1.1 Column Parameters

In GFC, maximum resolution is obtained with long columns. The ratio of the length of the cylindrical column to its diameter may vary from 20 to 100 [17]. A wide variety of pre-packed columns with different materials and sizes are available. Packing instructions are given below; however, for efficient packing, one needs to strictly follow the product manual's instructions. The loosely packed column with gaps either on top or bottom severely affects the resolution, pressure, etc.

8.5.1.2 Packing the Column

A column needs to be properly packed to obtain good resolution of protein separation through gel filtration chromatography. For proper packing of a column, the protocol to be followed is provided below:

According to the manufacturer's recommendation, add an appropriate amount of double distilled autoclaved water to the gel or dry gel powder. The gel can be pre-swollen at 90 °C for 1–5 h or kept at room temperature for 3–72 h. Never use a magnetic stirrer as it damages the beads. Gently mix the pre-swollen slurry and degas it. Fix the column vertically and pass the buffer to remove air bubbles in the outlet tube. Block the outlet tube and pour the gel slurry to the required height with the help of a glass rod. A gel reservoir can be used for smooth and continuous pouring of the gel. Allow the gel to settle, remove the excess buffer, and put the adaptor. Nowadays, a wide variety of pre-packed columns are commercially available that cater to specific separation requirements, which might be used for obtaining higher resolution [12].

8.5.1.3 Air Bubbles, Uneven Packing, and Cracks

Air bubbles in the column affects its packing and hence care should be taken to prevent air bubbles or remove them if found. The presence of air bubbles can be checked via transmitted light. It is recommended to use colored protein markers such as blue-dextran 200, cytochrome-c, and myoglobin to check the uneven packing, air bubbles, and cracks present in the column [12].

8.5.1.4 Choice of Eluent

For separation of proteins of interest through GFC, a favorable eluent condition is to be maintained. Although the elution profile is independent of the type of buffer used, it is important to provide a stable environment to the macromolecules. This is achieved on a case-to-case basis with the help of additives such as metal ions, cofactors, and protease inhibitors. It is to be noted that high concentrations of salts or solvents that give peaks at 215 nm or 280 nm wavelengths should be avoided [12].

8.5.1.5 Effect of Flow Rate

Since resolution is inversely proportional to the flow rate, a high resolution in GFC is achieved through use of very low flow rate. The optimum flow rate depends on the column and pump systems mentioned by the manufacturer. However, in GFC, the approximate flow rate range of 2 mL/cm^2/h to 30 mL/cm^2/h is preferably used [12].

8.5.1.6 Column Cleaning and Storage

To maintain a standard resolution as well as performance, gel filtration matrices must be cleaned according to the manufacture's instructions. Three basic cleaning protocols are recommended—simple, rigorous, and harsh. Simple is recommended with a low concentration of acetic acid or NaOH when there is an increase in back-pressure due to presence of contaminants. Rigorous cleaning is recommended when the following are observed: an increase in back-pressure; a color change at the top of the column; and a loss of resolution. Harsh cleaning is recommended when the column is contaminated mainly due to microbial growth. The use of 0.02% sodium azide in autoclaved water has proven to be useful to prevent microbial growth and is highly recommended for column storage [3].

8.5.1.7 Sample Preparation

A series of precautionary steps are required during sample preparation to achieve the best possible resolution. A sample or protein concentration of up to 50 mg/mL can be used for a non-viscous protein sample. The sample must be clear and free from any particulate matter, which is usually achieved by filtration using 0.22 μm syringe filter, or centrifugation at 10,000 × *g* for 15–30 min prior to injection. Furthermore, sample stability test can be performed before injecting into the gel filtration column under required salt, pH, and temperature conditions. This will prevent the sample from aggregating or precipitating, which further leads to column clogging and contamination or bacterial growth. High sample viscosities should be avoided. The sample volume should be 3–5% of the total column volume [12].

8.6 Applications of Gel Filtration Chromatography

As discussed earlier in this chapter, gel filtration chromatography has immense applications in protein biochemistry. Apart from serving as the last important step in recombinant protein purification, it is also used for desalting of proteins and determination of their molecular weights, hence oligomeric properties.

Some of the major applications with appropriate protocols are provided below.

8.6.1 Molecular Weight Determination

Gel Filtration Chromatography is one of the simplest tools to determine molecular weight (hence oligomeric property) of a protein of interest. The easy availability and user-friendly attributes of a gel filtration system make it one of the most popular and widely used tools in protein biochemistry laboratory. Since the proteins are run under their native conditions, this technique is also capable of determining the oligomeric property of a protein [18, 19].

An elaborate protocol for molecular weight determination using GFC is provided below.

8.6.1.1 Operating Procedure

The setup is as follows.

GFC System

1. *Chromatography system*: Any commercially available FPLC system with a UV detection system and a fraction collector.
2. *Gel filtration column* (choice should be based on the application and molecular weight of the desired macromolecule—refer to Table 8.1).
3. *Necessary accessories*:
   (a) Vacuum filtration unit (for filtering buffer and protein samples).
   (b) 0.22 μm syringes and filters.

Reagents

1. *Protein of interest* (usually 1 mL of solution with a final concentration of ~2 mg/mL).
2. *Molecular Weight Standards*: Can be made in the lab or purchased; MW range is usually between 12 and 200,000 kDa (for example: 1 mg/mL BSA, 1 mg/mL MBP, 1 mg/mL β-casein, and 1 mg/mL lysozyme).
3. *Blue Dextran* for determination of void volume.
4. *Molecular weight standard buffer:* 50 mM Tris–HCl, 100 mM salt, pH 7.5 (Buffer A).
5. *Sample elution buffer*: 50 mM Tris–HCl, 150 mM salt, pH 7.5. It is advisable to add a reducing agent such as Dithiothreitol or DTT (since the markers and the protein of interest might have multiple cysteine residues). Variation in buffer composition is possible (e.g., 20 mM Na_2HPO_4/NaH_2PO_4 and 200 mM NaCl, 1% (v/v) Glycerol at pH 7.5). Glycerol can be used to maintain stability of the protein of interest. ~200 mM salt is added to the buffer to maintain stability of the protein as well as to prevent it to stick to the column wall.
6. Distilled water (1 L).
7. 20% *Ethanol in distilled water* (1 L)—for cleaning.

Standard Operating Tools

Read the manufacturer's instructions for standard operating procedure and maintenance-related specifications. Every system operating window consists of a system controller, method editor, and evaluation workspace.

1. *System controller*: This wizard is for connecting and running the system manually and to calibrate pumps and detectors.
2. *Method editor*: For creating a new method, defining flow rate, amount of water or buffer to pass, equilibration time, injection time and fraction collection time, etc.
3. *Evaluation*: For analyzing the data, comparing the peaks and exporting data, etc.

The automated system has pump modules, precise UV detectors, conductivity measures, and a pH meter. The system is connected with its supporting software that runs under user-friendly operating systems.

Running the Experiment-Instrumental Setup

1. Turn on the chromatographic system followed by software on the computer and connect it with the system.
2. Create method: Define the parameters such as pressure limit, UV monochromators, flow rate (~0.5 mL/min), volume of the equilibration buffer (~2 column volume), injection time, fraction volume, time of collection, and other required information.
3. It is preferred to inject the protein after equilibration is over to prevent protein aggregation in the loop.
4. Check the tubing connections prior to injecting the protein. System pump is connected to the column directly.
5. Set up the fraction collector: Using the fraction collector, define the type of rack, collection scheme, and fraction size in mL.

Running Analyte Through the GFC Column

Step 1:

1. Column Standardization:
   Before injecting the standards, wash the column with at least 1 column volume of autoclaved double distilled water and equilibrate using 2 column volume of a MW marker buffer. If the protein is being run for the first time, the UV absorbance needs to be monitored at different wavelengths (215 nm, 254 nm, and 280 nm) and then standardized. The flow rate should not exceed ~0.5% of the column volume per minute. The UV monitor needs to be set to "zero" once the reading becomes stable.
2. Obtaining the standard curve:
   (a) Take 1 mg/mL blue dextran (~ 1 mL volume) and inject into the column and elute with Buffer A. Calculate the volume of the buffer required to reach the middle of the peak (as seen on the computer monitor). This measure gives the value of the void volume (V_0).
   (b) Either use commercially available protein standards or use 1 mg/mL BSA, 1 mg/mL MBP, 1 mg/mL β casein, and 1 mg/mL lysozyme and run them individually through the column and elute with 2 column volume of Buffer A. Since the elution volume includes the volume of the protein, the volume can be reset to "zero" prior to each injection. Measure elution volume of each standard macromolecule (V_e).
   Note: If more than three protein markers are used, mixing may cause peak interference because BSA might form a complex with other proteins. It is advisable to prepare an individual or multiple marker groups of 2–3 proteins with significant size differences.

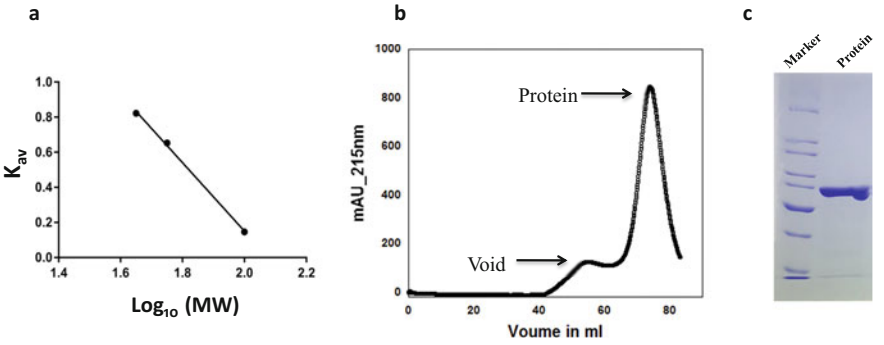

Fig. 8.5 (**a**) Using standard molecular weight markers, a standard curve is plotted and K_{av} values of the marker proteins are determined experimentally. (**b**) Chromatogram of the protein of interest as well as the void volume is shown in the graph obtained from the GFC system. (**c**) The collected fractions were pooled, concentrated, and run on SDS-PAGE. The protein band shows >95% purity. *Marker*: Molecular Weight marker; *Protein*: Protein of interest

(c) Plot K_{av} (Y-axis) versus the logarithm of molecular weight, i.e., $\log_{10}$MW (on the X-axis) for the protein standards (refer to Eqs. (8.1, (8.2, (8.3, and (8.4). Fit the data to a straight line, which is the standard curve (Fig. 8.5a). Once the column is standardized, the values obtained can be used multiple times until there is a major protocol change or the column repacking has been done.

Note: If a problem arises regarding UV signal stabilization, column cleaning is recommended.

Step 2: Determination of the MW of the protein of interest:

1. Wash the column with at least 0.5 to 1 column volume of double distilled water and Buffer A.
2. Equilibrate the column with 2 column volumes of elution buffer. Set the UV reading to "zero" once the signal gets stabilized.
   Note: For an unknown protein, use of multiple wavelengths to measure the peak intensity is advisable (including 215 nm, 260 nm, and 280 nm). This will help choose the best wavelength to use for the particular protein sample.
3. Prior to loading onto the column, concentrate the protein to 1 mL volume with a final concentration not less than 1 mg/mL.
4. Elute the protein with at least 1 column volume of the elution buffer. Calculate V_e/V_o and plot the point on the standard curve to obtain the MW (Fig. 8.5b).
5. The eluted protein (corresponding to the peak observed) is collected in the fraction collector and run on SDS-PAGE for checking its purity (Fig. 8.5c) and further storage.
   Note: If the protein has no tryptophan (that absorbs at 280 nm or 295 nm), set the UV measurement wavelength to either 260 nm (for Tyrosine) or 215 nm (peptide bonds).

Note: The plot of K_{av} versus $\log_{10}$ (MW) yields a straight line. It is important to note that, K_{av} value of the protein of interest should ideally fall between 0 and 1 and can be obtained from the graph. $K_{av} > 1$ indicates some interaction between the sample and the column. On the other hand, $K_{av} < 1$ indicates the formation of channels in the column. In any case, where there is a deviation from linearity, consider replacing the column.

8.6.2 Purification of Recombinant Proteins

Proteins expressed and purified in bacterial system are mainly used for biochemical, biophysical, and structural characterizations. Therefore, large amount of protein with >95% purity is preferred for these studies. Proteins obtained from the bacterial system are usually run through affinity chromatography, and if required followed by ion-exchange chromatography (IEC). This gives the protein of interest a reasonable level of purity (might vary from protein-to-protein). The last polishing step is then achieved by running the protein through an appropriate GFC column.

The protocol for protein purification though similar is much simpler than the one discussed above. The protein of interest is used as the loading sample and the eluent from the peak (as displayed in the chromatogram on the system computer) is collected in the fraction collector. However, for an unknown protein, it is important to have a rough estimate of the elution volume, which is achieved by calculating its elution volume from an already determined standard curve [20, 21].

8.6.3 Desalting and Buffer Exchange

"Desalting" of proteins refers to removal of salt or other undesirable contaminants such as nucleotides and chemicals from a protein solution. On the other hand, "buffer exchange" refers to replacement of the existing buffer with a final buffer that might be required to store the protein under stable and native conformation and/or conducive for certain biochemical, biophysical, and structural analyses [22].

Although the principle behind both the procedures is very similar, there are certain subtle differences in approach as well as applications. For desalting purpose, the GF column is equilibrated with water, while in buffer exchange, the column is equilibrated with the desired buffer solution for the protein. In both the cases, the existing buffer solution within the column is replaced with either water or a different buffer system. Along with the previous buffer, other impurities also pass out of the column.

In both the techniques, the size of the column plays a crucial role and hence standardization of the column size becomes necessary. This is because a very large column might lead to dilution of the protein sample and a smaller one will lead to inadequate separation or exchange.

8.6.4 Miscellaneous Applications

The versatility of gel filtration chromatography has led to a variety of applications that encompass various scientific disciplines and their interfaces. It has also created a niche both in industries and in clinical applications. The various other applications of GFC include group separation that allows fast separation of macromolecules from natural extracts and chemicals [22–24], separation of proteins through PEGylation [25], separation of proteins from peptides [26, 27], separation of viruses [28, 29], and separation of oligonucleotides from contaminants [30, 31]. Furthermore, GFC has found immense application in the fields of enzymology, endocrinology, and clinical pharmacology [32–36] as well.

8.7 Troubleshooting Tips for Running GFC

Different problems encountered while running a gel filtration column and their troubleshooting tips are provided in Table 8.2 below.

Table 8.2 Troubleshooting tips for running various experiments using gel filtration chromatography

Sr. no.	Problem	Recommendation
1.	Column gets dried	Pass 20% methanol followed by water and observe for void volume change and back-pressure. If it changes, then repack the column according to the manufacturer's instructions. The dried column produces channels within the column and perturbs the resolution and hence repacking becomes imperative
2.	Poor resolution	a. Decreasing the flow rate might improve resolution b. Revisit your choice of column and type of matrix for the particular protein of interest since fractionation range and pore-size diameter play important roles in resolution. Therefore, switching to a more appropriate column-matrix combination might help c. Clean the column with appropriate buffers and detergents, organic solvents, denaturing agents (within allowed concentration and tolerable range of the matrix) in an increasing manner as suggested in the users' manual. If there is no improvement, disassemble the column and repack it after washing the beads and other column components
3.	UV lamp is unstable and making noise	Pass at least two column volumes or more buffer by keeping UV "*ON*" while enabling auto-zero option frequently in between. The buffer should not contain components that give signals or noise. Always make fresh buffers if you are adding DTT (reducing agent), which often gets oxidized over time and creates this issue. The column and flow system should be clean to prevent growth of any microorganism

(continued)

Table 8.2 (continued)

Sr. no.	Problem	Recommendation
4.	Air bubbles in column, or in the eluent	Air bubbles are found in the gel bed when eluents are not thoroughly degassed or the column temperature is increased. Since air bubbles affect the resolution to a greater extent, extensive washes with degassed buffer might solve the problem. Consider repacking the column if the problem persists after washes
5.	Very high column pressure	The column and flow system should be rigorously cleaned to eliminate the possibility of growth of microorganisms that sometime lead to high column pressure
6.	Broad peaks	Might be due to loss of column efficiency. To take care of this problem: use mobile phase of lower viscosity; lower the flow rate; pack the column with smaller particle-sized beads; inject smaller sample volume; and elevate column temperature. However, if the problem still persists, then replace the column

8.8 Conclusions

Gel Filtration Chromatography has become an indispensable tool in protein purifications. Due to its simplicity, robustness, and versatility, it has found applications beyond separation of macromolecules and created a place for itself in biomedical research. The huge popularity of GFC has eventually led to significant advancements in its instrumentation as well as broadening of the gamut of its applications at the interface of academia and industry.

Upgradation in the classical GFC technique include recent development of leading-edge tools such as Absolute Size-Exclusion Chromatography (ASEC) and SEC-MALS. The former technique combines principles of dynamic light scattering with that of size exclusion chromatography to obtain absolute size of proteins and other macromolecules. GFC/SEC coupled with DLS is capable of enhancing DLS resolution and is used for aggregation studies [37]. Furthermore, SEC-MALS, which combines the power of size-exclusion chromatography with multi-angle light scattering, offers an advanced and faster analytical technique that bypasses the lengthy column calibration step [37].

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Problems

Multiple Choice Question

1. In Gel Filtration Chromatography the best resolution is usually achieved with the following combination:
   (a) Faster flow rate, short and wide columns, small pore-size gel, and less sample volumes (1–5% of the total column volume).
   (b) An optimum flow rate (slow or medium), long and narrow columns, small pore-size gel and sample volumes (1–5% of the total column volume).
   (c) Faster flow rate, short and wide columns, large pore-size gel, and less sample volumes (1–5% of the total column volume).
   (d) An optimum flow rate (slow or medium), long and narrow columns, large pore-size gel, and high sample volumes (10–25% of the total column volume).
2. Which one of the following statements is true?
   (a) K_{av} value of the protein of interest should fall between 0 and 1 and can be obtained empirically by substituting the values (known total, void, and elution volumes) in the standard equation.
   (b) K_{av} value of the protein of interest should fall between 0 and 1 and can be obtained experimentally from the standard graph (known molecular weight markers).
   (c) K_{av} value of the protein of interest should be any integer and can be obtained experimentally from the standard graph (known molecular weight markers).
   (d) K_{av} value of the protein of interest should be any integer and can be obtained empirically by substituting the values (known total, void, and elution volume) in the standard equation.
3. A gel filtration column is filled with Polyacrylamide media (Bio-Gel P-150) with a molecular weight separation range of 15–150 kDa. A mixture of four different proteins: Protein A (MW 55 kDa); Protein B (MW 35 kDa); Protein C has a molecular weight (MW 200 kDa), and Protein D (MW 10 kDa). Protein B has a tendency to form aggregates (>300 kDa) in small proportions. Therefore, the order of their elution time would be:
   (a) Protein D will be eluted first, followed by B, A, C and aggregates will be eluted last.
   (b) Aggregates will be eluted first, Protein C will be the second to elute, followed by A, B, and D.
   (c) Aggregates and Protein C will elute simultaneously in the void volume, followed by D, B, and finally A.
   (d) Aggregates and Protein C elute simultaneously in the void volume, followed by A, B, and finally D.

Subjective Question

1. A researcher was trying to characterize the native oligomeric status of one of the least studied member "**PROTEIN X**" of a member of a serine protease family. "**PROTEIN X**" has been purified using Ni-NTA chromatography and subsequently subjected to gel filtration chromatography using Superdex 200 10/300 HR column ($V_t = 120$ mL) (GE Healthcare, Uppsala, Sweden). The elution volumes with other necessary information are provided in the table below. Blue Dextran was used to determine the void volume of the column. The standard proteins BSA, MBP, and lysozyme were run on the same column and their elution volumes are provided. Answer the following two questions from the following information:
   (a) Calculate the K_{av} of the standards.
   (b) Find the K_{av} values and thereafter calculate the molecular weights and oligomerization status of newly characterized "**PROTEIN X**."

S. no	Protein	Mol. wt.	Elution volume
1	Blue Dextran	2000 kDa	46.5 mL
2	BSA	66.5 kDa	77 mL
3	MBP	45 kDa	86.5 mL
4	Lysozyme	14 kDa	111.5 mL
5	"**PROTEIN X**"	?	Peak 1: 64 mL (major peak) Peak 2: 87 mL (minor peak)

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