Size-Exclusion Chromatography

How to separate molecules by hydrodynamic size alone, without binding, for aggregate analysis, buffer exchange, and size-based fractionation.

LT Biotech

Resource Series
No. 01

01 What SEC separates

Size exclusion chromatography separates molecules according to their hydrodynamic volume, the effective size they occupy in solution, by exploiting differential access to the pore network of a porous stationary phase. It is also called gel filtration when operated in aqueous mobile phases for biological macromolecules, and gel permeation chromatography when operated in organic solvents for synthetic polymers.

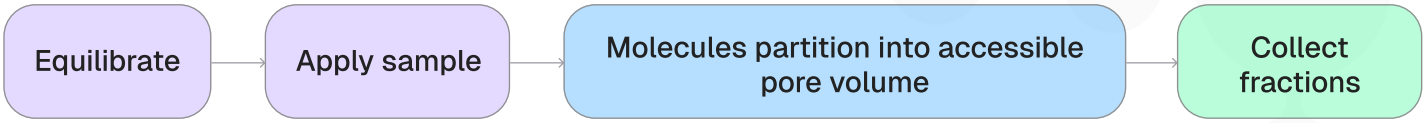
The most important conceptual distinction is that SEC separates by hydrodynamic volume, not by molecular weight directly. These are not equivalent. A globular protein and an elongated protein with the same molecular weight occupy different hydrodynamic volumes in solution and will elute at different positions on an SEC column. A denatured or partially unfolded protein may elute earlier than its native form because unfolding increases the hydrodynamic radius. Aggregated species, including relatively small oligomers, elute significantly ahead of the monomer because their combined hydrodynamic volume is much larger. SEC retention is therefore a readout of molecular size-in-solution under the specific conditions used, not a direct measurement of mass.

The separation mechanism is purely steric and entropic: molecules that are too large to access the pore volume are excluded and travel only through the interstitial volume between particles, reaching the detector first. Smaller molecules partition into the pore volume, spending more time within the stationary phase, and elute progressively later. The smallest molecules that can access the full pore volume elute at or near the total column volume. There is no binding interaction between analyte and stationary phase under ideal SEC conditions; selectivity arises entirely from the pore-size distribution of the resin and the hydrodynamic sizes of the analytes.

SEC is the principal analytical tool for protein aggregate and fragment profiling, and a preparative tool for desalting, buffer exchange, and size-based fractionation. Its non-binding nature means mobile-phase composition can often be chosen to maintain protein stability without concern for the effects of ligand chemistry on the target.

02 How the separation works

A typical SEC process is:



Unlike affinity, IEX, or HIC, there is no intentional binding and elution cycle. The mobile phase carries all sample components through the column isocratically. What changes between components is how much time each spends within the pore network.

Species larger than the pore exclusion limit cannot enter the pores and travel only through the inter-particle void volume, the shortest path through the column. They elute first, near the void volume (V0). Species small enough to access some fraction of the pore volume elute at intermediate volumes. Species small enough to access all available pore volume elute near the total accessible volume (Vt). The most useful fractionation occurs between V0 and Vt, for molecules whose hydrodynamic volumes fall within the resin's fractionation range.

Matching the resin's fractionation range to the hydrodynamic sizes of the species of interest is therefore the first and most important criterion in resin selection. A resin optimised for large proteins (fractionation range up to several million Daltons) will not resolve small proteins or peptides; a resin designed for small molecules will completely exclude a high-molecular-weight target.

For desalting and buffer exchange, the size difference between the target (a protein or nucleic acid) and the small molecules to be removed (salts, detergents, small-molecule reagents) is so large that selectivity is straightforward. The target is fully excluded from the pores and elutes near V0, while salts and small molecules enter the full pore volume and elute near Vt. This application does not require high resolution between species of similar size; it exploits a large size difference and can be operated at higher flow rates on shorter, wider columns.

Sample volume is a critical process variable that affects resolution. Loading a larger sample volume broadens the starting zone and reduces resolution between adjacent peaks. As a practical guideline, analytical SEC is typically performed with sample volumes of 1–5% of the column volume; preparative fractionation may tolerate somewhat more, at the cost of resolution. Flow rate, particle size, and column length influence band dispersion and resolution independently of sample volume. All of these variables must be considered together when optimising a preparative SEC process.

03 Applications and method development

Analytical SEC is the standard tool for quantifying aggregate and fragment content in protein preparations, including monoclonal antibodies and other biopharmaceuticals. Retention time or elution volume relative to molecular weight standards is used to characterize the size distribution of a sample. Results must be interpreted with the understanding that standards and unknowns may have different hydrodynamic properties at the same molecular weight, particularly when they differ in shape, glycosylation, or conjugation. Orthogonal techniques such as multi-angle light scattering (MALS) coupled to SEC provide more absolute molecular weight information than SEC alone.

Desalting and buffer exchange exploit the large size difference between macromolecular targets and small solutes. Short, wide columns achieve rapid processing; high resolution between species is not required because the size difference is large.

Preparative fractionation separates proteins, nucleic acids, or complexes whose hydrodynamic sizes fall within the resin's fractionation range. This includes separation of monomeric protein from soluble aggregates, fractionation of protein mixtures by size, and enrichment of a desired oligomeric state. Because SEC capacity is inherently lower than binding-mode techniques and because column volume requirements can be large, preparative SEC is most efficiently placed late in a purification workflow, after a higher-capacity capture step has already reduced the volume and complexity of the feed.

Method development should address:

- Hydrodynamic size of the target and key impurities or contaminants: this determines resin selection.
- Resin fractionation range and pore size.
- Column dimensions: length determines resolution; diameter determines loading capacity per unit time.
- Sample volume: must be minimised relative to column volume for adequate resolution.
- Sample concentration: high concentrations can cause viscous fingering and band broadening.
- Mobile-phase composition: chosen to maintain product stability and minimize nonspecific interactions with the resin.
- Flow rate: higher flow rates reduce resolution; lower flow rates increase run time and may not be practical at scale.
- Resolution between target and key impurity peaks at the intended operating conditions.

04 Strengths, limitations, applications

Strengths

Purely size-based — no binding chemistry or ligand interaction is required.

Mobile-phase composition — can be freely selected to maintain product stability, independent of ligand constraints.

Suitable — for desalting and buffer exchange applications with large size differences.

Useful — as a polishing step after higher-capacity capture operations.

Conditions are typically mild — and compatible with native protein structure.

Limitations

Lower loading capacity — than binding modes, making SEC poorly applicable as a capture technique.

Sample volume directly limits resolution — large-volume samples require proportionally large columns.

Range-limited — only molecules within the fractionation range of the selected resin are efficiently resolved.

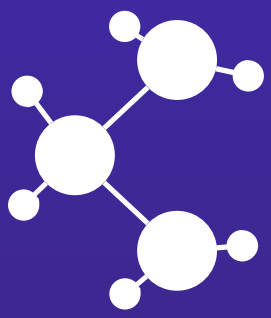
Specificity — species with similar hydrodynamic volumes are difficult or impossible to separate regardless of other differences. Nonspecific interactions with the resin can occur if mobile-phase composition and resin chemistry are not appropriately matched.

Sensitivity — SEC does not directly measure molecular weight and is sensitive to molecular shape, conformation, and sample conditions.

Best-fit applications

Choose SEC when the separation objective is size-based: desalting, buffer exchange, aggregate or fragment separation, size-based fractionation, or analytical size-distribution profiling. It is best positioned as a polishing or characterisation step following a higher-capacity capture operation, not as a primary capture tool. Where absolute molecular weight determination is required, SEC should be combined with a detector that provides molecular weight information independently of column calibration.

Product-specific operating ranges, capacities, cleaning conditions, and regulatory claims should be taken from current LT Biotech resin documentation and product-specific validation data.



LT Biotech

UAB LT Biotech
Phone: +370 5 2160227
E-mail: info@ltbiotech.lt
Address: Mokslininku 2, Vilnius, Lithuania, LT-08412

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