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Size-exclusion chromatography in Biotech Industry

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Review Article

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ABSTRACT

Chromatography, although primarily a separation technique, is most employed in chemical analysis in biotech plant is SEC which is very powerful technique for protein purification, polymer characterization etc. As the principal advantage of SEC is its gentle non-interaction with the sample, enabling high preservation of biological activity, so SEC has lot of importance in biotech industry. In this review the basic theory, principle & applications of SEC in biotech industry are included.

INTRODUCTION

Chromatography is probably the most powerful analytical technique available to the modern chemist. Size-exclusion chromatography (SEC) is a chromatographic method in which molecules in solution are separated by their size, and in some cases molecular weight. It is usually applied to large molecules or macromolecular complexes such as proteins and industrial polymers. Typically, when an aqueous solution is used to transport the sample through the column, the technique is known as gel-filtration chromatography, versus the name gel permeation chromatography which is used when an organic solvent is used as a mobile phase. SEC is a widely used polymer characterization method because of its ability to provide good molar mass distribution(Mw) results for polymers [1].

Grant Henry Lathe and Colin R Ruthven was the pioneer of size exclusion chromatography who started this technique for separation of analytes of different size with starch gels as the matrix, later Jerker Porath and Per Flodin introduced dextran gels.

SEC is a widely used technique for the purification and analysis of synthetic and biological polymers, such as proteins, polysaccharides and nucleic acids. Biologists and biochemists typically use a gel medium usually polyacrylamide, dextran or agarose and filter under low pressure. Polymer chemists typically use either a silica or crosslinked polystyrene medium under a higher pressure. These media are known as the stationary phase [2].

The Stationary Phase

In SEC the selectivity is aimed to be dependent solely of the inherent porosity of the material.The matrices used in SEC are often composed of natural polymers such as agarose or dextran but may also be composed of synthetic polymers such as polyacrylamide. Gels may be formed from these polymers by cross-linking to form a three-dimensional network. Different pore sizes can be obtained by slightly differing

amounts of cross-linking. The degree of crosslinking will define the pore size. The first commercial SEC media, Sephadex, composed of dextran that was crosslinked with epichlorohydrin. Many gels are now commercially available in a broad range of porosities [3].

The Mobile Phase

In contrast to other types of media the selectivity of a SEC matrix is not adjustable by changing the composition of the mobile phase. Optimally there is no adsorption involved, and the mobile phase should be considered as a carrier phase and not one which has a large effect on the chromatography. However, the sample may require a buffer solution with a well defined pH and ionic composition chosen to preserve the structure and biological activity of the substances of interest [3].

Theory & Principle

Principle

Size exclusion chromatography (SEC) is the separation of mixtures based on the molecular size (more correctly, their hydrodynamic volume) of the components. Separation is achieved by the differential exclusion or inclusion of solutes as they pass through stationary phase consisting of heteroporous (pores of different sizes) cross linked polymeric gels or beads. The process is based upon different permeation rates of each solute molecule into the interior of gel particles. Size exclusion chromatography involves gentle interaction with the sample, enabling high retention of biomolecular activity [4].

The basic principle of size exclusion chromatography is quite simple. A column of gel particles or porous matrix is in equilibrium with a suitable mobile phase for the molecules to be separated. Large molecules are completely excluded from the pores will pass through the space in between the gel particles or matrix and will come first in the effluent. Smaller molecules will get distributed in between the mobile phase of in and outside the molecular sieve and will then pass through the column at a slower rate, hence appear later in effluent [4].

Theory [2][5]

SEC is used to separate and analyze a variety of chemicals, including both synthetic polymers and biopolymers. It is a powerful and popular method to purify and determine the molecular weight of proteins. The total volume of a SEC column may be regarded as containing three compartments, Vo, Vi and Vg. The total volume is calculated by equation,

$$Vt = V_g + V_i + V_0$$

The void volume Vo represents the volume outside of the beads. Vo is determined by using a very large molecule that is larger than the exclusion range for the gel. Blue Dextran is most often used for this purpose.Blue dextran is a very large polysaccharide with a blue dye covalently bonded which has a molecular weight of about 2,000,000 Da. Vg is the volume occupied by solid matrix of gel or beads. The space within the beads is referred to as the inclusion or internal volume, Vi. To determine this quantity, a very small molecule is used. Typically, an amino acid linked to a fluorescent molecule such as dinotrophenyl or ascorbic acid is used to find Vi. The solutes which are smaller in size enter freely into the interstics of the gel. The band maxima for small molecules appear at the end of the column because of the higher degree of retardation, at an elution volume corresponding to (Vi+Vo).The molecules which are of intermediate size and are able to enter the interstics of the gel in some fraction kd, require the elution volume solute Ve , which is expressed by equation,

$$V_e = V_0 + K_d Vi$$

This equation describes that the behaviour of column with reference to all solutes , which get eluted from column. For molecules that can enter the interstices of the gel, kd=0 & Ve = Vo

Applications

a) **SEC for purification of biomolecules:** Size-exclusion chromatography (SEC) is a popular method to separate biomolecules based on their size. Primarily, it is applied to the separation of biopolymers

such as proteins and nucleic acids, i.e. water-soluble polymers. This system is also called gel filtration, typically with beads of dextran or agarose serving as gel matrix. Smaller molecules pass significantly slower through the column than larger molecules. Not to be mixed up with gel electrophoresis, there are big differences in terms of the separation principle. SEC does not require electric current and the sieving effect will not separate small molecules first.[6]

b) **Determination of Molecular Size by Size-Exclusion Chromatography (Gel Filtration):** Proteins in solution, or other macromolecules, are applied to a column with a defined support medium. The behavior of the protein depends on its size and that of the pores in the medium. If the protein is small relative to the pore size, it will partition into the medium and emerge from the column after larger proteins. Besides a protein's size, this technique can also be used for protein purification, analysis of purity and study of interactions between proteins.[7]

c) **Evaluation of meningococcal C oligosaccharide conjugate vaccines by size-exclusion chromatography/multi-angle laser light scattering:** The mean molecular masses of three different meningococcal C saccharide (MenC)-protein conjugate vaccines and their constituent proteins were estimated using HPLC size-exclusion chromatography (SEC) with multi-angle laser light scattering (MALLS) and refractive-index (RI) detection (SEC/MALLS).[8]

d) **SEC & SE-HPLC for proteins:** SE-HPLC is an alternative approach of getting good resolution of macromolecules within a short time. The fundamental principal remains the same for both SEC and SE-HPLC. The advantage offered by HPLC are good resolution and speed of analysis, reusability of column without repacking & regeneration, high reproducibility due to the close control of parameters which effecting the efficiency of the separation, easy automation of the instrument operation and data analysis.[9]

e) **Molecular Characterization of Multivalent Bioconjugates by Size-Exclusion Chromatography with Multiangle Laser Light Scattering:** The degree of substitution and valency of bioconjugate reaction products are often poorly judged or require multiple time- and product-consuming chemical characterization methods. These aspects become critical when analyzing and optimizing the potency of costly polyvalent bioactive conjugates. In this study, size-exclusion chromatography with multiangle laser light scattering was paired with refractive index detection and ultraviolet spectroscopy (SEC-MALS-RI-UV) to characterize the reaction efficiency, degree of substitution, and valency of the products of conjugation of either peptides or proteins to a biopolymer scaffold, i.e., hyaluronic acid (HyA). Information obtained using this technique can improve macromolecular engineering design principles and help to better understand multivalent macromolecular interactions in biological systems.[10]

f) **Analysis of biothearpeutics & their aggregates:**In recent years, the use and number of biotherapeutics are increased significantly. For these largely protein based therapies, the quantitation of aggregates particular concern given their potential effect on efficacy & immunogenicity. This need has renewed interest in SEC.[11]

g) A high performance SEC system is described that minimizes the ionic & hydrophobic interactions of proteins with the stationary phase. The system can be used to determine reliably M*r* 10000 & 70000. A further application would be to separate proteins on a preparative (milligram) scale. [12]

h) **Desalting:-** A common use of SEC is for desalting protein or nucleic acid samples. The molecule of interest is eluted in the void volume, while smaller molecules are retained in the gel pores. To obtain the desired separation, the gel should have an exclusion limit significantly smaller than the molecule of interest.[1]

i) **Characterization of Starch by Size-Exclusion Chromatography:**Shear degradation is examined in size-exclusion chromatography (SEC, or GPC) of native starch in an eluent system (dimethylsulfoxide and LiBr) in which the starch is completely dissolved. Changes in apparent size distribution with flow rate suggested extensive shear scission of the amylopectin region. For smaller sizes, largely amylose, there was no significant scission for lower flow rate. [13]

j) **A routine high-performance size-exclusion chromatography to determine molecular size distribution of *Haemophilus influenzae* type b conjugate vaccines:**High-performance size exclusion chromatography has been used to determine the molecular size distribution of *Haemophilus influenzae* type b (Hib) conjugate vaccines. Both high molecular weight preparations of native Hib capsular polysaccharide coupled to tetanus toxoid and low molecular weight vaccines with Hib oligosaccharides linked to the CRM_{197} nontoxic mutant diphtheria protein were analysed. Columns with different fractionation ranges were used for the two kinds of vaccines. This method showed to be rapid, accurate and reproducible for different lots of Hib vaccine of different composition produced by various manufacturers. It could replace more time-consuming chromatographic methods enabling control authorities to employ a single methodological approach for different Hib vaccines. [14]

k) SEC is standard technique for the analysis of monoclonal antibodies and their aggregates.[15]

l) **Liposome retention in size exclusion chromatography**:Size exclusion chromatography is the method of choice for separating free from liposome-encapsulated molecules. Liposomes are self-assembled phospholipids enclosing a droplet of the aqueous medium in which they are formed. Liposomes have numerous applications namely as *in vivo* drug delivery vehicle. Drugs interact with liposomes in several different ways depending on their solubility and polarity characteristics. Recently, enzymes are encapsulated in liposomes to enhance the enzyme stability with respect to dilution and proteases. Size exclusion chromatography (SEC) is an old and widely used tool to separate small solutes from liposomes or to narrow the size distribution. [16]

m) **Other applications are** [4]

- Protein folding studies.
- Concentration of sample.
- Copolymerisation studies.
- Relative molecular mass determination
- Protein-ligand binding studies.

CONCLUSION

Chromatography plays important role in the discovery, development and manufacture of products in biotech industry. Size Exclusive Chromatography is probably the most universal, most sensitive analytical method and is unique, in that it is easily used for purification of biomolecules such as proteins and nucleic acids, molecular size determination.

REFERENCES

1. Douglas A. Skoog, F. James Holller & Stanley R. Crouch, Instrumental analysis, 8th edition 2011, p. 923-926.
2. Dr. Supriya S. Mahajan, " Instrumental Methods of Analysis", Popular Prakashan Private Ltd, 1st edition 2010, p. 262-263.
3. Douglas A Skoog, Donald M. West, "Fundamental of Analytical Chemistry," 8th edition, p.988.
4. Dr. Vikas Kumar Dubey, Proteimics & Genomics, Lecture 5 size-exclusion (gel filtration) chromatography, IIT Guwahati, p.1-6
5. Wallart and Provost lab, Jan.2004, Size Exclusive Chromatography protocol/Guide , Dragnotech, p-1.
6. Applichem, Appication No.6, Size-exclusion chromatography for purification of biomolecules.
7. G Brent Irvine, 2001, Current protocol in cell biology, Determination of Molecular Size by Size-Exclusion Chromatography, p.1.
8. Jumel K, Ho MM , Bolgiano. Evaluation of meningococcal C oligosaccharide conjugate vaccines by size-exclusion chromatography/multi-angle laser light scattering. Biotechnol App Biochem. 2002;36:219-226.
9. Saad Tayyab, Seema Kumar and Mozafarul Islam, Size Exclusive Chromatography & Size Exclusive-HPLC of Proteins. Biochem Edu. 2010;19:1-4.
10. Jacob F. Pollock, Ronald S. Ashton, Nikhil A. Rode. Molecular Characterization of Multivalent Bioconjugates by Size-Exclusion Chromatography with Multiangle Laser Light Scattering, Bioconj Chem. 2012;23(9):1794-1811.
11. Paula Hong, Stephan koza et.al. A review of size –exclusive chromatography for the analysis of biothearpeutics & their aggregates. J Liq Chromatogr Rel Technol. 2012;35:2923-2950.
12. Franz Hefti. High-performance size-exclusion chromatography: A buffer for reliable determination of Molecular weights of proteins. Anal Chem. 1982;121(2):378-381.
13. Richard A Cave, Shane A. Characterization of Starch by Size-Exclusion Chromatography: The Limitations Imposed by Shear Scission, Biomacromol. 2009;10(8):2245-2253.
14. C Von Hunolstein, L.Parisi, S Recchia. A routine high-performance size-exclusion chromatography to determine molecular size distribution of *Haemophilus influenzae* type b conjugate vaccines. Vaccine. 1999;17(2):118-125.
15. Paula Hong and Kenneth J. Fountain, Waters Corporation, Method Development for size exclusive chromatography of monoclonal antibodies and higher order aggregates, p.1-4.
16. Tristan Ruysschaert, Audrey Marque, Jean-Luc Duteyrat. Liposome retention in size exclusion chromatography. BMC Biotechnol. 2005;5(5):11.