Journal of Pharmaceutical and Biomedical Analysis 101 (2014) 161–173

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Review

Theory and practice of size exclusion chromatography for the analysis of protein aggregates

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ARTICLE INFO

Article history:
Received 12 February 2014
Received in revised form 3 April 2014
Accepted 8 April 2014
Available online 19 April 2014

Keywords:
Size exclusion chromatography
Therapeutic proteins
Aggregates
Biopharmaceuticals

ABSTRACT

Size exclusion chromatography (SEC) is a historical technique widely employed for the detailed characterization of therapeutic proteins and can be considered as a reference and powerful technique for the qualitative and quantitative evaluation of aggregates. The main advantage of this approach is the mild mobile phase conditions that permit the characterization of proteins with minimal impact on the conformational structure and local environment. Despite the fact that the chromatographic behavior and peak shape are hardly predictable in SEC, some generic rules can be applied for SEC method development, which are described in this review.

During recent years, some improvements were introduced to conventional SEC that will also be discussed. Of these new SEC characteristics, we discuss (i) the commercialization of shorter and narrower columns packed with reduced particle sizes allowing an improvement in the resolution and throughput; (ii) the possibility of combining SEC with various detectors, including refractive index (RI), ultraviolet (UV), multi-angle laser light scattering (MALLS) and viscometer (IV), for extensive characterization of protein samples and (iii) the possibility of hyphenating SEC with mass spectrometry (MS) detectors using an adapted mobile phase containing a small proportion of organic modifiers and ion-pairing reagents.

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http://dx.doi.org/10.1016/j.jpba.2014.04.011

1. Introduction

The clinical use of therapeutic proteins has enabled the treatment of a wide range of life-threatening diseases. Many of these diseases were considered incurable or untreatable only a few decades ago. Almost half of the new drugs recently approved by the United States Food and Drug Administration (FDA) are therapeutic proteins, and the drug-pipeline landscape is now shifting even more toward this drug class of drugs [1]. There are several hundred potential biopharmaceuticals that have been evaluated in clinical trials for the treatment of genetic diseases, cancer and infectious diseases [2–4]. Because the development of biopharmaceuticals and biosimilars is complex, regulatory bodies such as the FDA and European Medicines Agency (EMA) require comprehensive drug substance characterization, lot-to-lot and batch-to-batch comparisons, stability studies, impurity profiling, glycoprofiling and related protein and excipient determination, such as polysorbate or protein aggregate elucidation [5,6].

Due to the unique physical and chemical properties of therapeutic proteins, they are prone to a number of changes during their preparation, formulation or storage [7]. These changes include several possible modifications, such as oxidation, deamination, glycosylation, aggregation, misfolding, or adsorption [8–11]. These modifications could lead to the potential loss of therapeutic efficacy or unwanted immune reactions [7]. Aggregate levels in protein-based drugs are a critical quality attribute due to their potential immunogenicity [12,13]. In recent studies, cytotoxic effects have also been observed with several biotherapeutic proteins due to significant denaturation or chemical alterations of the native protein [8,9,14]. In this context, the characterization of protein modifications requires a number of analytical methods because one single technique does not allow for the assessment of all the required parameters [6].

Protein aggregates are generally characterized using sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) [15], size-exclusion liquid chromatography (SEC-HPLC) [16–18], asymmetrical field flow fractionation (AF4) [19], fluorescence spectroscopy [20], circular dichroism (CD) [21] or light-scattering-based methods, such as multi-angle laser light scattering (MALLS) [22]. In addition, native mass spectrometry appears to be an alternative, emerging method to identify and characterize small oligomeric protein aggregates [23–25].

Among these techniques, SEC-HPLC is employed today as the standard separation technique for the quantification of protein dimers, trimers and oligomers. The main advantage of this approach is the mild elution conditions that allow for the characterization of the protein with minimal impact on the conformational structure and the local environment. Recently, some developments have been made in SEC-HPLC via the introduction of columns packed with sub-2 μm particles or the tandem and parallel interlaced approaches that enable a new level of chromatographic performance or throughput [26,27]. Conversely, several detection techniques are now available, ensuring improved sensitivity. The aim of this study is to review the possibilities and new developments in SEC-HPLC applied to the analysis of protein aggregates.

2. Theory of SEC separation

SEC separates biomolecules according to their hydrodynamic radius. The stationary phase consists of spherical porous particles with a carefully controlled pore size, through which the biomolecules diffuse based on their molecular size difference using an aqueous buffer as the mobile phase.

Basically, SEC is an entropically controlled separation process in which molecules are separated on the basis of molecular size differences (filtering) rather than by their chemical properties [28]. In SEC, the partitioning is ideally driven by entropic processes without any adsorption, thus the generally used Gibbs free-energy equation becomes

$$\ln\ K_D = -\frac{\Delta S_0}{R} \tag{1}$$

where K_D is the thermodynamic retention factor, ΔS_0 is the change in the system entropy and R is the gas constant. However, it is important to notice that in practice, temperature can indirectly impact the elution time to a small degree by altering the conformations of the proteins and affecting mobile phase viscosity and solute diffusivity. The thermodynamic retention factor in SEC is different from other chromatographic modes. Here, the thermodynamic retention factor is the fraction of the intraparticle pore volume that is accessible to the analyte [29].

Conversely, an analogous retention factor, k^*, was suggested by Engelhardt as the ratio of the probabilities of the sample staying in the stagnant mobile phase inside the pores and in the moving mobile phase between the particles (interstitial volume) [30]. In SEC, this k^* value is limited, and its maximum value is given by the ratio of the pore volume (V_p) and the interstitial volume (V_z) of a column or similarly by the pore porosity (ε_p) and the interstitial porosity (ε_z):

$$k^* = \frac{V_{elu} - V_z}{V_z} \tag{2}$$

$$k^*_{\max} = \frac{V_p}{V_z} = \frac{\varepsilon_p}{\varepsilon_z} \tag{3}$$

Therefore, the ratio of the pore porosity to the interstitial porosity determines the achievable retention range for a given separation. Using currently available state-of-the-art columns, this maximum retention factor is around $k^* \sim 1.6$–1.8. These parameters – and the associated selectivity – can be altered by packing a column more densely (or decreasing particle size) or by using particles with larger pore volumes.

In SEC, the size-based separation allows the construction of a calibration curve based on a set of known analytes, which can be used to estimate the molecular weight of an unknown analyte. Typical calibration curves are based on proteins or polymers of known molecular weights. By plotting $\log M$ vs. the retention volume, one typically obtains a third order polynomial with a linear region providing the highest resolution and molecular weight accuracy [29]. The largest proteins, which are excluded from the pores, elute first. Subsequent proteins elute in order of decreasing size. As proteins shapes could also vary (e.g., globular, rod-like or flexible chains), their Stokes radii do not correlate exactly with molecular weight. Another source of error in the calibration curve is that non-ideal adsorption may alter the retention volume [31–33].

The slope of the line in the linear portion of the calibration curve is a measure of the selectivity of the stationary phase, which can be defined by the relationship

$$\log\ M = m \cdot K_D + b \tag{4}$$

where m and b are the slope and intercept of the line, respectively. As the pore size distribution of the particle becomes narrower, the slope becomes less steeper, which results in a greater selectivity to discriminate analytes of similar size. A typical SEC calibration curve is shown in Fig. 1.

Similarly to other separation techniques, the main interest of modern SEC is to improve the analysis throughput by reducing the analysis time. The analysis time in SEC is mostly determined by the mobile phase flow rate with a given column configuration because all of the analytes of interest are eluted before the total void time (volume) of the column (there is no partition and retention) [34]. To shorten the separation time in SEC, the ratio of the column void