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Determination of pore size distributions in capillary-channeled polymer fiber stationary phases by inverse size-exclusion chromatography and implications for fast protein separations

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ABSTRACT

Capillary-channeled polymer (C-CP) fibers have been utilized as liquid chromatography stationary phases, primarily for biomacromolecule separations on the analytical and preparative scales. The collinear packing of the eight-channeled C-CP fibers provides for very efficient flow, allowing operation at high linear velocity ($u > 100$ mm s^{-1}) and low backpressure (<2000 psi) in analytical-scale separations. To take advantage of these fluid transport properties, there must not be mass transfer limitations as would be imposed by having an appreciably porous phase, wherein solute diffusion limits the overall mass transport rates. To better understand the physical nano-/micro- structure of C-CP fibers, inverse size exclusion chromatography (iSEC) has been employed to determine the pore size distribution (PSD) within C-CP fibers. A diversity of test species (from metal ions to large proteins) was used as probes under non-retaining conditions to obtain a response curve reflecting the apparent partition coefficient (K_d) versus hydrodynamic radii (r_m). A mean pore radius (r_p) of 4.2 nm with standard deviation (s_p) of ±1.1 nm was calculated by fitting the K_d versus r_m data to model equations with a Gaussian pore size distribution, and a pore radius of 4.0 ± 0.1 nm was calculated based on a log-normal distribution. The derived mean pore radius is much smaller than traditional support materials, with the standard deviation showing a relatively uniform pore distribution. van Deemter plots were analyzed to provide practical confirmation of the structural implications. Large molecules (e.g., proteins) that are fully excluded from pores have no significant C-terms in the van Deemter plots whereas small molecules that can access the pore volumes display appreciable C-terms, as expected. Fitting of retention data to the Knox equation suggests that the columns operate with a characteristic particle diameter (d_p) of ~53 μm.

1. Introduction

High performance liquid chromatography (HPLC) plays a crucial role in the identification, characterization, and processing of proteins, ranging in scale from chip-based proteomics studies to the industrial scale in biotechnology and pharmaceutical applications [1,2]. Numerous new packing materials, including organic and inorganic polymers, have been introduced as stationary phases to improve biomolecule separation quality [3]. While exquisite surface chemistries have been developed to affect high levels of chemical selectivity, the chromatographic efficiencies, throughput, and binding capacities of a system are dictated by the physical characteristics of a given phase. High levels of porosity within small particles provide for bed uniformity, short diffusion distances, and large phase ratios, leading to enhanced efficiencies for small molecule separations; but mass transfer limitations hinder their utility for macromolecule applications. Preparative macromolecule separations are met with the conflicting metrics of high equilibrium binding capacities of porous media, versus their low processing rates/throughput.

Virtually all chromatographic packing materials (i.e., support phases) have porosity on some level. As both the geometry and density of pores can affect the adsorption of analytes and transport behavior of fluid flow, characterization of the pore structure of new chromatographic packing materials is necessary for all candidate stationary phases, with regards to the particular application at hand [4]. The pore size distribution (PSD) of a given stationary phase reflects the density of pores within a certain radii range, making it a critical parameter in describing pore structures [5]. Several methods can be used to determine the PSD of a stationary phase,

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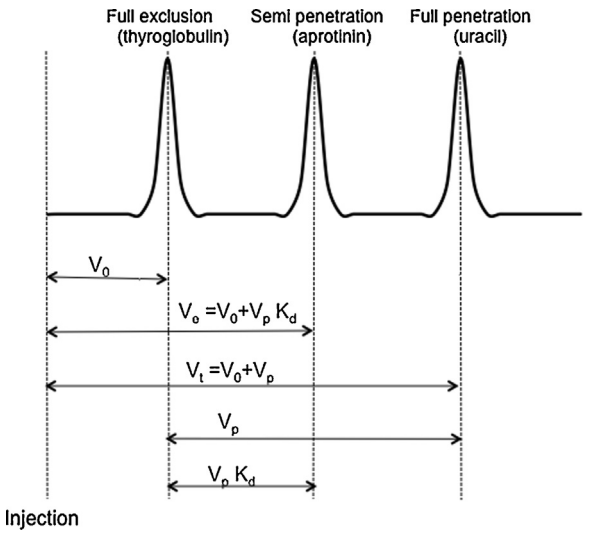

Fig. 1. Extraction of iSEC parameters from the retention volumes (*Ve*) of probes having different hydrodynamic sizes.

including mercury intrusion [6], nitrogen adsorption [7], atomic force microscopy [8], and more recently, ellipsometric porosimetry [9]. Of greater practical relevance, inverse size exclusion chromatography (iSEC) is commonly used because it probes the material under chromatographic conditions [10–12].iSEC has several advantages in comparison to the other methods. For example, in mercury intrusion and nitrogen absorption determinations, a dry experimental environment with high pressure and low temperature are necessary [6,7], which would cause the structural damage of some materials such as polymer beads. In iSEC, intact structural information of materials can be achieved without morphological changes. In addition, iSEC is operated under typical chromatographic conditions, so it is a relatively convenient and inexpensive approach [5]. The method is a simple variation of the size-exclusion chromatography (SEC), used to entropically separate macromolecules based on their hydrodynamic volume or relative size [13]. In SEC, solutes of unknown size/molecular weight (MW) are sequenced using a packing material of known pore dimensions. Conversely, iSEC uses molecules of known size to determine the packing material's unknown pore dimensions [10].

The technique of iSEC was first utilized by Aggerbrandt and Samuelson in 1964 [14] to determine the pore size distribution (coincidently) of cellulose fibers. Later, Knox and Scott [15] and Svec et al. [16] made substantial contributions to refine and apply the theory. With the mathematical models established by Gorbunov in 1988, both the theory and practice of iSEC was strengthened and extended [17]. Yao and Lenhoff have presented an excellent review of the methodology [5]. In the basic experiment, a set of probe species having different hydrodynamic radii (r_m) are injected into a HPLC system to determine their retention times (t_r) and volumes (V_e) under mobile phase conditions which they are not enthalpically retained (i.e., non-retaining conditions). In iSEC, as in SEC, the large molecules are excluded from the pores and therefore elute first, as small molecules become entrapped in the pores of the stationary phase to various extents and elute later. The operational aspects of iSEC are presented in Fig. 1, where V_0 is indicative of the column void volume obtained by the retention volume of a solute too large to penetrate the pores, and V_t is the total permeable volume of the column obtained from the retention volume of the solute that is retained for longest time (presumably the smallest r_m). Solutes of intermediate hydrodynamic radii elute at corresponding retention times.

The distribution coefficient, K_d, also called the partition coefficient [18,19] or the exclusion coefficient [15], represents the fraction of the pore volume accessible to the various solutes and ranges from zero to unity [20]. Chromatographically, this can be calculated by [21]

$$K_d = \frac{V_e - V_0}{V_t - V_0} \tag{1}$$

In iSEC, the response curve of K_d vs. r_m graphically provides important information about the PSD. Ideally, the pores in a given material are assumed to be of the same shape, but having different geometric cross-sections [21]. The pore size distribution function f(*r*) represents the pore cross-section dimensions, thus the total pore volume whose cross-section is in the range between pore radius (*r*) and *r* + d*r* can be calculated. A Gaussian function is commonly used to model the PSD, with the PSD function presented as:

$$f(r) = \exp\left[-\frac{1}{2}\left(\frac{r - r_p}{s_p}\right)^2\right] \tag{2}$$

where r_p is mean pore radius and s_p is standard deviation of the distribution.

While the Gaussian distribution is mathematically straight forward, there are occasions where it provides negative distributions for *r*, which of course have no physical meaning. By the same token, the *a priori* assumption of a Gaussian distribution should be taken with some caution without physical evidence of such. To avoid the negative distribution in the lower range of *r*-values, a log-normal distribution has been used [21,22]:

$$f(r) = \frac{1}{2}\exp\left[-\frac{1}{2}\left(\frac{\log(r/r_p)}{s_p}\right)^2\right] \tag{3}$$

where r_p and s_p have less physical meaning than the Gaussian distribution, but still represent the centroid of the distribution and breadth respectively. Use of a log-normal representation of retention data (log molecular weight/molecular radius vs. retention volume) is a very common means of visualizing the size cut-off characteristics of porous phases [5,10,23,24]. In this work, both of the methods will be presented so as not to pre-suppose the actual pore size distribution function.

The interpretation of K_d in a separation is model-dependent realizing that neither probe molecules nor the probes can be adequately described mathematically in three dimensions. The most generic description, a spherical probe in a cylindrical pore, is used here, so the distribution coefficient for a single pore, *K*, is presented as [15]

$$K = \left(1 - \frac{r_m}{r}\right)^2 \tag{4}$$

Integrated and normalized across the probe and pore populations, the accessible pore volume is presented by the Gaussian distribution [21]

$$K_d = \frac{\int_{r_m}^{\infty} f(r)K dr}{\int_0^{\infty} f(r) dr} = \frac{\int_{r_m}^{\infty} e^{-\frac{1}{2}\left(\frac{r-r_p}{s_p}\right)^2}\left(1 - \frac{r_m}{r}\right)^2 dr}{\int_0^{\infty} e^{\frac{1}{2}\left(\frac{r-r_p}{s_p}\right)^2} dr} \tag{5}$$

and is further described by the log-normal distribution [21]

$$K_d = \frac{\int_{r_m}^{\infty} f(r)K dr}{\int_0^{\infty} f(r) dr} = \frac{\int_{r_m}^{\infty} \frac{1}{r}\exp\left\{-\frac{1}{2}\left[\frac{r-r_p}{s_p}\right]^2\right\}\left(1 - \frac{r_m}{r}\right)^2 dr}{\int_0^{\infty} \frac{1}{r}\exp\left\{-\frac{1}{2}\left[\frac{r-r_p}{s_p}\right]^2\right\} dr} \tag{6}$$