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Kinetic plots in aqueous size exclusion chromatography of monoclonal antibodies and virus particles

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ABSTRACT

The growing importance of monoclonal antibodies and virus particles has led to a pressure for faster size exclusion chromatography. In recent years, numerous small particle columns for size exclusion chromatography of biologicals have been introduced. Small particles are a strategy to reduce analysis time. In the following study, opportunities of small particles in size exclusion chromatography of large biomolecules are investigated. Poppe plots reveal that the lower particle size limit depends on the size of the sample molecule. Hydrodynamic radii of monoclonal antibody monomer, aggregates and H1N1 as well as the diffusion coefficients were determined. Considering this sample compound dependency, kinetic plots referring to the resolution of a distinct compound pair instead of the plate number of a single analyte are more meaningful. Plate times were found to be equivalent with 4 and 2 μm particles for a monoclonal antibody aggregate separation at resolutions smaller than 1.8. Quantification of a H1N1 in clarified cell culture can be accomplished with 17 μm and 13 μm particles at equal plate times at resolutions smaller than 2.5. Virus polydispersity is likely to be affected by run times of several hours at room temperature and shear forces resulting from particles smaller than 10 μm. Comparatively high flow rates should be applied in size exclusion chromatography of the 100 nm H1N1 virions.

1. Introduction

Size exclusion chromatography (SEC) is a versatile chromatographic technique for the qualitative and quantitative analysis of biomolecules. Benefits of the method are high reproducibility, straight-forward method development and mild analysis conditions. In recent years, numerous columns and column formats for SEC of biomolecules have been introduced. Smaller particle sizes are promising with regards to resolution and plate numbers. Knox suggested in 1977 the use of 1 to 2 μm particles for economic and efficient liquid chromatography [1,2]. The trend toward smaller particles has originally emerged in reversed phase chromatography. Numerous publications [3,4] describe advantages of the sub 2 μm particles, which include also protein applications of reversed phase chromatography [5]. After years of mostly theoretical considerations, columns with nonporous 2 μm particles made of silica became commercially available in 1996 [2,4]. Smaller particles allow accomplishing given reversed phase separation in shorter analysis times. Popovici and Schoenmakers came to the conclusion that shorter analysis times in SEC can also most likely be realized when using smaller particles, and reduced analysis times are the most important motivation to use small particles in SEC. Smaller particles allow either to reduce the column length or to increase flow rates [6]. Their conclusions were based on results obtained with polystyrenes and packed particle beds. A good tool to compare the separation performance of different columns and column formats are kinetic plots [7]. Various authors have employed different formats of kinetic plots [8–11]. For instance, Poppe plots were employed to compare superficially and fully porous packing material with monoliths in reversed phase chromatography [12]. Separation efficiency of convective media had earlier been evaluated in the light of increasing flow rates by Rodrigues and co-authors [13,14]. However, to our best knowledge the use of monoliths for SEC is yet limited to smaller proteins and has comparatively lower efficiency than SEC columns based on small fully porous particles [15–17]. Superficially porous particles have a lower pore volume than fully porous particles. Since separation in SEC relies on pore volume, superficially porous particles would at the same column volume provide less separation efficiency.

Popovici and Schoenmakers constructed Poppe plots for gel permeation chromatography of high molecular weight polystyrenes [18] based on a reduced van Deemter plot. They found that diffusion of large polystyrenes is not as unfavorable as formerly thought, which is mainly due to the coupling of mass transfer

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effects and eddy diffusion as introduced by Knox, Parcher and Giddings [19,20]. Poppe plots for small molecules strive asymptotically toward a plate time minimum at high flow rates. According to the results of Popovici and Schoenmakers, this does not hold true for polystyrenes with diffusion coefficients in the range of 10^{-7} cm^2/s [18]. The plate time decreases with decreasing plate numbers even at high flow rates, which means that less challenging separations can be realized at high flow rates and shorter analysis times [18]. This may be a general principle for molecules with similar diffusivity. Diffusion coefficients of typical biopharmaceutical molecules like monoclonal antibodies (mAbs) and virus particles are small compared to small molecules. Typical diffusion coefficients for small molecules in aqueous solution are in the range of 10^{-6} cm^2/s [21]. Diffusion coefficients for IgG molecules are one order of magnitude smaller [22]. This paper intends to discuss the applicability of Poppe plots for SEC of typical biologicals by means of separations with different particle sizes. Practical limits of fast SEC, namely column stability at increased flow, are considered.

2. Theory

SEC discriminates compounds regarding their hydrodynamic radius. The diffusion coefficient is inversely proportional to the hydrodynamic radius of a globular protein by the Stokes Einstein equation [23]. Tanford developed an equation that links the hydrodynamic radius r_h of a globular protein to the cube root of its molecular weight M_W (Eq. (1)) [23,24].

$$r_h \approx 0.081 \cdot \sqrt[3]{M_W} \tag{1}$$

The molecular weight of a protein is logarithmically related to the distribution coefficient K_D of a sample in size exclusion chromatography with k and c as empirical constants [23].

$$K_D = -k \cdot \log M_W + c \tag{2}$$

Eqs. (1) and (2) and the Stokes Einstein equation yield a logarithmic cubic relationship between the diffusion coefficient and the selectivity determining constant K_D in size exclusion chromatography (Eq. (3)).

$$K_D \approx -k \cdot \log\left(\left(\frac{5.7 k_B \cdot T}{6\pi\eta D}\right)^3\right) + c \tag{3}$$

T represents the Temperature, η the dynamic viscosity and k_B the Boltzmann's constant. The diffusion coefficient of H1N1 is smaller than the diffusion coefficient of mAb monomer by one order of magnitude. The diffusions coefficient of mAb may be considered representative for host cell proteins in the virus sample. For a given column and sample type, k and c can be considered constant. Eq. (3) predicts that changes in D will have more impact on K_D if sample diffusivity is low, such as for large molecules. Values between K_D are in the range of 0.0 and 1.0. The impact of K_D and D on H with relation to particle size d_p is given by the following equation [25].

$$H = \frac{2D(1+K_D)}{u} + 2d_p + 0.6u\left(\frac{K_D}{(1+K_D)^2} \cdot \frac{d_p^2}{D}\right) \tag{4}$$

Similar equations correlating *HETP* with sample diffusivity and particle size have been formed by different authors and can be found in literature [17,26]. A more basic correlation of *HETP* and u is given by the van Deemter plot, which is frequently used to construct kinetic plots. Kinetic plots relate the plate time or column void volume to the plate count or the peak capacity [9]. Poppe plots represent a particular kinetic plot, where the plate time t_p, which is the time required to achieve one plate, is plotted on the y-axis. The plate time can be described by the following equation:

$$t_p = \frac{H}{u} \tag{5}$$

The plate time is plotted against the number of plates N required for a given separation. N depends on the column length L and H as can be described by the following equation:

$$N = \frac{L}{H} \tag{6}$$

N and H are related by the Darcy equation (Eq. (7)) at a given flow rate u_1. Plate times of small molecule separations are nearly constant until a critical required plate number is reached. At this threshold value, plate times increase exponentially and strive toward an infinite value at the maximum plate number for a given column and sample. Flow rates or backpressures providing a good compromise between column efficiency (that is plate number) and fast separations can be obtained from Poppe plots and vice versa. A comparative approach allows evaluating different column formats and stationary phases. In the current study, regression of actual $H(u)$ data yields correlations for each sample and column. The Darcy equation can be used to calculate $N(u)$ for a given column, eluent, pressure and flow rate (Eq. (7)).

$$\Delta p = \frac{u\emptyset\eta L}{d_p^2} = \frac{u\emptyset\eta HN}{d_p^2} \tag{7}$$

Δp is the pressure drop across the column and depends on the flow rate. Dynamic viscosity η of water at 298 K is 0.891×10^{-3} kg/(ms) [27]. Flow resistance factors $\emptyset$ are calculated by the Darcy equation (Eq. (7)) at the standard flow rate of every column and the resulting pressure. The Darcy equation is further used to calculate the corresponding Δp at a given flow rate. A pair of variates for N and H/u is calculated for u_0, $u_1 \ldots u_{\max}$ and Poppe plots are constructed numerically.

3. Materials and methods

3.1. Samples & standards

p-Amino benzoic acid (pABA) (Sigma Aldrich, Taufkirchen, Germany) was used for TSKgel SW column performance tests. A solution of 5% acetone (Sigma Aldrich) in water was used to test performance of TSKgel PW SEC columns. A mAb from Chinese hamster ovary cell culture was purified by preparative Protein A chromatography with TOYOPEARL AF-rProtein A-HC 650F. The mAb was eluted from Protein A with 100 mM sodium acetate buffer at pH 3.5. pH of the post-Protein A pool was elevated to pH 6.5 by addition of 0.5 M disodium hydrogen phosphate. The solution was diluted to 0.71 g/L and stored at 2–8 °C.

A pandemic influenza type A H1N1 virus was produced in adherent Madin-Darby Bovine Kidney bioreactor cell culture. This feedstream was kindly provided by IDT Biologika GmbH, Dessau-Rosslau, Germany. The feedstream was concentrated 20-fold volumetrically and contained 4.11 log hemagglutination units/100 μL. Inactivation was accomplished with β-propriolactone. The applied hemagglutination assay protocol by Kalbfuß [28] was adapted from Mahy and Kagro [29].

3.2. Protein size exclusion chromatography columns

TSKgel SW columns were chosen for mAb analysis. Packing material of these columns is based on diol bonded silica particles. Pore size of the TSKgel SW columns used in this study was 250 Å. TSKgel SuperSW3000 and TSKgel UP-SW3000 (all Tosoh Bioscience GmbH, Griesheim, Germany) are available in 4.6 mm ID × 30 cm L.