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Critical evaluation of fast size exclusion chromatographic separations of protein aggregates, applying sub-2 μm particles

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ABSTRACT

A new size exclusion chromatography column packed with 1.7 μm particles and possessing 200 Å pore size has been critically evaluated for the determination of proteins and monoclonal antibody aggregates.

In a first instance, the kinetic performance of this column was compared with that of a conventional column packed with 5 μm particles and with a recently launched 3 μm material, also possessing 200 Å pore size.

In average, 2–5 times lower plate height were achieved on the 1.7 μm packing, compared with the conventional 5 μm particles. It was also demonstrated that elevated mobile phase temperature (up to 50 or 60 °C) allows improving the kinetic efficiency by 20-40% in size exclusion chromatography, compared to 30 °C. On the other hand, the new 3 μm material performed only slightly lower kinetic efficiency than the 1.7 μm one. When considering the upper pressure and temperature limits of these three columns, the 1.7 μm column systematically outperforms the 5 and 3 μm materials in the "practical" plate number range ($N < 30{,}000$) and analysis times could be cut by 2–4 times. The column packed with 5 μm particles was only beneficial for plate counts beyond 100,000 plates, while the 3 μm packing could be considered as a good compromise between speed, efficiency and pressure.

Besides the excellent kinetic performance of 1.7 μm size exclusion material under high temperature conditions, some artifacts were observed when quantifying protein aggregates. Indeed, both high pressure observed with 1.7 μm particles (shear forces, frictional heating) and elevated temperature produce some non negligible amount of on-column additional protein aggregates.

1. Introduction

When dealing with characterization of proteins, one of the most common aspect is the evaluation of aggregation using size exclusion chromatography (SEC) [1,2]. Basically, SEC is an entropically controlled separation process in which molecules are separated on the basis of molecular size differences, rather than by their chemical properties [3]. SEC is frequently employed in the biopharmaceutical industry to characterize purified or recombinant proteins but is also a widely used method, to estimate the molecular weight and molecular weight distribution of polymers [4,5].

The analysis time in SEC is mostly determined by the mobile phase flow rate with a given column configuration, since all analytes of interest are eluted before the total void time (volume) of the column [6]. To shorten the separation time in SEC, the ratio of the column void volume to the flow rate needs to be decreased. Reduction of the column size and increase of flow rate are the straightforward ways for performing fast SEC analysis [6]. Shortening the column length has been the major direction for fast SEC analysis, to keep the backpressure at a reasonable level under high flow rate [7,8]. However, decreasing the column length proportionally reduces the number of theoretical plates. Popovici and Schoenmakers reported several considerations for the fast separations using different commercial SEC columns at room temperature [8]. The effects of particle size, column length, and mobile phase flow rate on the SEC separation were studied in a systematic way [8,9]. The main difficulty in achieving both high speed and high resolution separation in SEC is the slow mass transfer of the large solutes between the interstitial space and the pore space [9]. To increase the mass transfer rate, temperature could be a valuable parameter. As temperature increases, the mobile phase viscosity decreases and the analytes diffusivity improves. Furthermore, this approach allows the use of high flow rate for a given column dimension. As example, Park et al. showed some very fast high temperature SEC separations of polystyrene standards,

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performed at 110 °C [6]. The analysis time in SEC can be shortened by applying small particles, short columns and elevated column temperatures. Today, various packings of 3–20 μm particles are commercially available with different pore-sizes [3]. The most common column dimension in analytical SEC is 30 cm column length and 4.6–8 mm internal diameter. Applying these "conventional" SEC columns, the analysis time is generally comprised between 15 and 50 min. Wätzig et al. currently showed a 15 min long SEC separation of IgG1 antibody aggregates using a conventional 30 cm long column packed with 5 μm particles and demonstrated the precision and repeatability of monomer and aggregate quantitation [10].

Recently a new sub-2 μm SEC material was introduced and makes possible to perform very fast SEC separations [11]. Using this state-of-the-art 1.7 μm SEC material, the aggregates of insulin were recently separated in 7 min [11]. Different variants of monoclonal antibodies were separated in mixed mode liquid chromatography by using this 1.7 μm SEC column, within 10-30 min [12]. Diederich et al. found this column to be suited for antibody aggregate quantification [13]. The authors presented a more than two fold improvement in throughput when comparing this column to conventional TSKgel columns, moreover the throughput was increased by using parallel interlaced size exclusion separation [13].

However, on very fine particles (sub-2 μm), the separation quality is improved at the cost of pressure. It has been established in reversed phase liquid chromatography (RPLC) that high pressure might cause important frictional heating effects inside the column and this is probably also true for SEC. Therefore, temperature sensitive proteins might suffer from on-column aggregation or denaturation [14]. Similarly, when using elevated temperature, there is a risk of on-column aggregation. Finally, when reducing the particle size into the sub-2 μm range, increases the risk of shear degradation [15]. Therefore, using this recent sub-2 μm SEC material, especially at very high pressures and temperatures may raise a number of questions when measuring protein aggregates.

The aim of this study was to perform a critical evaluation of the practical possibilities and limitations of this new commercially available sub-2 μm SEC column. Measurements were performed with three different model proteins (having different molecular weight), to assess the achievable plate counts, separation impedance and analysis time. All the characteristics of this new material were systematically compared to a "conventional" SEC column (a reference column that is widely used in pharmaceutical industry) and to a recently launched 3 μm stationary phase. For reliable comparison, the three stationary phases possess similar pore-size of 200 Å. This 200 Å average pore-size is widely used in the pharmaceutical industry to separate aggregates of therapeutic proteins, including monoclonal antibodies (mAbs). To the best of our knowledge, this study represents the first systematic comparison of 1.7, 3 and 5 μm SEC materials, by applying separation impedance plots and kinetic plot methodology. Finally, the effects of column pressure and temperature on the possible on-column protein aggregation were also evaluated and some practical examples are presented, to highlight the potential of this new sub-2 μm SEC packing for the separation of protein aggregates.

2. Experimental

2.1. Chemicals, columns

Disodium hydrogen-phosphate (BioXtra) and ortho-phosphoric acid 85% (puriss) were purchased from Sigma–Aldrich (Buchs, Switzerland).

Water was obtained with a Milli-Q Purification System from Millipore (Bedford, MA, USA).

IgG monoclonal antibody, panitumumab (Vectibix), was purchased from Amgen (Switzerland). Albumin (from hen egg white), β-lactoglobulin (from bovine milk) and uracil were obtained from Sigma–Aldrich (Buchs, Switzerland).

Acquity UPLC BEH200 SEC column (1.7 μm, 150 mm × 4.6 mm, 200 Å) was purchased from Waters (Milford, MA, USA), YMC-Pack-Diol-200 column (5 μm, 300 mm × 6 mm, 200 Å) was obtained from Lab-Comp Ltd. (Budapest, Hungary) and Phenomenex Yarra SEC-3000 (3 μm, 300 mm × 4.6 mm, 200 Å) was purchased from Brechbühler (Echallens, Switzerland).

2.2. Equipment, software

The measurements were performed using a Waters Acquity UPLC™ system equipped with a binary solvent delivery pump, an autosampler and fluorescence detector (FL) (excitation at 280 nm, emission at 360 nm, 20 Hz). The Waters Acquity system includes a 2 μl sample loop and a 2 μl FL detector flow-cell. The connection tube between the injector and column inlet was 0.13 mm I.D. and 250 mm long (passive preheating included), and the capillary located between the column and detector was 0.10 mm I.D. and 150 mm long. The overall extra-column volume (V_{ext}) was about 15 μl as measured from the injection seat of the auto-sampler to the detector cell. The average extra-column peak variance of our system was found to be around $\sigma^2_{ec} \sim 6 - 8\ \mu l^2$ (depending on flow rate, mobile phase composition and solute). This extra-column peak variance in isocratic elution mode was found to be negligible compared to the columns peak variances obtained with these standard bore (4.6 mm and 6 mm ID) columns. Therefore, no correction for extra-column peak dispersion was applied. Data acquisition and instrument control were performed by Empower Pro 2 Software (Waters).

When studying the effect of pressure on aggregation, column pressure was increased by connecting capillary tubes of 25 μm I.D. and lengths of 5, 10, 15, and 20 cm, to generate the desired pressure. The tubes were connected between the columns outlet and the detector cell inlet, using zero dead volume connectors. The volume of the longest tube was around 0.10 μl, which was negligible compared to the total extra-column volume of the instrument. Therefore, the addition of thin, short restrictor tubing prior to the detector is not expected to affect column efficiency and elution (elution time). The capillary tubes were purchased from SGE Analytical Science (Kiln Farm Milton Keynes, UK).

Calculation and data transferring was achieved using Excel and OriginPro 8 templates.

2.3. Apparatus and methodology

2.3.1. Mobile phase composition and sample preparation

For all experiments, 20 mM disodium hydrogen-phosphate buffer at pH 6.8 (adjusted with ortho-phosphoric acid) was used as mobile phase. This mobile phase ensured appropriate peak shape and peak width for the three model proteins on both columns.

Intact native panitumumab was injected (0.5 μl) without dilution, directly from the concentrated commercial solutions (20 mg/ml panitumumab).

Heat-stressed aggregated panitumumab samples were produced by incubating the commercial solution at 90 °C for 2–10 min. The final samples contained 2–20% (area percentage) aggregates, depending on the incubation time.

The albumin from hen egg and the β-lactoglobulin were injected from their 0.5 mg/ml solutions prepared in water.