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Application Note

Pore Size Distribution Study of GTxResolve™ SEC Columns for Analysis of Genetic Medicine Drug Substances and Products

Mateusz Imiolek[a], Szabolcs Fekete[a], Stephan Koza[b], Matthew Lauber[b]

[a]*Waters Corporation, Switzerland*

[b]*Waters Corporation, United States*

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Abstract

Size exclusion chromatography (SEC) is increasingly used to quickly assess the aggregate content and integrity of genetic medicine drug substances and products. The introduction of new generation widepore SEC columns has enabled robust analyses of large modalities such as lipid nanoparticles (LNPs), plasmids, nucleic acids, viral vectors and similar particles. With a diverse library of biologics under development, it is imperative to deliberately choose a SEC column with appropriate pore properties to facilitate effective size variant analysis. Pore size (PS) and pore size distribution (PSD) dictate which analytes can be accommodated or excluded, directly determining the quality of separation. Various approaches can be used to characterize SEC packing materials to obtain that necessary information. Mercury intrusion porosimetry (MIP) is a popular technique informing on average pore diameter and volume. However, being based on forcing non-wetting mercury into pores, MIP may not provide results that are applicable to and interpretable for complex biological molecules.

Here, a more straightforward procedure is demonstrated involving calibration curves (***i.e.,*** fitting a quantitative

model to elution times) using a set of flexible (nucleic acid) and rigid (gold nanoparticle) analytes of known size that provides the most direct particle size distribution (PSD) and effective median pore measurements. This is highly relevant in the context of many genetic medicines.

Benefits

- Detailed insights into physicochemical properties of 3 widepore SEC packing materials (nominally 450 Å, 1000 Å, and 2000 Å), allowing well-informed choice of the optimal column for a given analyte
- Application of porous materials characterization method based on chromatographically derived models for average PS and PSD determination

Introduction

SEC is highly established in the biopharmaceutical industry, where size variant analysis of monoclonal antibodies and antibody-drug conjugates has been identified as a critical measurement.[1] In recent years, a range of new biologics, collectively known as genetic medicines, have been entering the market and posing new analytical challenges, in large part due to their considerably larger sizes (20–200 nm). The assessment of their high molecular weight species content, heterogeneity and purity plays an even greater role for ensuring safety and efficacy, considering their complex nature and potential for the introduction of genomic alterations.[2] Historically, SEC columns with suitably large pore sizes (>50 nm) have only been available with low efficiency (>5 µm) particles, often packed in stainless steel hardware which make them inadequate to establish robust, high-resolution platform methods essential for analysis of such novel molecules and complexes.[3]

In response to the emerging need, column manufacturers have designed a new generation of efficient (sub-3 µm) SEC columns enabling accelerated and sensitive aggregate analysis of large biologics. Featuring fit-for-purpose chemistry together with low-adsorption hardware that minimize undesired secondary (non-specific) interactions, a set of new particles with increasingly large pores have become available (***e.g.*** GTxResolve Column family of 450 Å, 1000 Å, 2000 Å).[4] One must appreciate that both the pore size and PSD govern the characteristics of a SEC separation, as those metrics describe the accessible pore population that dictates the equilibrium constant of the sizing separation and thus analyte elution times.[5] Packing materials are usually characterized to determine PS, PSD and pore volume using techniques such as nitrogen adsorption (up to 50 nm) and MIP (from 7.5 nm).[6] However, these data are not widely available from all of the manufacturers. SEC

columns are typically only defined by restricted information related to the packing material's average or perhaps median pore size diameter.[7] At times, polymeric or protein molar mass calibration curves are made available, too. Nonetheless, it can be difficult to extrapolate these data to the analysis of a genetic medicine drug substance. Therefore, selection of an SEC column can be problematic and involve trial-and-error testing.

In this application note, this issue has been addressed by further expanding the amount of information available for GTxResolve SEC Columns through the presentation of a simple workflow to determine the median PSD based on calibration curve fitting. For a nucleic acid dsDNA ladder and a set of different size functionalized gold nanoparticles (AuNPs), the elution times of those multiple species in standard SEC separations are determined. Multiangle light scattering (MALS) and dynamic light scattering (DLS) mediated measurements were performed to ascertain accurate analyte sizes under each applied experimental condition. Together, these data are the basis of an inverse size exclusion chromatography approach that yield empirical information on pore properties.

Experimental

Gold nanoparticles functionalization

The AuNPs were functionalized with two different ligands to achieve particles of varying size in solution. Bovine serum albumin (BSA, Sigma-Aldrich) or 11-mercaptoundecanoic acid (MUA, Fluorochem) was employed for surface modification in the following way: concentrated BSA (40 µL, 10 mg/mL in 1X PBS) or MUA (40 µL, 100 mM in EtOH) stock was added to AuNPs solution (360 µL, as received). The resulting mixture was sonicated for 0.5 hour, incubated for 1 hour at ambient temperature and overnight in a chilled autosampler (6 °C). Just before injection, AuNPs solutions were briefly vortexed to suspend sedimented material.

Calibration curve fitting

First, SEC elution times of the calibrants were transformed to partitioning coefficients, K_{SEC}

$$K_{SEC} = \frac{t_e - t_i}{t_M - t_i},$$

where t_e is the analyte's elution time, t_i is the elution time of species eluting in the interstitial volume of the column and t_M is the time of species eluting at the total hold-up time. t_i and t_{M-} can be measured by totally

excluded and fully penetrating markers, respectively. Next, calculated K_{SEC} values and analyte diameter, d_a were fitted to a modified Richard's model[8]:

$$\log d_a = \log d_{50} + \frac{1}{n}\log\left(\sqrt[m]{\left(\frac{1}{K_{SEC}}\right)} - 1\right),$$

in which the values of unknowns: n (fit parameter), m (asymmetry parameter), and d_{50} (the analyte diameter for which K_{SEC} = 0.5) were fitted to the experimental data using Microsoft Excel™ Solver™ Software, via minimization of squared differences sum method. The data were replotted to visualize the K_{SEC} vs log d_a relationship. To obtain accessibility-weighted PSD, the derivative of $f(1 - K_{SEC})$ was calculated and plotted against effective pore size d_p, which in turn is correlated for spherical solutes and cylindrical pores following the Ogston model[9]:

$$d_p \approx 3.14\, d_a.$$

LC Conditions

LC system:	ACQUITY™ UPLC™ H-Class Bio QSM System
Detection:	ACQUITY UPLC TUV Detector with 5 mm Titanium Flow Cell, 260 nm for nucleic acids or 280 nm & 520–580 nm (for AuNPs @ 2 points/s, Wyatt DAWN™ Instrument with a Wyatt-QELS™ Dynamic Light Scattering (DLS) Module.
Vials:	QuanRecovery™ with MaxPeak™ HPS 12 x 32 mm Screw Neck Vial, 300 µL, 100/pk, (p/n: 186009186)
Samples:	1) dsDNA 50 bp to 1350 ladder (p/n: 186010778) 2) AuNPs from 5 nm–60 nm, (suspension in 0.1 mM PBS, reactant free, Sigma-Aldrich)

	functionalized with BSA or MUA ligands
Column:	1) GTxResolve Premier BEH SEC 450 Å Column, 2.5 µm, 4.6 x 150 mm, 7.8 x 300 mm 2) GTxResolve Premier SEC 1000 Å Column, 3 µm, 4.6 x 150 mm, 7.8 x 300 mm 3) GTxResolve 2000 Å SEC Column, 3 µm, MaxPeak Premier Technology, 4.6 x 150 mm
Column temperature:	25 °C
Sample temperature:	6 °C
Injection volume:	1.0 µL for nucleic acids or 10.0 µL for AuNPs
Flow rate:	0.05–0.6 mL/min
Mobile phase:	1) 2X strength PBS buffer for nucleic acids 2) 0.1X strength PBS mobile phase, 2% isopropanol, 0.02% SDS for AuNPs Mobile phases were 0.2 µm sterile filtered before use.

Results and Discussion

SEC separation of dsDNA and AuNPs

The analysis of pore properties using inverse size exclusion chromatography is based on the correlation of analyte diameters with analyte elution properties. In this study, two sets of large-sized analytes were employed – a flexible random coil biopolymer ladder (nucleic acids) and rigid spherical nanoparticles (gold nanoparticles).

The analysis of dsDNA on a widepore SEC column has been optimized in a previous study, in which coupling to MALS detection allowed precise determination of the eluting species size.[10] The experiment with a 50 bp DNA ladder sample was repeated for other available SEC GTxResolve Columns using 2X strength PBS as the mobile phase and the flow rate was adjusted in order to resolve all components of the ladder.

Conversely, the analysis of AuNPs usually requires careful method development and adequate surface treatment of the metal nanoparticles due to prominent secondary interactions with both packing material and column hardware components. Two previously used strategies were used to mitigate non-ideal interactions: 1) addition of anionic surfactant (SDS) into the mobile phase[11] and 2) use of a charged bearing ligand (carboxylate[12] or protein). Additionally, the adoption of such a functionalization strategy creates a set of AuNPs with varying size, extending the calibration. Method development studies revealed the need for salt-deficient and organic solvent containing weakly buffered mobile phase (0.1X PBS, 0.02% SDS, 2% IPA), to efficiently elute the AuNPs analytes from the studied columns, despite the differences in the packing material chemistry (diol bonded 450 Å vs PEO bonded 1000 Å and 2000 Å particles). A DLS detector was used to determine the average hydrodynamic radius of the eluting species close to the peak apex.

The results of SEC separations for both sets of analytes are shown in Figure. 1, wherein the practical elution zones are shown (from full exclusion to full inclusion, as approximated by elution times of large DNA aggregates and a small molecule marker).

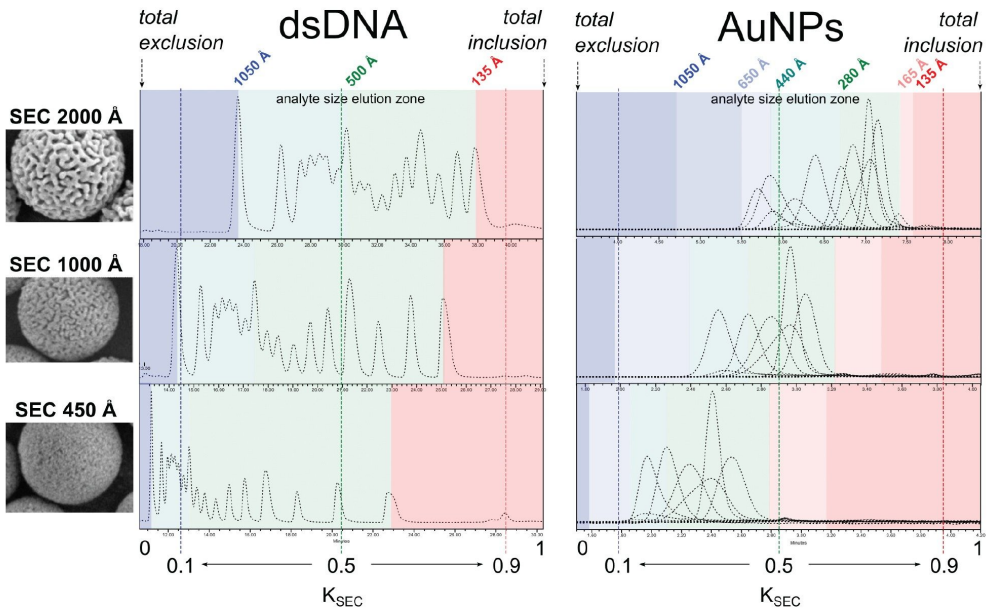

Figure. 1 SEC chromatograms for three GTxResolve Columns (2000, 1000, 450 Å with SEM micrograph of corresponding representative packing material particle on the left) showing separation of dsDNA (left) and AuNPs (right) analytes. Zones in the chromatograms are color coded to indicate regions where analytes of specific size are predicted to elute, while the x-axis was converted to partition coefficient K_{SEC} with characteristic limits of 0.1, 0.5, and 0.9 indicated by vertical lines.

Analysis of the chromatograms revealed agreement between measurements made with the two different analytes. The dsDNA ladder (containing species with sizes ranging from 135 to 1050 Å) could be separated using all three columns, albeit at different conditions (extremely low flow and/or large column dimensions) highlighting the flexibility in SEC method development and importance of packing materials median PS vs PSD. Nevertheless, in accordance to the indicated average pore size, most of the species separated on the GTxResolve 2000 Å SEC Column have K_{SEC} >0.5 (larger pore penetration), confirming the ultrawidepore characteristics of this packing material. The opposite was true for separations with the GTxResolve 450 Å SEC Column. The analytes elute in an intermediate zone on the GTxResolve 1000 Å SEC Column, which is in agreement with its intermediate pore properties.

Calibration curve fitting and pore properties evaluation

The obtained elution and size data were used to construct calibration curves (Figure. 2A). Meanwhile, PSD data and fitting to the Richard's model with Ogston model assumptions allowed us to calculate median PS and visualize the PSD (Figure. 2B, line). A comparison to mercury porosimetry data is plotted with the dotted line traces (Figure. 2B).

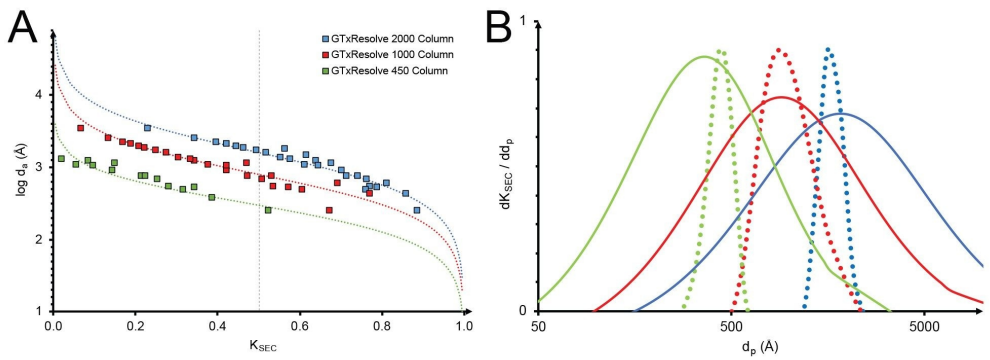

Figure. 2 (A) The logarithm of hydrodynamic diameter (d_a) vs partitioning coefficients (K_{SEC}) of the analytes with a fitted curve following the Richard's model. (B) Derivative of cumulative relative frequency pore diameter plot illustrating log-normal shape of pore accessibility and comparison to normalized mercury porosimetry data (dotted lines). Color legend for both graph: green - 450 Å, red - 1000 Å, blue - 2000 Å.

Based on the fitted data, the median of accessible pores and the characteristic values of K_{SEC} of 0.9, 0.5, and 0.1 was determined for each of the columns (Table 1). This delimited range (0.1–0.9) corresponds to analyte sizes for which the column delivers optimal SEC separation efficiency, while the middle value is usually where the highest selectivity is expected, especially for material with a narrow PSD (flat curve).

Column	Median of accessible pores (Å)	d_a for K_{SEC} = 0.1, (Å)	d_a for K_{SEC} = 0.5, (Å)	d_a for K_{SEC} = 0.9, (Å)
2000 Å	2010	2457	589	97
1000 Å	953	1095	279	54
450 Å	482	308	141	24

Table 1. Calibration curve fitting derived pore properties and effective separation range K_{SEC} (0.1–0.9) for a given diameter d_a analyte.

The data suggests that nominal designation of the column pore size matches the practically determined median of accessible pores within 10% accuracy. Obtained ranges confirm experimental suitability of both ultrawidepore columns (1000 Å and 2000 Å) for the separation of large analytes (>50 nm) and the GTxResolve 2000 Å SEC Column for species >100 nm.

These results suggest that the GTxResolve 2000 Å SEC Column offers the best selectivity for analytes of about 500 Å (in the middle of the elution window and $K_{SEC} \approx 0.5$), while the GTxResolve 450 Å Column provides only limited resolution for species larger than this (blue zones Figure. 1). This can be intuitively confirmed through interpretation of the chromatograms from Figure. 1.

Similar experiments were conducted for MaxPeak Premier Protein 250 Å SEC Columns, for which obtained calibration curves can guide method development for separation of smaller analytes (<15 nm).[13]

Comparison of pore size distribution graphs reveals the same trend and results in similar median values, though inherent differences between the two methods illustrate the difficulty in assessing the relative width of a PSD. For example, based on Hg intrusion data, it would have been difficult to predict the relative potential of the GTxResolve 2000 Å Column for separating AuNPs (165–650 Å). This discrepancy reflects the fundamental methodological differences: MIP measures geometric pore diameters under high-pressure intrusion, primarily probing the narrowest constrictions of connected pores, whereas iSEC reflects the hydrodynamic accessibility of analytes in solution, effectively integrating effects of pore connectivity, tortuosity, and size heterogeneity along the flow path. As a result, iSEC-derived PSDs emphasize functional accessibility under chromatographic conditions, while MIP provides a more structural but narrower view of pore entry sizes.

Conclusion

This application note demonstrates that the effective PSD of an SEC column can be estimated based on the elution volumes of analytes with known size (d_a). Chromatographic calibration curves were constructed with nucleic acid and metal nanoparticle analytes. That elution data could be then converted into detailed information about the packing material pore properties following simple modelling. Both flexible and rigid analytes could be separated on three different widepore packing materials (GTxResolve 2000 Å, 1000 Å, and 450 Å SEC Columns), informing on their most suitable size ranges and justifying nominal pore size nomenclature for these columns. It is worth noting that the obtained total PSD should be viewed as the analysis of the accessible pore population (as dead-end pores do not contribute to a separation). Such an approach can guide design of SEC separations for new analytes of known hydrodynamic diameter, which can be determined via batch DLS experiments.

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