HPLC columns

Achieving consistent SEC performance through the use of 3 µm, 550 Å monodisperse media in novel bioinert column hardware

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Abstract

Purpose: Demonstrate the benefits of novel bioinert coating and the consistency of lot-to-lot results achieved using Thermo Scientific™SurePac™ Bio 550 SEC MDi™ 3 µm Columns.

Methods: Size exclusion chromatography (SEC) with ultraviolet (UV) and refractive index (RI) detection.

Results: Improved sample recovery and reproducibility with the use of advanced monodisperse particle platform with novel bioinert hardware (MDi) technology.

Introduction

Size exclusion liquid chromatography has been widely used for detecting and quantifying aggregates due to its ability to differentiate molecules based on size. SEC effectively separates aggregates from their native state by exploiting the ability of analytes to access the pores of the stationary phase, with larger molecules being more pore-restricted and eluting earlier than smaller ones.

A 3 µm monodisperse media with narrow pore size distribution centered at 550 Å was specifically engineered for the high-resolution analysis and precise measurements of adeno-associated virus (AAV) monomeric capsids from high molecular weight species (HMWS) and was recently introduced to the market [1]. This media features monodispersed silica particles covalently modified with a proprietary diol hydrophilic layer, which minimizes secondary interactions and works perfectly together with the hydrophilic-coating of the column hardware. These properties reduces secondary interactions and ensures the optimal performance for all analytes from the first injection.

The consistent size of the monodisperse particles maintained from batch-to-batch synthesis allows for precise control over column packing, resulting in reliable lot-to-lot performance. The narrow 550 Å pore size distribution, ensures a wide separation range, providing accurate, and reproducible analysis. This enables exceptional and robust separation of various macromolecules, not only AAVs.

This study investigated the physical parameters of the 3 µm, 550 Å monodisperse media and shows it's excellent batch-to-batch consistency. The media packed in the novel bioinert column hardware provided superior recovery of analytes compared to a conventional one. Additionally, the optimal testing conditions were determined by performing a Van Deemter curve study and were then used for protein and other macromolecule analysis to create a calibration curve based on both molecular weight and hydrodynamic radius.

This study underscores the capabilities of the 3 µm, 550 Å, monodisperse media packed in coated hardware to provide robust and accurate characterization of various protein aggregates, making it an invaluable tool for researchers in the field of large molecule separation.

Materials and methods

Sample List

- 4-Aminobenzoic acid (P/N A9878, Sigma-Aldrich)
- Albumin from porcine serum (P/N A2764, Sigma-Aldrich)
- Blue dextran (P/N MB32165, Biosynth Ltd)
- Cytochrome C from equine heart (P/N C2506, Sigma-Aldrich)
- EasiVial PEG/PEO (P/N PL2080-0200, Agilent)
- Gibco™ Insulin (P/N 12585014, Thermo Fisher Scientific)
- NativeMark™ Unstained Protein Standard (P/N LC0725, Thermo Fisher Scientific)
- Thyroglobulin from bovine thyroid (P/N T1001, Sigma-Aldrich)
- Recombinant streptavidin, Bovine Serum Albumin, Carbonic anhydrase II, Ribonuclease, Thioredoxin samples are generated in house in TFS (Vilnius, Lithuania)

Columns

SurePac Bio 550 SEC MDi 3 µm Column, 4.6 × 150 mm (P/N 43903-154631)

R&D prototype of SurePac Bio 550 SEC MDi 3 µm Column in 4.6 × 150 mm conventional Stainless-steel hardware

Sample Preparation

Samples were prepared in the mobile phase, unless stated otherwise. Blue dextran was dissolved to 0.2% (w/v) using water. EasiVial PEG/PEO was prepared according to manufacturer recommendations. 4-aminobenzoic acid (4-ABA) was prepared in the concentration of 2.5 µg/mL, while remaining proteins - 0.5 mg/mL.

Test Method

Mobile phase: 50 mM phosphate buffer with 300 mM sodium chloride, pH 6.5.

Isocratic method parameters:

Flow: 0.35 mL/min

Column compartment: 30 °C

Inj. Volume: 1 – 20 µL

UV: 280 nm

RI was used only for PEG/PEO analysis

Data Analysis

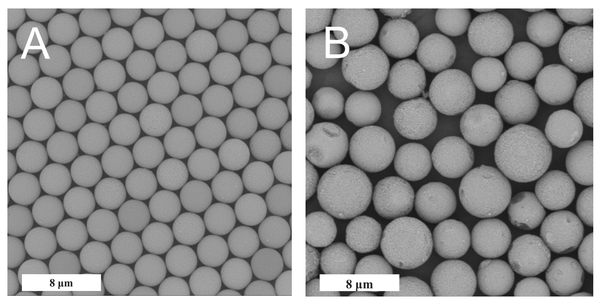
Thermo Scientific™ Chromeleon™ 7.3.2 CDS software was used for data acquisition and analysis.

Results

Monodisperse vs. Polydisperse Silica Particles

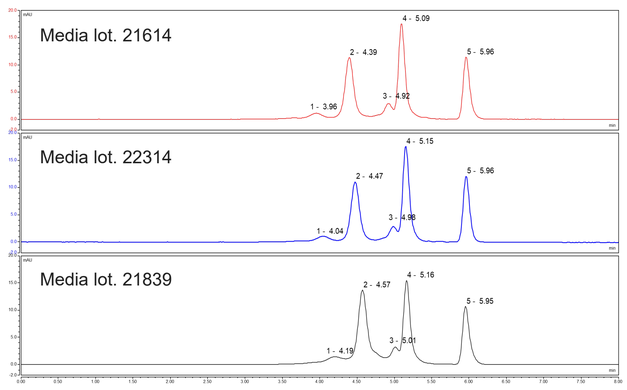
The SurePac Bio 550 SEC MDi 3 µm column monodispersed silica particles are covalently modified with a proprietary diol hydrophilic layer. This proprietary process brings an extremely low level of non-desired interaction sites. Compared to traditional polydisperse particles (Figure 1B), the consistent size distribution of the monodisperse particles (Figure 1A) not only facilitates precise control over media synthesis and column packing, but also significantly improves column-to-column and lot-to-lot reproducibility.

Figure 1. SEM image of 3 µm monodisperse silica particles (A) vs. 3 µm polydisperse particles (B).



Three different batches of media were chosen for lot-to-lot reproducibility assessment based on their proximity to the specification limits, with the aim of evaluating the impact on the chromatographic results. Testing was performed with a test mix of three analytes: fully retained analyte – 4-ABA, intermediate size protein – albumin and thyroglobulin – a well-researched large protein (Figure 2).

Figure 2. Chromatographic results of three different batches. Peaks: 1 – thyroglobulin dimer, 2 – thyroglobulin, 3 – albumin dimer, 4 – albumin, 5 – 4-ABA

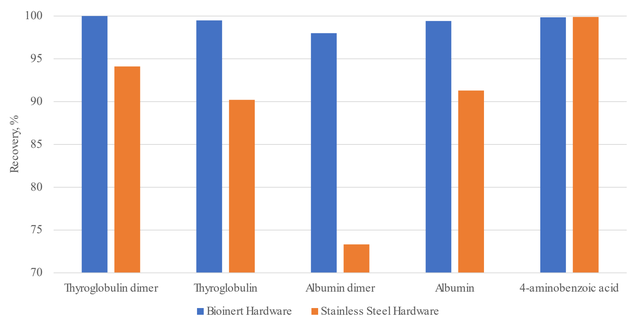


Retention time depends on the pore volume of stationary phase. The fully retained analyte in all three batches has identical retention times, while other analytes show only a slight variations despite the batches being chosen based on their proximity to specification limits.

Media batch, #	Pore Volume, cm³/g	Δt (4-ABA – TG2), min
21614	Upper limit of specification	2.00
22314	Middle point of specification	1.92
21839	Lower limit of specification	1.76

The stationary phase was housed in state-of-the-art hydrophilic-coated stainless-steel hardware. The hydrophilic bioinert coating reduces secondary interactions, ensuring optimal performance during the initial injection (Figure 3). Two columns of the same media batch were compared: SurePac column and R&D prototype of a SurePac column in conventional stainless steel hardware.

Figure 3. Recovery comparison of first injection

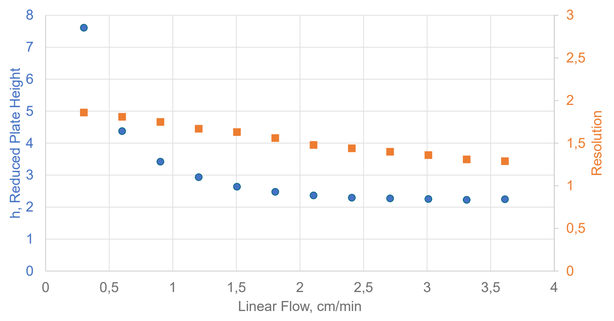


The use of high salt additives in the mobile phases of SEC analysis to reduce secondary interactions is widely reported. However, results of this study show that secondary interactions with column body persist in these conditions, unless bioinert hardware is used. Moreover, decreasing salt content in the mobile phase would greatly increase secondary interactions with conventional hardware.

Analysis optimisation

The identification of the optimal flow rate provides benefits for efficiency and resolution of chromatographic separation. This optimization enhances the separation performance, minimizes analysis time, and ensures reproducibility across different batches. Various flow rates ranging from 0.05 mL/min to the recommended limit of 0.60 mL/min were selected. Two parameters were monitored: the reduced plate height of a fully retained analyte and the resolution of the thyroglobulin dimer (Figure 4). Resolution is slightly decreasing with increased flow rate; however reduced plate height indicates that optimal performance is observed around 2.1 cm/min flow rate (0.35 mL/min). This flow rate of 2.1 cm/min was chosen for testing, as it provides fast and efficient separation.

Figure 4. Flow rate influence on the reduced plate height of 4-ABA and resolution of the TG2

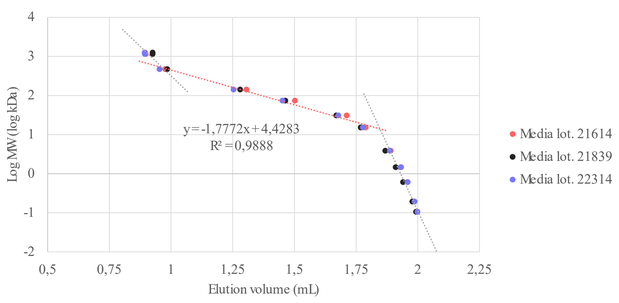


Separation range

Key parameter of size exclusion columns is the separation range. The SurePac Bio 550 SEC MDi 3 µm column utilizes the use of 550 Å pores which is designed for AAV analysis. As pore size is oriented towards bigger molecules, smaller molecules will not be effectively separated by these pores.

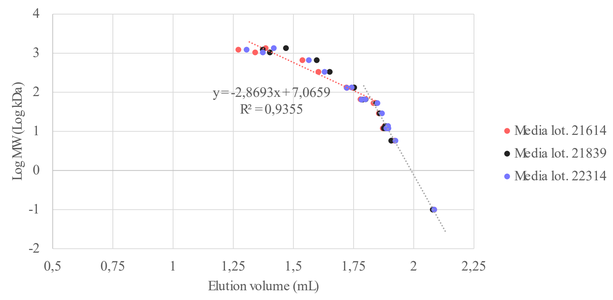
Separation range can be determined using known size standards, like polyethylene glycol/polyethylene oxide calibration kit (Figure 5). This kit contains PEG/PEO ranging 106 – 1,250,000 g/mol in size.

Figure 5. Calibration curve of PEG/PEO (molecular weight vs. Elution volume) obtained with 3 different media batches of the SurePac Bio 550 SEC MDi 3 µm Column



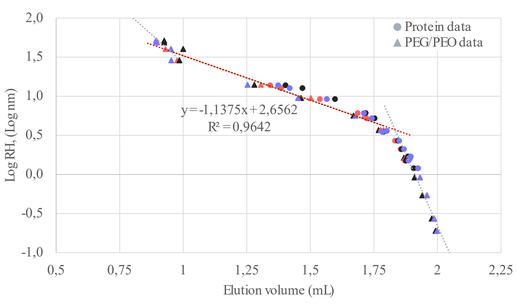
PEG/PEO calibration kit allows determining the limits of separation; however, the nature of polymers significantly differs from proteins. Proteins tend to fold in very compact structures, so even with bigger molecular weight, their hydrodynamic radius is smaller – calibration curve is steeper (Figure 6).

Figure 6. Calibration curve of proteins (molecular weight vs. Elution volume) obtained with 3 different media batches of the SurePac Bio 550 SEC MDi 3 µm column



Separation is based on the size of the molecule, characterized by its hydrodynamic radius, rather than its molecular weight. Consequently, using molecular weight for the calibration curve of PEG/PEO and proteins is not feasible. Therefore, a combined calibration curve for proteins and PEG/PEO was established using the hydrodynamic radius (Figure 7).

Figure 7. Calibration curve of PEG/PEO and proteins (Hydrodynamic radius vs. Elution volume) obtained with 3 different media batches of the SurePac Bio 550 SEC MDi 3 µm column



Results indicate that the SurePac Bio 550 SEC column can separate proteins starting from 50 kDa, although the upper exclusion limit could not be determined, as the thyroglobulin dimer (1,350 kDa) lies on the calibration curve.

Conclusions

The SurePac columns utilize the use of monodisperse particles and bioinert hardware. This technology shows both batch-to-batch and column-to-column consistency. Novel bioinert hardware provides stable results from 1st injection.

- Bioinert hardware allows for nearly complete recovery of 100% from first injection, significantly outperforming conventional hardware.
- The monodisperse particle technology provides consistent results from batch-to-batch, even when media with proximity to limits of specification are taken into account.
- The calibration curves of SurePac columns were introduced for PEG/PEO and proteins samples and show good linear dependency (R^2>0.9). The extensive study of proteins and PEG/PEO indicates a separation range of 4–30 nm in hydrodynamic radius.

References

1.Ma, K.; Ashworth, J.; Nieves, V.; Koontz, A.; Bechler, S., "Size-exclusion chromatography of adeno-associated viruses with the SurePac Bio 550 SEC MDi column", Application Note 003089, Thermo Scientific, 2024

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