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Application News

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Biopharma / Nexera Bio UHPLC / Shim-pack Bio Diol

Optimizing Aggregate Analysis – Pore Size

❑ Introduction

Size exclusion chromatography (SEC) is one of the chromatographic separation modes used for large molecules such as proteins and polymers by their sizes. SEC is one of the crucial technique for characterizing protein-based therapeutic products. It is used for qualitative and quantitative analysis of fragments, monomers, dimers, and higher aggregates of therapeutic proteins. The variations in aggregates impact the applicability, efficacy, and safety of these therapeutic proteins.

In SEC, smaller molecules enter the pores of the particle and proceed slowly along the axis direction of the column while molecules larger than the pore size are excluded from pores and elute at first from the column. In this application, we demonstrate the effect of pore size in SEC analysis. The analysis was performed using Shim-pack Bio Diol columns with different pore sizes on a Nexera Bio UHPLC system.

❑ Experimental

Bio-rad's gel filtration standard mixture was diluted in water. The sample was analyzed by a Shimadzu Nexera Bio UHPLC with a UV detector. Sodium phosphate buffer was prepared by mixing 0.1 mol/L NaH_2PO_4 + 0.1 mol/L NaCl and 0.1 mol/L Na_2HPO_4 + 0.1 mol/L NaCl stock solutions to pH 7. Analytical conditions are shown in Table 1.

Table 1: SEC analytical conditions

LC system	: Nexera Bio UHPLC
Column	: Shim-pack Bio Diol, 300 mm x 4.6 mm I.D., 3 µm Diol-60 (P/N: 227-31007-01) Diol-120 (P/N: 227-31008-01) Diol-200 (P/N: 227-31009-03) Diol-300 (P/N: 227-31010-03)
Column temp.	: 25 ℃
Mobile phase	: 0.1 mol/L(sodium) phosphate buffer + 0.1 mol/L NaCl (pH 7)
Flow rate	: 0.5 mL/min
Elution mode	: Isocratic flow
Injection vol.	: 8 µL
Detector	: UV, 280 nm

Table 2: Other consumables

Item description and P/N*
1.5ml Screw-thread amber vial with write on spot, caps with PTFE/white silicone septa (P/N: 226-54111-41)
1L Solvent bottle (P/N: 226-88583-02)
Solvent safety caps kit (P/N: 226-50319-01)
LC solvent waste kit (P/N: 226-50330-00)
PEEK fitting (P/N: 226-50106-02)

*Please contact your local Shimadzu representative for more information on these consumables.

❑ Results & Discussions

Table 3 lists the components of the gel filtration standard mixture. Figure 1 shows the analyses of GFC standard mixture on Shim-pack Bio Diol columns with different pore sizes.

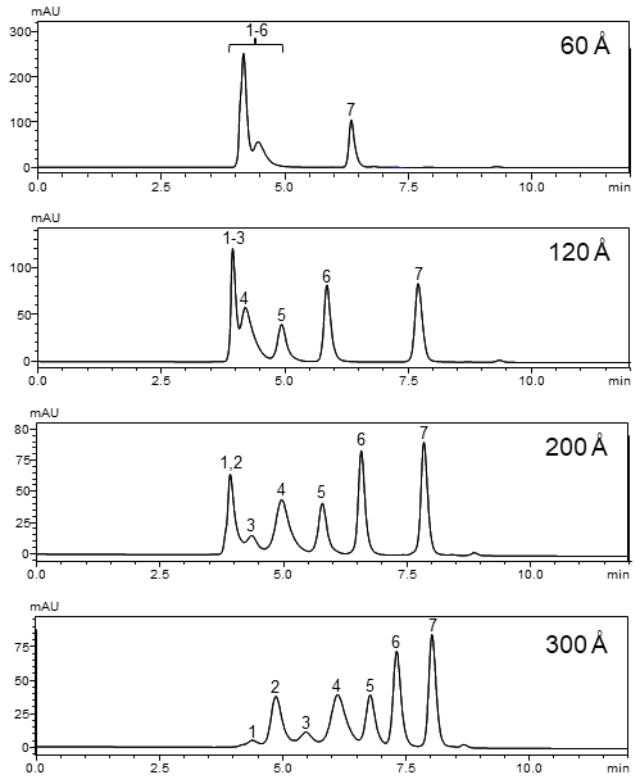

Figure 1. Analysis of GFC standard mixture on Shim-pack Bio Diol columns with different pore sizes. Peaks: 1. aggregates of thyroglobulin, 2. thyroglobulin, 3. degradation product of thyroglobulin, 4. ɣ-globulin, 5. ovalbumin, 6. myoglobin, 7. vitamin B12.

Table 3: Gel filtration standard components

Component	Estimated molecular weight	Concentration
Thyroglobulin (bovine)	670,000	1.2 mg/mL
γ-Globulin (bovine)	158,000	1.2 mg/mL
Ovalbumin (chicken)	44,000	1.2 mg/mL
Myoglobin (horse)	17,000	0.6 mg/mL
Vitamin B12	1,350	0.12 mg/mL

Table 4: Resolution value between proteins of Shim-pack Bio Diol columns with various pore sizes.

	Column			
	60 Å	120 Å	200 Å	300 Å
Aggre/Thyro	-	-	-	1.02
Thyro/Degrade	-	-	1.08	1.23
Degrade/γ-glo	-	0.72	1.14	1.12
γ-glo/Ovalb	-	1.74	1.93	1.38
Ovalb/Myo	-	3.14	2.71	1.61
Myo/Vit B12	5.07	6.95	4.93	2.44

Table 4 summarizes resolution values of adjacent proteins on each column. The 60 Å column was unable to resolve the high molecular weight proteins (thyroglobulin, γ-globulin, ovalbumin, and myoglobin). The resolutions of the proteins were improved on the 120 Å column with good resolution between ovalbumin, myoglobin, and vitamin B12. Thyroglobulin and γ-globulin were not resolved on the 120 Å column. The 200 Å column was able to separate all components in the GFC standard mixture except for thyroglobulin and its high molecular weight aggregates. Similar result was observed on the 300 Å column with better resolution on the high molecular weight thyroglobulin aggregates.

As expected, pore size of SEC column affected the peak resolution. Thus, SEC column should be chosen according to the target molecular weight range (Table 5). Small pore size columns (Diol-60 and Diol-120) separated low molecular weight components. The high molecular weight proteins were bigger than the pores in the particle, so they were excluded from the pores and unresolved from each other. Columns with larger pore size (Diol-200 and Diol-300) allowed the high molecular weight proteins to enter the pores and elute at different retention times. As a result, large pore size columns improved the resolution of the high molecular weight components.

On the other hand, resolutions of adjacent low molecular weight components (vitamin B12, myoglobin, and ovalbumin) decreased as pore size increased. The molecular size of these molecules are smaller than the pore size. Therefore, these molecules can diffuse into the pores. The difference in elution time decreased as the pore size increased.

Table 5: Target molecular weight range of Shim-pack Bio Diol columns.

Shim-pack Bio	Target molecular weight range
Diol-60	Below 10,000
Diol-120	1,000 – 100,000
Diol-200	5,000 – 300,000
Diol-300	20,000 – 1,000,000

Shim-pack Bio Diol columns are available in various pore sizes. Good repeatabilities were shown in Figure 2 and Table 6. The repeatabilities in elution time and peak area of six consecutive analyses were less than 0.3% RSD for all proteins.

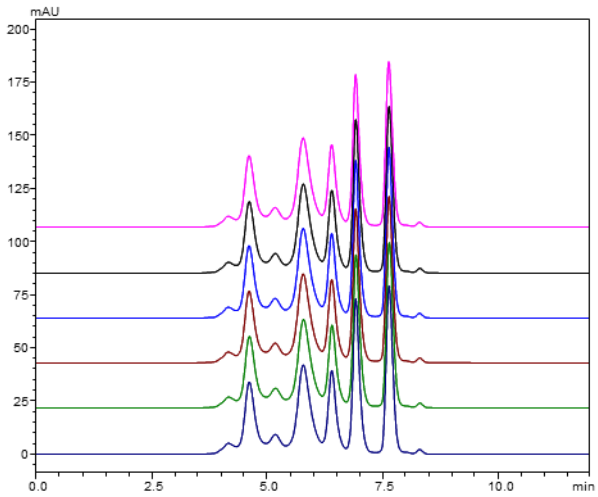

Figure 2. UHPLC-UV (280 nm) chromatograms of six injections of gel filtration standard mixture on Shim-pack Bio Diol-300.

Table 6: Repeatabilities of elution times and peak areas (n = 6) of proteins on Shim-pack Bio Diol-300.

	Elution time		Area	
	Average (min)	%RSD	Average	%RSD
Thyroglobulin	4.63	0.07	5,803,656	0.22
γ-Globulin	5.80	0.05	8,894,182	0.22
Ovalbumin	6.42	0.04	5,198,125	0.18
Myoglobin	6.94	0.04	7762140	0.07
Vitamin B12	7.66	0.05	8473126	0.06

❑ Conclusion

SEC is crucial for characterization of protein-based therapeutic products. It is important to select a column with the appropriate pore size that matches the molecular weight of analytes for achieving the best resolution. The various pore sizes of Shim-pack Bio Diol columns provide easy method development in SEC analysis of aggregates and fragments of mAb, oligonucleotides, and carbohydrates.

Disclaimer: The data and instruments presented in this Application News are intended for Research Use Only (RUO). Not for use in diagnostic procedures.

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