Application Note

Biopharmaceuticals

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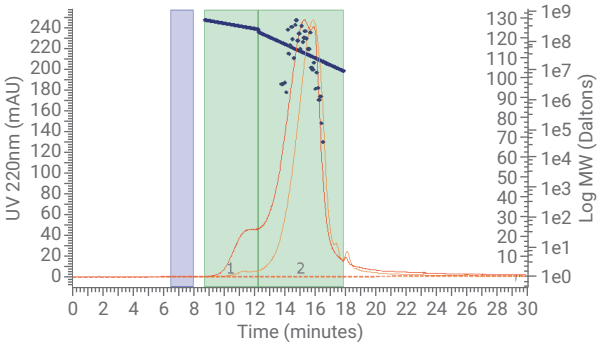
Determination of Hydrodynamic Radius (Rh) of Extracellular Vesicles Using the Agilent 1290 Infinity II Bio LC System and Bio-MDS



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Abstract

Exosomes are a type of extracellular vesicle (EV) that function as a vehicle to deliver proteins, nucleic acids, etc. and are used to deliver drugs to target organs. Exosomes range in size from 40 to 160 nm, and size verification is a key quality control (QC) measure to differentiate them from non-exosomal components.

The Agilent 1260 Infinity II Bio-SEC multidetector suite (Bio-MDS) supports the measurement of static light scattering (SLS) and dynamic light scattering (DLS). This technique shows high reliability when used in conjunction with size exclusion chromatography. Using this methodology, information on the size distribution of biologically derived macroparticles and hydrodynamic radius (Rh) can be confirmed.

In this application, the Rh of exosomes was confirmed using the Agilent 1290 Infinity II Bio LC system and Bio-MDS, and the relative standard deviation of Rh was confirmed to be within 2%. Additionally, SLS results measured at a 90° angle showed higher sensitivity for exosomes when compared to the UV detector.

Introduction

Extracellular vesicles (EVs) are vesicles derived from cells that are released to maintain homeostasis in the body and contain various intracellular substances such as proteins, nucleic acids, and lipids. Depending on the method of excretion, these substances are classified into ectosomes and exosomes, and among these, exosomes are reported to have a size of 40 to 160 nm. Exosomes are used for various purposes, including cosmetics and pharmaceuticals. Research is actively being conducted on procedures to load drugs into exosomes, starting with the use of naïve exosomes. Exosomes are not only more biocompatible than artificially manufactured lipid nanoparticles but also have the advantage of being able to specify target organs, depending on the cell type of origin. Exosomes are therefore being studied as drug transporters for highly toxic drugs, such as anticancer agents, for delivery to target organs.

Exosomes are purified from cell-derived substances such as parent cells, proteins, and microvesicles and are utilized as naïve exosomes or as drug delivery vesicles. Measuring the size and quantity of exosomes in the purification process and in the final product is one of the key factors in product QC. The nanoparticle tracking analysis (NTA) method, which monitors the Brownian motion of individual particles is being utilized for analysis. However, there are challenges in reliability and usability, such as variable results depending on the operator skill level and large sample requirements for analysis. Dynamic light scattering (DLS) is a principle that measures Rh by collecting data through the autocorrelation function (ACF) that reflects the Brownian motion of particles. But DLS has limitations, such as susceptibility to interference from bubbles and impurities. Additionally, when measuring two or more types of dispersed distributions of samples (bimodal), distinct results cannot be obtained unless the size difference between the particles is more than twofold.

Size exclusion chromatography (SEC) is a technique that can separate analytes based on their size. In SEC analysis of exosomes, the difference in size between exosomes and other impurities can be utilized. Impurities such as soluble proteins, other than membrane proteins mixed in the sample, can be separated by SEC and a check of the chromatogram according to the size of the exosome helps to understand the general characteristics of the sample. However, exosomes have diverse protein and lipid compositions depending on their origin, exhibiting different physicochemical properties. These properties may cause unexpected interactions with SEC columns, which may distort the correlation between elution time and size in SEC. Therefore, it is necessary to increase the reliability of the results by utilizing the DLS detector to confirm the size of exosomes. Since the signal of the UV detector is affected by the sample chromophore, the area value obtained from the UV detector reflects the optical properties of the absorbance for each exosome sample. The UV result makes it difficult to quantify exosomes with different properties, whereas DLS utilizes light scattering, free from the chromophore, making it possible to quantify exosomes of different origins.

By using a size exclusion chromatography system, the limitations of general DLS such as bubbles and interference from impurities were eliminated. Also, by separating and analyzing samples with bimodal and multimodal polydispersity through SEC, the reliability of the results was increased. By injecting a small amount of sample through an automatic sample injector, sample consumption was reduced, and precision was increased. To minimize the interaction between exosomes and the column, a high concentration buffer was used as the mobile phase. The 1290 Infinity II Bio LC system was used considering the interaction between the sample, the system, and the use of a high salt concentration mobile phase.

Experimental

Standards materials and reagents

Sodium phosphate dibasic heptahydrate, sodium phosphate monobasic, and bovine serum albumin were purchased from Sigma-Aldrich, and sodium chloride was purchased from Merck. Exosome standard (HEK293 cell line, $>1\times10^{10}$ particles/vial) was purchased from Novus.

Analysis instrument

- Agilent 1290 Infinity II Bio Flexible Pump (G7131A)
- Agilent 1290 Infinity II Bio Multisampler (G7137A) with sample temperature control unit
- Agilent 1290 Infinity II Multicolumn Thermostat (G7116B) with Bio standard flow heat exchanger
- Agilent 1290 Infinity II Variable Wavelength Detector (G7114B) with Bio standard flow cell (G1314-60188)
- Agilent 1260 Infinity Multi-Detector Suite (G7805A) with 1260 Infinity Bio-inert Dual Angle and Dynamic Light Scattering Detector (G7809A)

Column

Agilent Bio SEC-5 5 µm, 2000 Å, 7.8 x 300 mm (part number 5190-2541)

Software

Agilent Bio-SEC software version A. 02.01

Mobile phase and sample preparation method

The mobile phase was prepared by dissolving sodium phosphate dibasic heptahydrate 34.58 g, sodium phosphate monobasic 2.47 g, and sodium chloride 17.53 g in HPLC grade deionized water and filtering three times through a 0.2 µm RC filter.

Bovine serum albumin (BSA) was dissolved in the mobile phase at 20 mg/mL and filtered through a 0.2 um Captiva Premium Syringe Filter with a regenerated cellulose membrane (part number 5190-5106), and the exosome standard was used by dissolving in 100 µL of deionized water at 1 mg/mL (>1 × 10^{11} particles/mL).

Table 1. Agilent HPLC analysis conditions.

Parameter	Value
Mobile Phase	150 mM phosphate buffer pH 7.4 + 300 mM Sodium chloride
Flow Rate	0.6 mL/min
Analysis Time	60 min
Injection Amount	20 µL
Sampler Temperature	4 °C
Column Temperature	40 °C
VWD	220 nm
LS	40 °C, 1 Hz

Table 2. DLS analysis conditions.

Parameter	Value
Correlator Run Time	5.0 s
Correlator Function Clip Time	10 µs
R2	0.80
Viscosity of Eluent	0.00065 P (adjusted based on standard)
Refractive Index of Eluent	1.333

Results and discussion

Chromatogram

To minimize interactions between exosomes and the column, a composition of 150 mM phosphate buffer + 300 mM sodium chloride was used as the mobile phase. The concentration of the mobile phase was set considering that low salt concentration buffers can cause peak tailing due to interactions and high salt concentration buffers can cause structural deformation of the analyte. To minimize interference between injections due to peak tailing, the analysis time was set to 60 minutes. The SEC chromatograms of the BSA standard at a concentration of 20 mg/mL and the exosome standard at a concentration of 1 mg/mL were checked by UV at 220 nm and light scattering at a 90° angle. The exosomes showed a lower peak height at UV but a higher peak height at LS 90° compared to BSA (Figure 1).

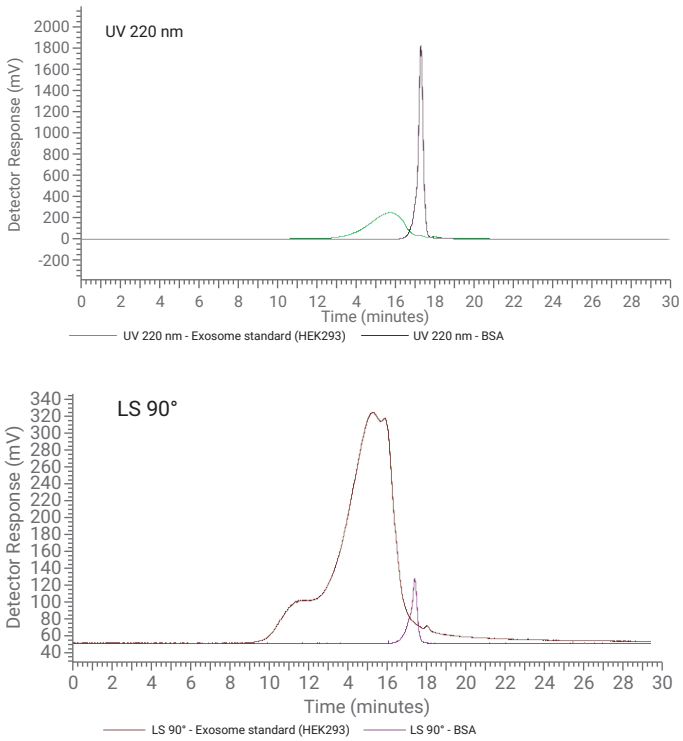

Figure 1. UV 220 nm, LS 90° chromatograms of BSA and exosome standard.

Determination of Rh of exosome standard

After sample analysis, parameters must be adjusted so that DLS produces the intended measurement values, preferably using standards with similar properties. To determine the Rh of exosomes, the eluent viscosity was set to 0.00065 P so that the Rh result matched the size of the exosome standard. The Rh of the main peak (Peak 2) of exosomes was identified from 67 to 130 nm, with an average measured at approximately 110 nm (Figure 2).

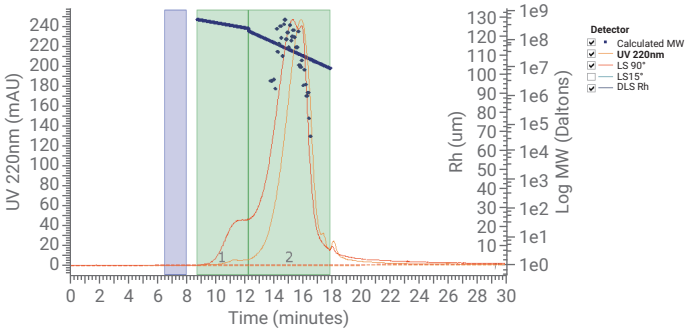

Figure 2. LS 90°, UV 220 nm chromatogram of exosome standard and Rh measurement results.

Confirmation of the repeatability of LS and DLS

A 20 µL aliquot of exosome standard solution was injected three times, and the peak area and Rh at LS 90° and UV for peak 2 were confirmed. Good repeatability was confirmed with the relative standard deviation of Rh of 1.86%, the relative standard deviation for the area at LS 90° of 3.48%, and the relative standard deviation for the area at UV 220 nm of 2.00%. (Table 3).

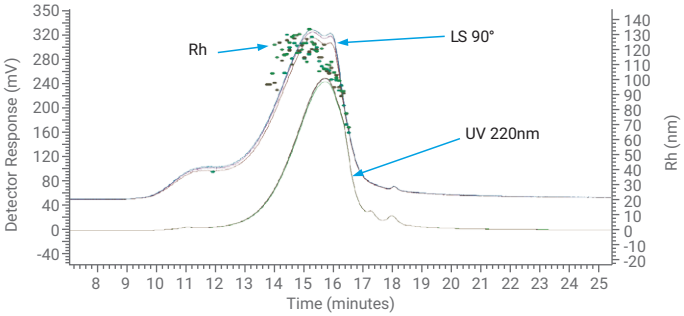

Figure 3. Repeatability chromatogram of exosome standard.

Table 3. Repeatability test results of exosome standard.

	Average Rh (nm)	LS 90° Area	UV 220 nm Area
1	108.56	43747.413	31735.621
2	109.30	46176.566	32780.112
3	112.41	46724.271	32927.401
Mean	110.09	45549.417	32481.045
%RSD	1.86	3.48	2.00

Conclusion

When the exosome standard was analyzed using the Agilent 1290 Infinity II Bio LC system and the Agilent Bio-MDS, the measured average Rh was confirmed to be 110 nm. The relative standard deviation for repeated tests of Rh was found to be 1.86%, and the results showed a high sensitivity LS 90° signal even at a concentration of 1×10^{11} particles/mL. Overall, the Bio-MDS was confirmed to exhibit excellent performance in analyzing biosamples with large sizes of approximately 100 nm.

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