Purification and SEC/MALS Analysis of Norovirus Virus-Like Particles

Introduction

In the fields of new modality, such as gene therapy drugs, virus vector, new vaccine, and drug delivery etc., applied researches on “bionanoparticles” are rapidly expanding and receiving the greatest interest these days. Due to their complex structures and distinct sizes, production and quality evaluation of their biological processes require integrated and multifaceted analytical techniques, even more than that of conventional biopharmaceutical compounds.

Virus-like particles (VLPs) are typical bionanoparticles. Since they are not infectious nor proliferated, but they can reproduce their original sizes and forms, they effectively elicit immune responses when used for vaccine. R&D for its commercialization is in progress as it would be an excellent platform.

This article presents the effectiveness of Size Exclusion Chromatography (SEC) for the analysis of bionanoparticles. As a proof of concept, method development for a chromatographic purification of norovirus cell surface layer binding protein originated VLP (NVLP) is discussed. This article also includes the evaluation of an effective method development using an SEC column with different analytical devices and a summary of how to select a suitable column for the monitoring of purification procedures.

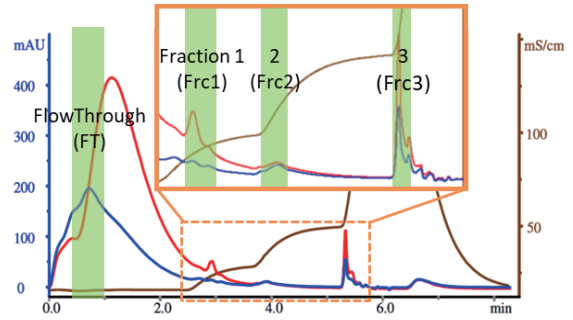
Purification and Analysis of NVLP Crude Extract

1. Purification of NVLP crude extract using a CIM monolith anion exchange column

NVLP crude extract was obtained from silkworms which were expressed with norovirus GII-4 cell surface layer VP1 protein (59 kDa) using the silkworm-baculovirus expression system. The sample was prepared as following: Grind the silkworm and ultrasonicate to obtain an extract. After centrifuging, filter the supernatant using a filter membrane (0.8 µm). The filtrate was then used as an NVLP crude extract sample.

We used a CIM monolith anion exchange column (BIA separations d.o.o, Slovenia) which has a high resolution and a high binding capability for bionanoparticles. The column is also known for its ability to be used at high flow rate while maintaining a low backpressure.

Figure 1 shows UV chromatograms of NVLP crude extract. Four fractions were obtained; the flow through (FT, the components not retained by the column) and fractions (Frcs) 1, 2, and 3. The components in Frcs 1 - 3 were eluted and collected at the set salt concentrations of the step gradient. The components of each fraction were analyzed by three different methods.



Sample : NVLP crude extract 2 mL, 50 µg/mL
Column : CIMmultus QA - 1, 2 µm, Bed vol. 1 mL
Eluent : (A) 10 mM Sodium phosphate buffer (pH 7.5)
(B) A + 2 M NaCl
Step gradient (5 column volume each)
7 B % → 14 B % → 26 B % → 100 B%
Flow rate : 5.0 mL/min
Detector : UV (260 nm, 280 nm)
Column temp.: Room temp.

Fig. 1. UV chromatograms of NVLP crude extract

2. SDS-PAGE

Small portions of NVLP SEC fractions were collected and boil-reduced for the SDS-PAGE analysis. Figure 2 shows the obtained SDS-PAGE image. A band, assumed to be the VP1 protein (59 kDa, indicated by orange arrows in Fig. 2) that composes NVLP was observed in the crude. A single band at the same position was also detected in Frc 2 (the second step of the salt gradient, 26 % B). There was rarely any other band observed in Frc 2 while few other bands, except the VP1 band, were seen in the FT fraction.

The results demonstrate that when NVLP crude extract was loaded to the CIM monolith anion exchange column, the majority of the impurities were not retained in the column, but eluted out. Meanwhile, NVLP binds to the column without any loss and it can be collected by changing the salt concentration to 26 % B.

Frc 3, the fraction obtained at the highest salt concentration (the third step, 100 % B) showed to contain components with strong UV absorbance (Fig.1). However, no observable band appeared in the SDS-PAGE image, which indicated the presence of impurities other than proteins in the crude extract. They could be the components mainly composited by the host genome originated DNAs.

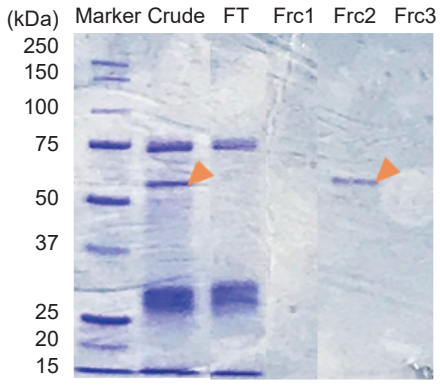


Fig. 2. SDS-PAGE image of NVLP crude extract

3. SEC-MALS

The components of each fraction obtained from the NVLP crude extract was analyzed by SEC-MALS. The column used was Shodex OHpak SB-805 HQ and the LS used was DAWN 8+ (Wyatt Technologies Corporation, USA).

Compared to the SDS-PAGE results, showing a single band of VP1 protein composing the NVLP, the UV chromatogram of the Frc 2 showed the main peak at 8 min as well as peaks at 9.5 min and 10.5 min (Fig.3 (a) brown line and (b) green line). The MALS chromatogram showed only one significant peak at 8 min, at where the main UV peak was observed (Fig. 3 (b) red line).

The intensity of the MALS significantly increases as the size of the target molecule increases. This response tendency is more obvious for nanoparticles, including VLP. The rms radius obtained by the light scattering around 8 min was about 22 nm. This is very close to the properly configured NVLP particle size. Therefore, MALS can be a strong tool to configure the attribution and to track the bionanoparticles, which is difficult by UV alone.

The presence of proteins that correspond to the sub peaks were not observed from the SDS-PAGE analysis. Therefore, those peaks are assumed to be the components related to the host originated genomic nucleic acid fragments. Moreover, SEC chromatogram (Fig. 3 (a) green line) and TEM image (Fig. 4) suggest that Frc 3 also does not contain proteins in this fraction.

On the other hand, the UV chromatogram showed a wide peak in-between the target NVLP peak, suggesting that the responsible components are also related to the nucleic acid fragments. This further suggests that the nanoparticles in the crude extract is not only NVLP.

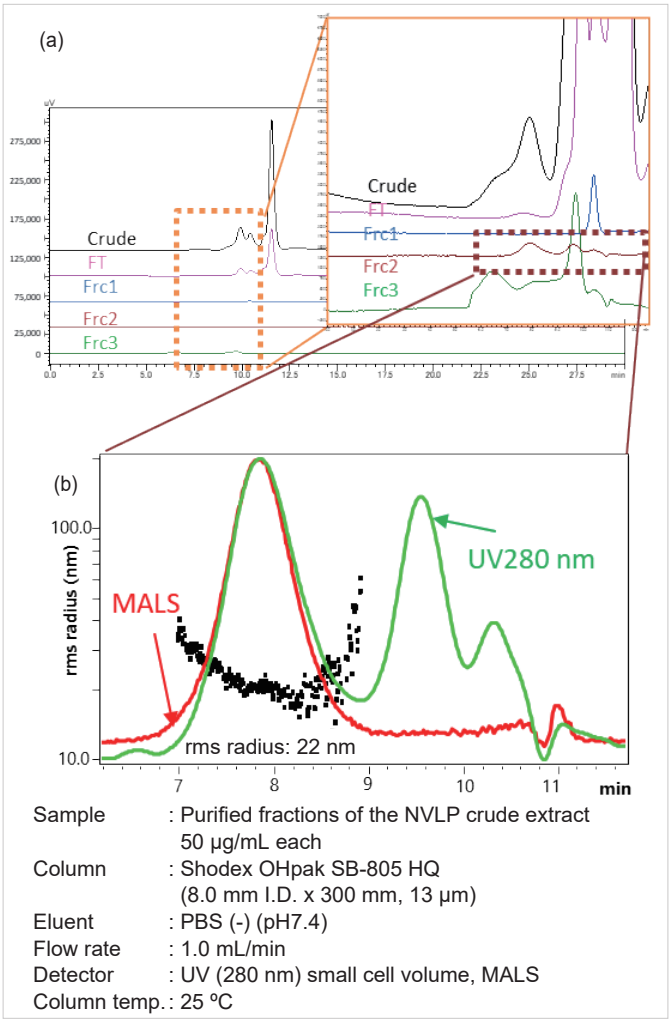


Fig. 3. SEC-MALS analysis of NVLP crude extract (a) SEC chromatograms of each fraction using SB-805 HQ (b) MALS analysis of Frc 2 (VP1 eluted fraction)

4. TEM study of Frc 2

A purified fraction of the NVLP crude extract (Frc 2) was dripped on a support film mesh, negatively stained, and observed by a transmission electron microscopy (TEM). Figure 4 shows the obtained image. The size observed matched well to the radius obtained by MALS, 22 nm. The presence of 40-nm diameter particles were observed, demonstrating that the purification achieved to maintain the higher-order structure of NVLP, which is expected for virus-like particles.

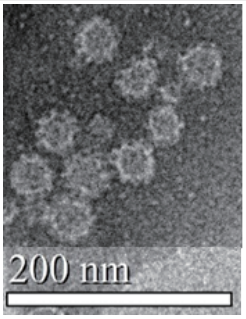


Fig. 4. TEM image of Frc 2

5. Effectiveness of the SEC-MALS

When developing a purification method for bionanoparticles, tracking of target components during the purification process can become complex and is a challenging task. Nanoscale particles cannot be observed by optical microscopy, since their main components are proteins, UV cannot differentiate them from various impurities produced during the culturing. Moreover, ELISA that selectively detects proteins, TEM observation, or analytical centrifugation (AUC) also have problems in their efficiencies such as analysis time, cost, and quantitative efficiencies.

The results in this application demonstrated a simple chromatographic method that allows to track the target nanoparticle in a short time when used with SEC-MALS. By combining the SDS-PAGE analysis, not only the targets in each fraction can be identified, but it also provides important guidance to improve the purification method by obtaining information about the existing impurities.

The components found in the UV peak at the lower molecular weight fraction is assumed to be consisted by the host genome originated nucleic acid etc. Thus, process improvements targeting the nucleic acid, e.g., more precise gradient steps for the fractionation or nuclease treatment to reduce the nucleic acid components, would be the next major improvement tasks.

6. Selection of a suitable SEC analytical column

Typical purposes of using SEC in the biological field are to prepare and collect the target compounds using its size exclusion effects. The size of bionanoparticles (10 to 100 nm) are relatively large compared to the host or culture originated components including some proteins and impurities. Thus, SEC is expected to separate the target particle by its size exclusion effects and elute them before other components.

As we have shown in section 3. SEC-MALS, the actual crude samples often contain non-negligible amount of impurities, genome originated nucleic acids, and the complexes of the target proteins which are similar or even larger than the target bionanoparticles.

The analytical SEC helps us properly monitor and understand the purification process that contains those various components. Therefore, mere exclusion of the higher molecular weight range components is not sufficient, but it is required to be able to fractionate the components in the size range of bionanoparticles. A selection of suitable SEC column becomes important.

We analyzed the NVLP crude extract using three columns having different molecular weight analytical ranges and compared their results (Fig. 5).

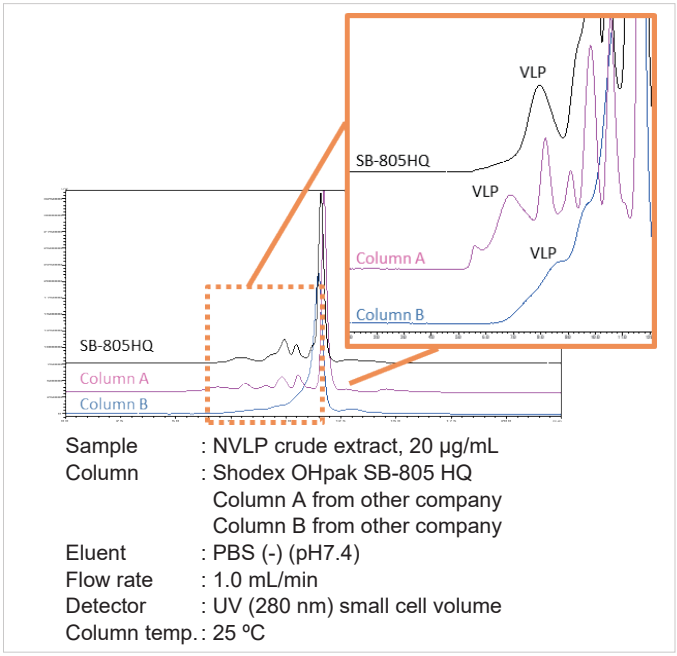


Fig. 5. A comparison of three columns for the analysis of NVLP crude extract

The result of column A shows that a part of target VLP was excluded since the column A's exclusion limit is smaller than the high-molecular weight end of the target VLP. This leads to a miss quantification of the target compound. Also, the presence of impurities with large molecular weights cannot be monitored accurately.

Meanwhile, the target VLP and the major impurities were not well separated by column B. This is because the working range designed for the column B is rather large for the target VLP fraction, separations in the lower molecular weight section became poor.

When selecting a suitable analytical SEC column, not only the analytical range for the fractionation, but adsorption of the target compounds, which influences the quantification and yield, should also be considered. Because of its sophisticated designed hydrophilic gels used and its analytical range covering the molecular weight of the target fractionation, the Shodex OHpak SB-805 HQ was a well-suited selection for the profiling of NVLP.

Conclusions

For an optimal profile monitoring of the bionanoparticle purification, it is important to select an analytical SEC column that covers the target fractionation molecular weight range and having a good separation efficiency in the range. In addition, combining the SEC with appropriate physiochemical and biochemical analytical techniques is also important. Especially, the use of MALS significantly improves the efficiencies of culturing and purification procedure development, as it simplifies to track bionanoparticles which are difficult to be differentiated from the impurities during the purification process.

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TA. NO. 015. (2) 20. D. OCT. P