Process Monitoring using Immunofluorescence and Multi Angle Light Scattering – tracking extracellular vesicles on analytical HPLC

K. Vrabec[1], T. Lojpur[1], P. Gagnon[1]
1 BIA Separations d.o.o., Mirce 21, 5270 Ajdovščina, Slovenia

INTRODUCTION

This poster shows how Multi-Angle Light Scattering detector and Fluorescence detector couppled to PATfix analytical HPLC system can be used to track extracellular vesicles through purification process.

Samples were analyzed by **analytical size exclusion chromatography (SEC)**. On SEC cell culture components diffuze into pores of chromatographic media and are separated (mostly) based on size. Particles larger than the media pore size are excluded in the void peak. This peak represents extracellular vesicles including apoptosomes, microvesicles and exosomes as well as cell debris and aggregates.

Multi-Angle Light Scattering detector (MALS) adds a valuable dimension to chromatographic analysis. Large particles like extracellular vesicles absorb very little UV light, which makes them difficult to detect with UV detector. MALS is used to discriminate vesicles from the majority of non-vesicle contaminants on SEC.

Immunofluorescence (IF) is used to detect vesicles carrying a specific antigen. SEC couppled to MALS can‘t discriminate between vesicle populations present in cell culture. Different vesicles carry versatile antigens which can be used as immunological markers. With immunofluorescence we can track exosomes using immunological markers present on exosome surface.

RESULTS

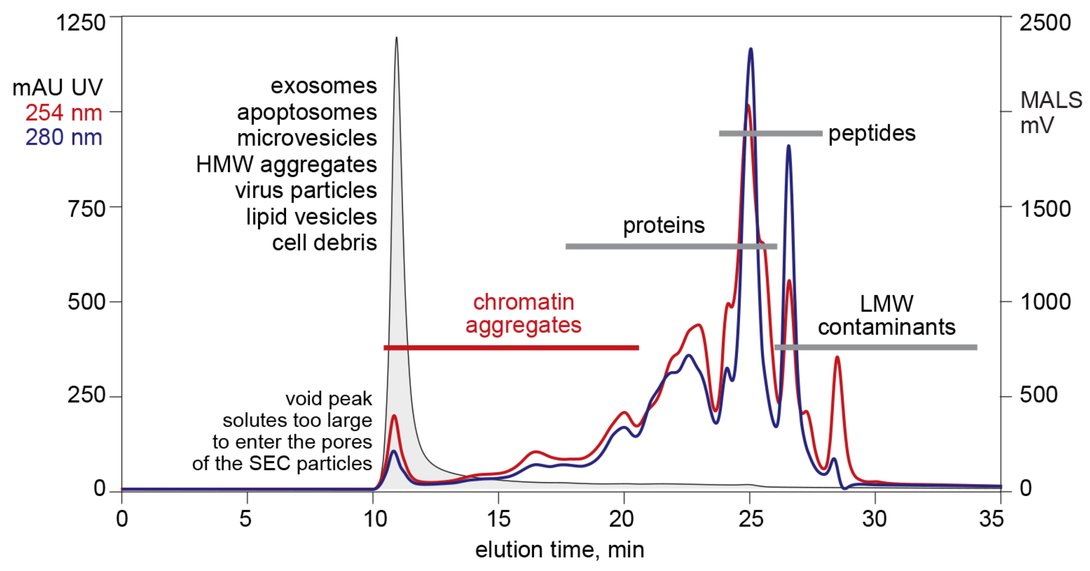

Figure 1. Analytical SEC-MALS of cell culture used for exosome isolation. Filtered serum-free HEK293T harvest was analyzed with SEC-MALS. Multi-Angle Light Scattering amplifies sensitivity for large species. SEC cannot discriminate exosomes from non-exosomal vesicles or other HMW species, but it provides a useful analytical perspective on overall contaminant size distribution.

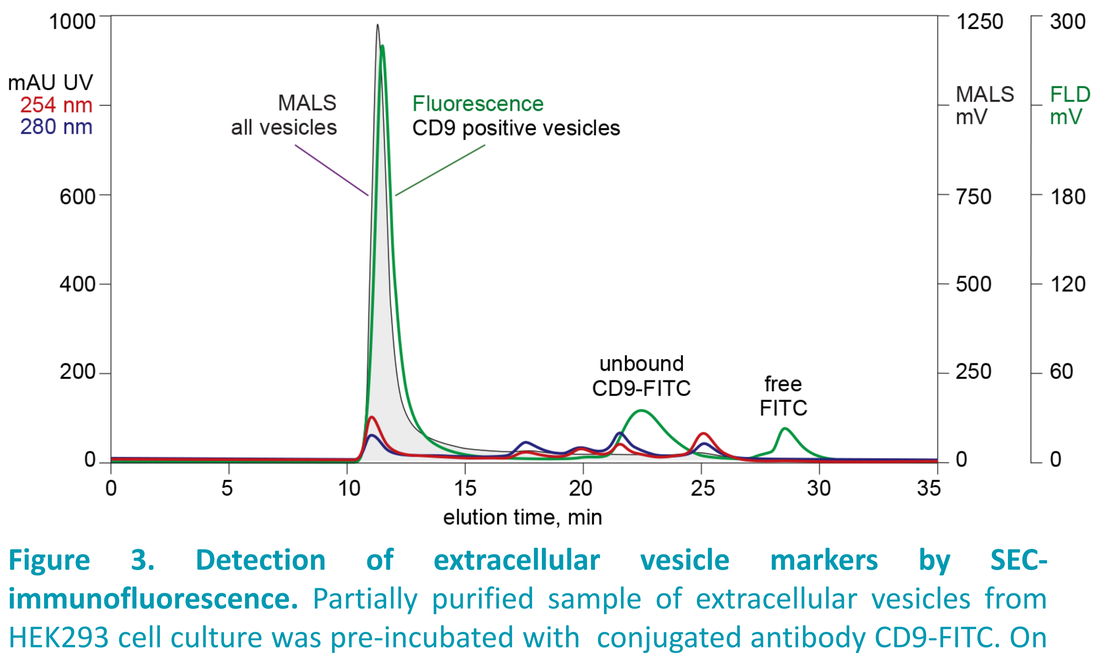

Figure 3. Detection of extracellular vesicle markers by SEC-immunofluorescence. Partially purified sample of extracellular vesicles from HEK293 cell culture was pre-incubated with conjugated antibody CD9-FITC. On SEC, the antibody that binds to vesicles is detected in the void peak at 11‘ and the free antibody elutes at 23‘. CD9 was used as experimental control for total extracellular vesicles.

MATERIALS AND METHODS

- Analytical chromatography methods were performed on a PATfix™ HPLC, model LPG, with 50 mm path-length UV cell (BIA Separations, Slovenia); equipped with a Dawn Heleos 365-H multi angle light scattering (MALS) detector (Wyatt Technology, USA).
- Analytical size exclusion chromatography (SEC) was performed on a TSKgel™ G4000SWxl column (Tosoh Biosciences, USA).
- Conjugated antibodies were obtained from BioLegend.
- Experimental work was performed with a HEK293T cell culture harvested in serum free media, obtained from FiberCell, Frederick, MD.
- Anion exchange chromatography was performed using CIMmultus™ EV (BIA Separations).

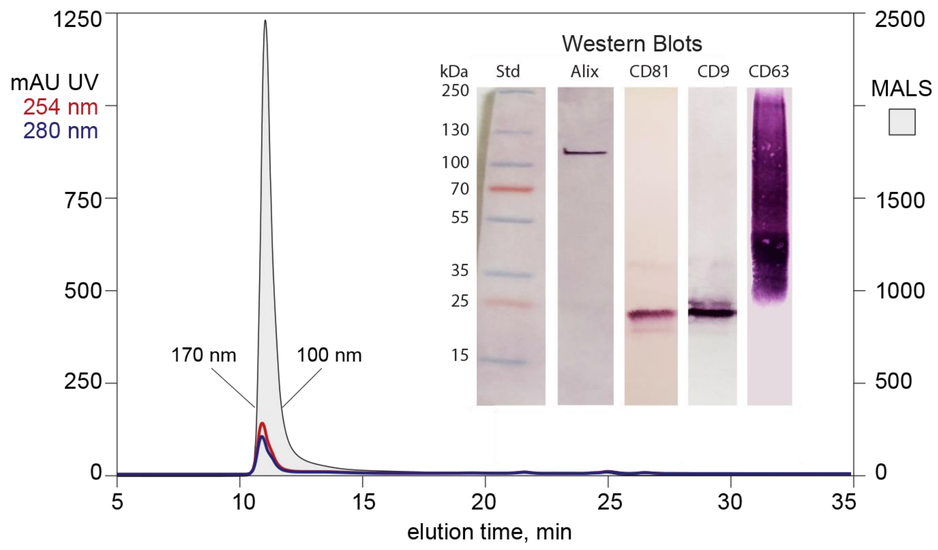

Figure 2. Analytical SEC-MALS of purified exosomes with confirmation by Western blotting. Exosomes carry different antigens on the surface and in the lumen. Because of the common origin they share many antigens with microvesicles, plasma membrane or apoptosomes. Confirmation of exosomes requires at least three immunological markers by full-gel Western blots. WB is a labor intensive and time-consuming method – after confirmation of antigen presence, SEC-IF was used for automated tracking of exosomes.

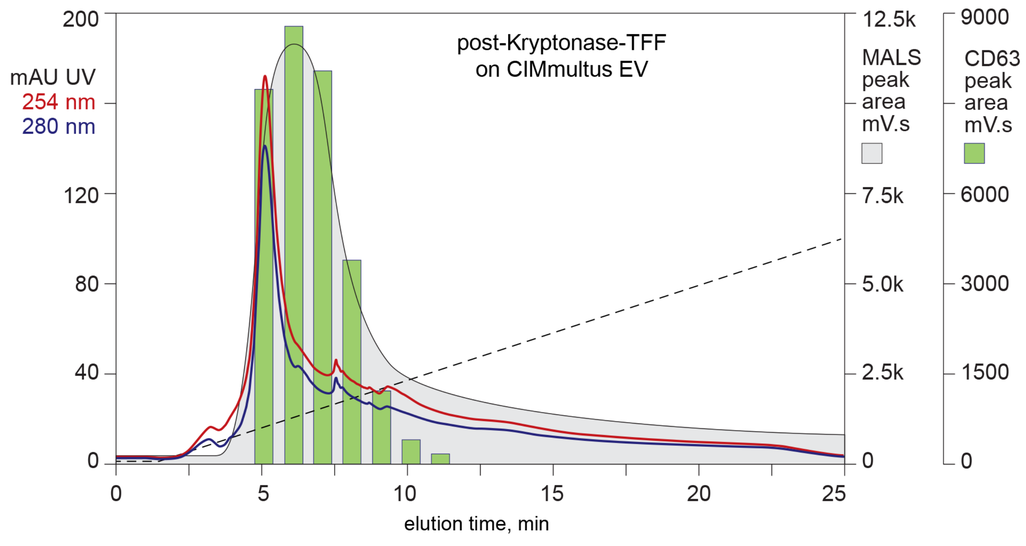

Figure 4: Tracking exosomal markers through purification with anion exchange chromatography (AEX). Partially purified cell culture was loaded to CIMmultus EV column. Fractions were collected, incubated with CD63-FITC antibody and analyzed off-line with SEC-IF. MALS and immunofluorescence agree well throughout the exosome fractions but persistent MALS indicates later eluting vesicles. MALS and IF both differ from UV. This is a warning that UV is a poor method for monitoring exosome elution. Based on IF, exosome recovery across the process is greater than 95%.

CONCLUSIONS

- Chromatography with simultaneous monitoring by UV, fluorescence, and MALS provides a range of valuable insights beyond conventional monitoring.
- Immunofluorescence provides fast, specific, quantitative results and enables tracking of recovery through purification process.
- These results provide valuable guidance for purification process development.

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