WHITE PAPER

WP2606: Exosome characterization with FFF-MALS-DLS

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Summary

Exosome characterization has become a hot topic in molecular biology and medical research. This white paper gives a summary of how the Wyatt Eclipse® Field-Flow Fractionation system coupled to MALS and DLS allows a more detailed and comprehensive characterization of exosomes. Key is a physical separation of exosomes from other components of the biological matrix (serum, urine, etc.) with online determination of size, composition and charge. Fractions can be collected for further off-line analysis, most importantly, sequencing of DNA contained in exosomes. A recent publication by Zhang et.al. in Nature Biology [1] has shown that biological function correlates with size and three distinct size populations could be separated for the first time using the Wyatt Eclipse FFF system. This white paper explains how the separation of exosomes can be achieved and which information is obtained by coupling MALS and DLS online.

Introduction

Recent studies have demonstrated that exosomes are a unique source of specific disease biomarkers. Exosomes are small vesicles released from all cells, including disease-affected and non-affected cells with a size range of 30-100 nm (Figure 1). They are to be distinguished from microvesicles, having a different mechanism of release from cells of origin, with nominal diameter of 100-1000 nm. Exosomes are of far higher interest with respect to biomarkers, because they originate from active cells, e.g. active tumor cells, whereas microvesicles come primarily from blebbing of dying cells and do not reflect the active disease status. Exosomes are characterized by a double lipid layer containing DNA, RNA, miRNA, soluble and transmembrane protein reflecting the biological repertoire of the cell. In order to use exosomes as a source of biomarkers, they need to be separated from soluble high abundance proteins and from microvesicles. Published studies commonly collect bulk exosomes using non-specific methods such as ultracentrifugation or precipitation. The method of choice is FFF, which is highly effective in size separation in the range of a few nanometers to a micron [2,3]. As the publication by Zhang et.al has demonstrated, the unique capability of FFF is to isolate exosomes from other material and to separate the exosomes themselves into three different populations which have distinct properties and carry different biomarkers.

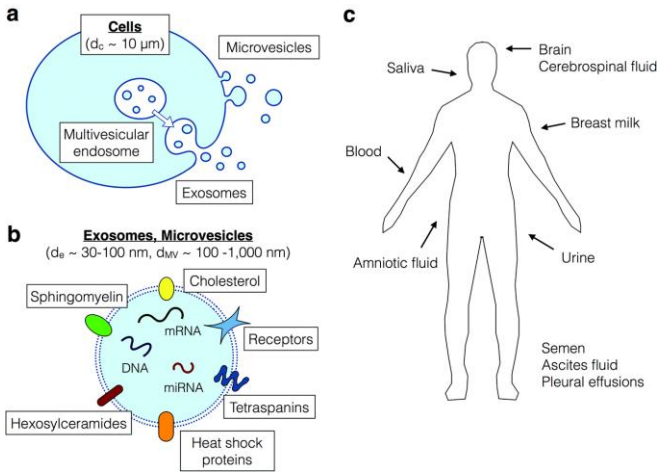

Figure 1. **Secretion of Exosomes from cells and their significance for biomarkers**. **a**. biogenesis of exosomes from multivesicular endosomes, in contrast to the microvesicles which are released from the cell surface via membrane budding. **b**. exosomes and microvesicles both contain transmembrane proteins, intracellular proteins, DNA, RNA and miRNA, which are potential biomarkers. **c**. exosomes are found in all body fluids. Figure from[2].

How Field-Flow Fractionation works

The sample is injected into a thin ribbon-like channel which has a porous bottom wall. Part of the flow passing through the channel towards the outlet permeates through the bottom, creating a cross-flow. This cross-flow concentrates the sample toward the accumulation wall by

exerting a friction force. Brownian motion acts as a counterforce working against the cross-flow. The molecules are constantly moving up, away from the bottom wall, but they are pushed back from above. This creates a particle cloud with an exponential decrease of the concentration as a function of the distance from the bottom wall. It is the same mechanism which leads to the decrease of the air pressure rising above sea level. In our case each particle size will have a different average distance from the accumulation wall which is given by its diffusion coefficient. Smaller particles with higher diffusion, will be higher up in the channel and the other way around for bigger particles. To give an idea of the dimensions, the concentration drops from the maximum value at the accumulation wall to zero within 10µm up into the channel. The typical height of the channel is 350 µm. It means that the sample does stays close to the bottom wall throughout the separation process.

If we consider the transport along the channel towards the outlet, smaller particles with more extended clouds will move faster. This is because the laminar flow profile has much higher transport velocity farther up as we go away from the accumulation wall. Therefore, the smaller particles will elute first, followed by the bigger ones as depicted in Figure 2, resulting in a very efficient separation.

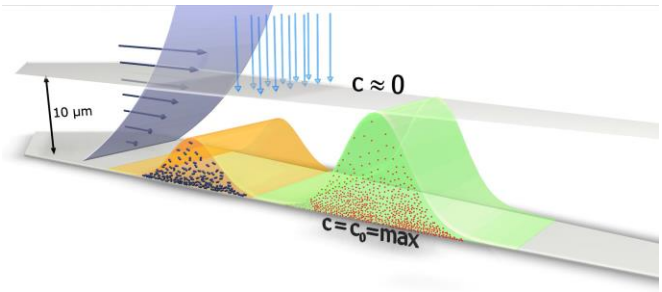

Figure 2. Principle of Flow-FFF separation. Shown are two particle clouds moving along the channel accumulation wall, which consists of a membrane supported by a frit. The cross flow is coming from the top and permeates through the accumulation wall exerting a friction force on the particles. The smaller (red) particles diffuse higher up against the cross flow compared to the bigger (blue) particles. The parabolic velocity flow profile of the channel flow transports the more extended particle cloud much faster, compared to the more compact particle cloud of the bigger particles.

FFF theory is well worked out and it gives us an equation which describes retention in a quantitative way. The FFF retention equation given below allows to calculate the diffusion coefficient D from a measured retention time t_R if the cross-flow rate F_x, channel flow rate F_c and the effective channel height w are known.

$$t_R = \frac{w^2}{6D_i} \ln\left(1 + \frac{F_x}{F_c}\right)$$

Based on the theory it is possible to predict the outcome of an experiment, which is what the software SCOUT DPS® does.

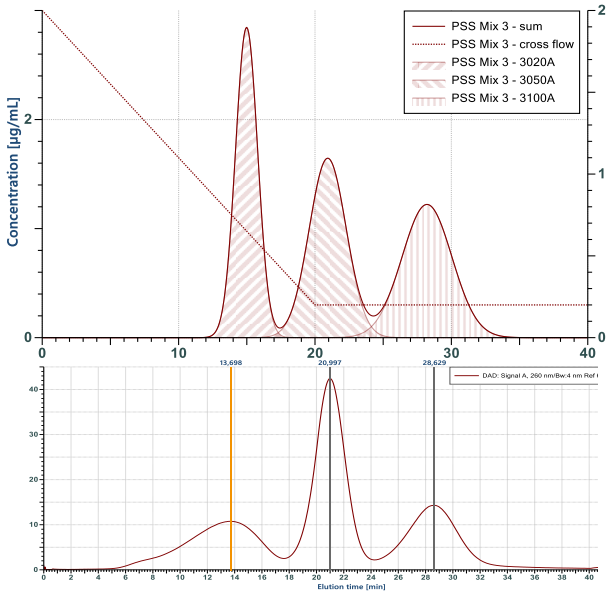

Figure 3. Simulation of a fractionation compared to the experimental outcome. Shown is the theoretical calculation of a fractogram from a mix of three particles with 10, 20 and 50 nm radius. Below is the experimental results. It comes very close to the prediction, with the exception of the wider peak for the 10 nm particle eluting first. The reason is, that this particle is not monodisperse as assumed in the theoretical calculation.

Materials and Methods

Exosome separation was performed on an Eclipse AF4 system using an Agilent 1260 HPLC equipped with a fraction collector. A DAWN® with WyattQELS™ installed at detector 12 was used for MALS and DLS detection. Instrument control was done with the Eclipse Plug-in for ChemStation.

The exact experimental protocol is described in a protocol exchange paper [4]. Separation was achieved using an SC (short) channel and a standard flow program, reducing the cross-flow from 0. 5 ml/min to 0 in 45 minutes.

Data Processing

Data processing was done with ASTRA® which has the unique capability of combining both Multi-Angle Light Scattering (MALS) and dynamic light scattering (DLS) in one integrated package.

Why MALS and DLS combined?

Both light scattering techniques measure nanoparticle size, but they are highly complementary:

MALS determines rms radius from 10 nm to 500 nm, and is about 20x more sensitive than DLS; DLS determines hydrodynamic radius for 0.5 nm- 200 nm (the upper limit is flow-rate dependent). The combination of MALS + DLS is indicative of shape and structure

The DAWN can incorporate an optional DLS module that utilizes the same flow cell and laser, making it versatile indeed.

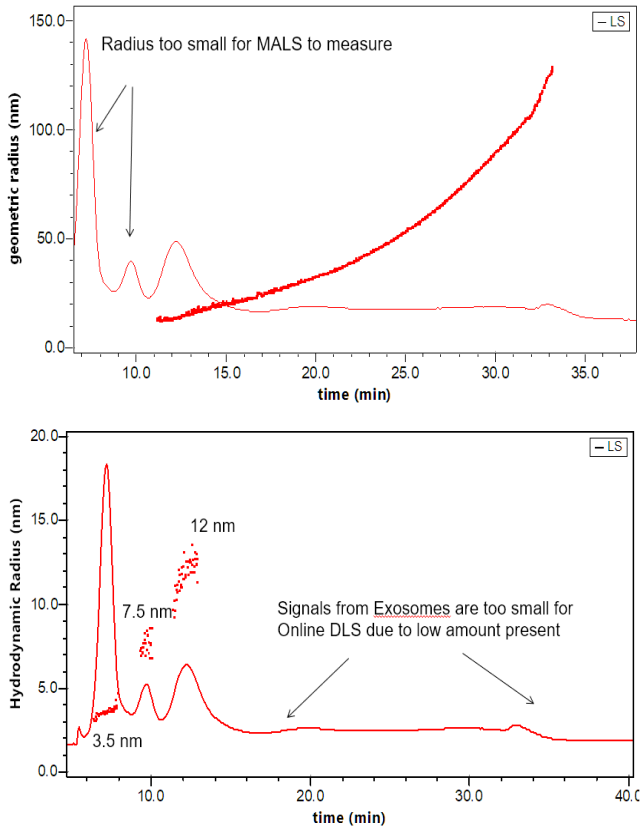

Figure 4. **Separation and characterization of exosomes by FFF-MALS-DLS.** The triple combination allows for unique characterization and understanding of these bionanoparticles. Utilizing MALS and DLS in tandem extends the range of size measurements, which has proven critical based on recent published results.

Results and Discussion

H. Zhang et al. in their Nature Cell Biology paper "Identification of distinct nanoparticles and subsets of extracellular vesicles by asymmetric-flow field-flow fractionation" have discovered that all exosomes do not have the same essential nature. Applying FFF-MALS-DLS, they have identified three distinct types of small extracellular vesicles, which they have termed:

- Exo-L, large exosome vesicles (90-120 nm);
- Exo-S, small exosome vesicles (60-80 nm); and
- Exomeres, non-membranous nanoparticles which are the smallest of the three—on average, about 35 nm in diameter.

These three types are grouped by size and other biophysical properties; they are also distinguished from each other by structure (outer membrane or not), protein content, degree of N-glycosylation, metabolite content, DNA/RNA profiles and corresponding function. However, these groupings are conserved across most cell lines, indicating that they are essentially independent classifications of bio nanoparticle with correspondingly distinct origins and function. The significance of these findings for cancer research are discussed in Scientists Discover New Nanoparticle, Dubbed Exomeres.

Conclusions and Outlook

FFF-MALS-DLS is a powerful platform for separating and characterizing exosomes and other nanoparticles. In addition to light scattering detectors, it supports additional online detectors such as UV/Vis absorption, fluorescence, differential refractometry with the Optilab® , and even mass spectrometry for extended analyses.

A new technology, electrical asymmetric-flow field-flow-fractionation (EAF4) uses the Mobility™ system to separate particles by size and charge. The Mobility can even determine zeta potential and so provide full, detailed distributions of size vs. zeta potential.

As mentioned earlier, while the Zhang paper represents a breakthrough in exosome research, it is not the first to make use of the Eclipse/DAWN system. In fact, the same setup had previously been employed by Yang et al. [5] to demonstrate that there is a significant difference in size between urinary exosomes from healthy controls and patients with prostate cancer, identifying a potentially life-saving biomarker. Additional publications making use of FFF-MALS-DLS to characterize exosomes have been published [6-8].

References

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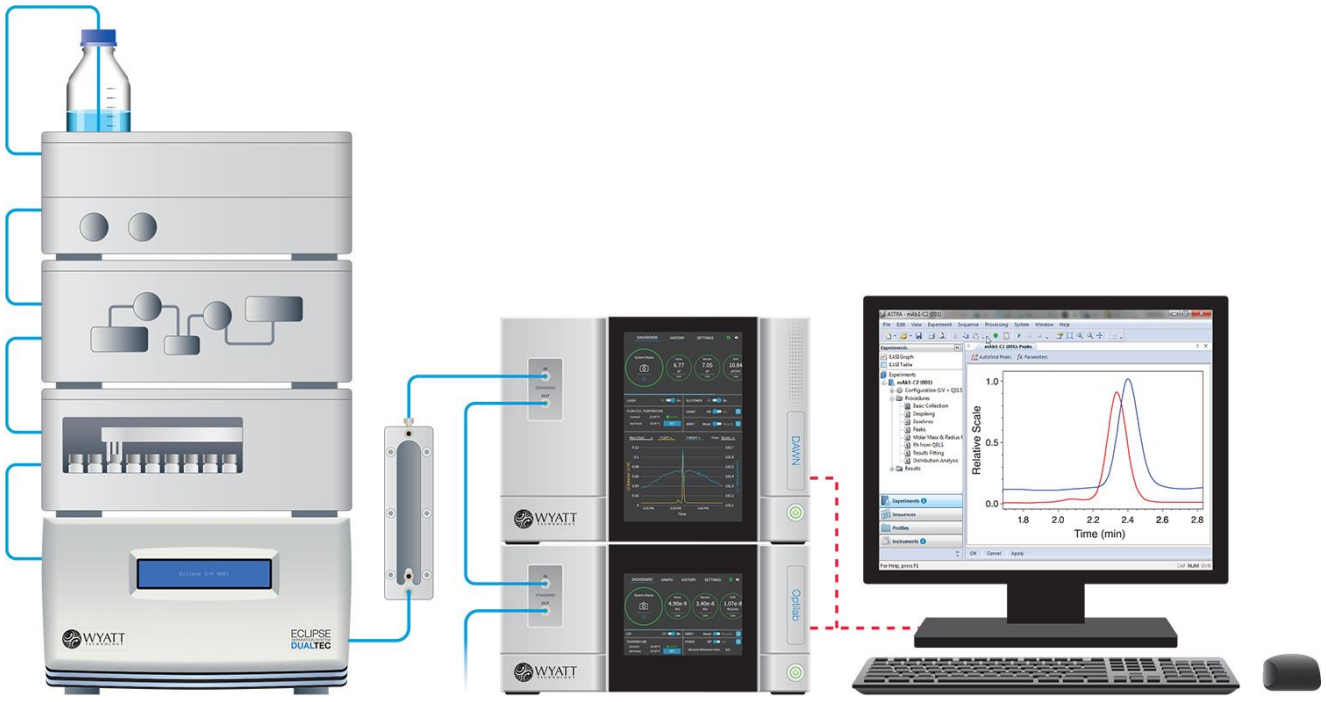

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