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WHITE PAPER

WP2606: Exosome Characterization with FFF-MALS-DLS

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Summary

Studies of exosomes, and extracellular vesicles (EV) in general, have reached prominence in molecular biology and medical research. Biophysical characterization and isolation are central to these studies, as they form the foundation for additional analyses. Field-flow fractionation coupled to online multi-angle and dynamic light scattering (FFF-MALS-DLS) is a versatile biophysical technique that provides detailed characterization of exosome physical properties such as size and charge, and in addition is useful for size-based isolation. Fractions can be collected for further off-line analysis such as imaging or genomic sequencing.

One of the key benefits of FFF is the physical separation of exosomes from other components of the biological matrix (serum, urine, etc.). Zhang et. al., in Nature Biology,[1] have shown that biological function correlates with size, and three distinct size populations were separated for the first time using the Eclipse™ FFF instrument. This white paper explains how the separation of exosomes is achieved and what information is obtained by coupling MALS and DLS online.

Introduction

Exosomes are small vesicles with a size range of 30 to 100 nm that are released from all cells, including disease-affected and non-affected cells. Exosomes are characterized by a double lipid layer containing DNA, RNA, miRNA, soluble and transmembrane proteins reflecting the biological repertoire of the cell. They are distinguished from microvesicles, that have a different mechanism of release from cells of origin and a nominal diameter of 100 to 1000 nm. Recent studies have demonstrated that exosomes are a unique source of specific disease 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.

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 describe collecting bulk exosomes using non-specific methods such as ultracentrifugation or precipitation. FFF is highly effective for size-based separation in the range of a few nanometers to a micron.[2,3] As the publication by Zhang et al. demonstrated, FFF is uniquely capable of isolating exosomes from other materials; it further separates the small EVs themselves into three different populations which have distinct properties and carry different biomarkers. This makes FFF the technique of choice for exosome isolation.

Figure 1. An FFF-MALS-DLS system comprising autosampler and pump (bottom left), Eclipse FFF instrument and separation channel (bottom right, foreground), and three detectors: UV (top left), MALS/DLS and refractive index (top right).

How field-flow fractionation works

Wyatt Technology's FFF system is based on the Eclipse FFF instrument in combination with a pump and autosampler, as shown in Figure 1. Separation takes place in a thin channel which has a porous bottom wall comprising an ultrafiltration membrane supported by a frit (Figure 2). Constriction of the channel outlet causes part of the flow to permeate through the bottom, creating a cross flow.

During sample injection, the cross flow concentrates the sample toward the membrane. Brownian motion acts as a counterforce working against the cross flow, moving the particles up and away from the membrane. The balance between cross flow and diffusion creates a particle cloud with an exponential decrease of the concentration as a function of the distance from the membrane. Smaller particles, with higher diffusion, will be higher up in the channel, whereas larger particles will remain closer to the membrane.

During elution, laminar flow of the carrier fluid along the channel leads to a velocity profile that depends on height above the membrane. Smaller particles with more extended clouds encounter faster flows, and will elute sooner than larger particles which primarily encounter slower flows. This results in a very efficient separation, yet the particles experience little to no shear or membrane interactions.

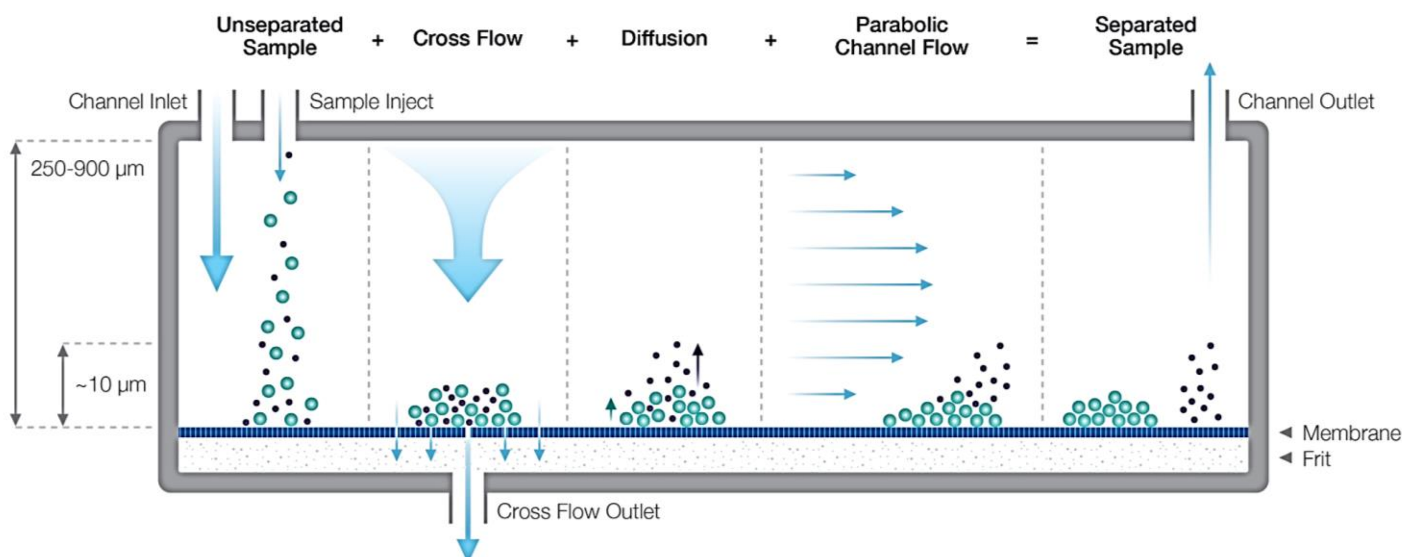

Figure 2: Principle of flow FFF separation. Cross flow pushes the particles towards the membrane, while diffusion causes them to move towards the center of the channel. The smaller (red) particles diffuse higher compared to the bigger (blue) particles. The parabolic velocity profile of the channel flow transports the more extended cload of smaller particles faster, compared to the more compact particle cloud of the bigger particles.

Simplified method development

FFF theory under the assumption of constant flow rates leads to Equation 1, which enables accurate prediction of retention time t_R based on particle size and flow rates. Here D is the particle diffusion coefficient (directly related to hydrodynamic size R_h), F_x the cross-flow rate, F_c the channel-flow rate and w the effective channel height. Conversely, if t_R is measured, R_h can be calculated.

$$t_{\mathrm{R}} = \frac{w^2}{6D_{\mathrm{i}}} \ln\left(1 + \frac{F_{\mathrm{x}}}{F_{\mathrm{c}}}\right) \qquad (1)$$

VISION™ DESIGN software is a computer-aided FFF method development tool which is based on rigorous FFF theory. Rather than running a matrix of experiments with different flow rates, channel heights etc. in order to determine the optimal method, one simply enters the expected particle sizes into the software and specifies flow conditions. The program simulates the elution and immediately displays a graph of expected retention times. By varying the flow program—primarily the cross-flow rate as a function of time—it is possible to quickly arrive at a near-optimal candidate method. Typically, a single physical run is then carried out, with the results fed back into the software for finalize method optimization.

In the example shown in Figure 3, FFF separation of a mixture of three particle sizes—10, 20 and 50 nm radius—is simulated and measured. The initial cross-flow profile, shown as a solid blue line, leads to an elution profile that is very close to the simulation with peak retention times at 13.7, 21.0 and 28.6 minutes. A small discrepancy arises in the broader 10 nm particle peak at 13.7 minutes, a result of polydispersity in that sample. By reading the result into VISION DESIGN it is possible to further refine the method, obtaining baseline separation of each peak.

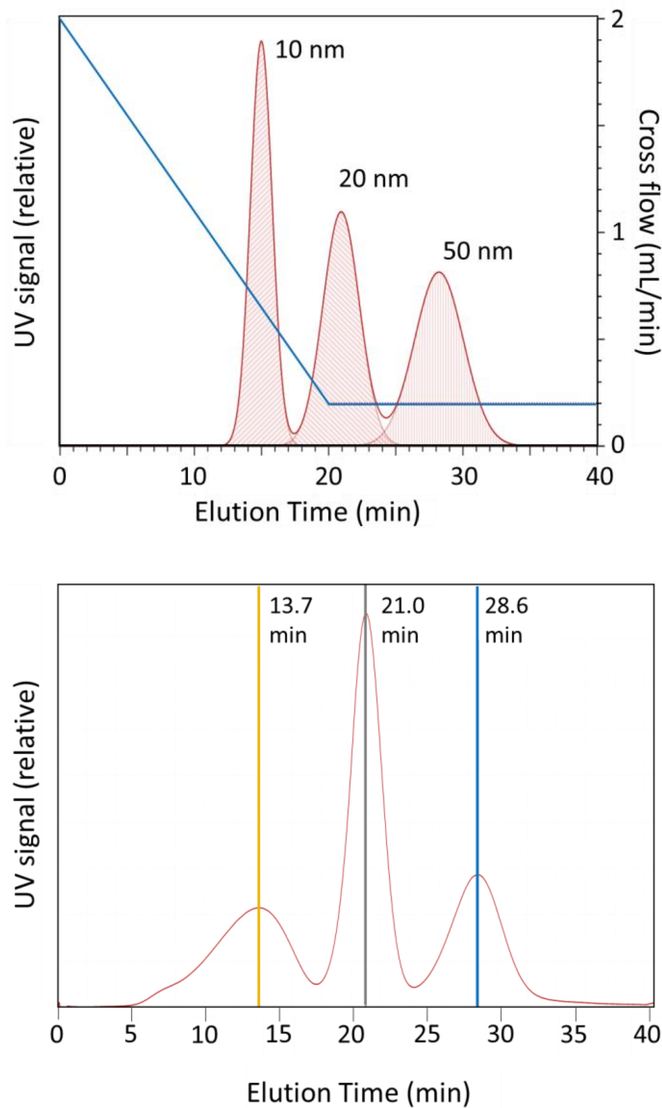

Figure 3. FFF method development using VISION DESIGN. Top: the theoretical fractogram simulated from a mix of three particles, with 10, 20 and 50 nm radius, using the cross-flow profile shown as a solid blue line. Bottom: the experimental results, which come very close to the prediction, with the exception of the wider peak for the 10 nm particle centered on 13.7 minutes - a result of polydispersity in the measured sample.

Once in-silico method development is complete, the method can be loaded into VISION RUN software which orchestrates the autosampler, pump, Eclipse FFF control system and inline detectors, performing fully automated and unattended FFF-MALS-DLS separations and analyses.

Enhancing fractions

As we have seen, FFF separates particles such as exosomes by size, with very good resolution. The addition of an autosampler enables isolation of narrow particle size ranges. However, the abundance of exosomes in a sample is often quite low, and all the more so upon separation. FFF inherently imparts a high degree of dilution when sample exits the channel and mixes with the top layer of carrier fluid. As discussed below, with the use of advanced technology, dilution is partially ameliorated, making FFF with a Wyatt system all the more effective for isolation.

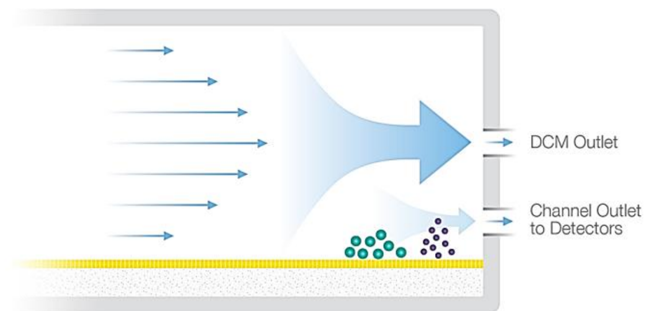

Figure 4. Schematic of the Dilution Control Module principle. Excess carrier fluid exits the DCM outlet while the detector flow is tightly regulated.

The Eclipse can incorporate a Dilution Control Module™ (DCM), reducing dilution by as much as 10x- 20x by siphoning off the top layer of carrier fluid before it mixes with the eluting sample, as indicated in Figure 4. While this concept is not novel, the implementation of DCM in the Eclipse includes a major innovation: active control of the flow to the detectors and autosampler, which ensures a very constant flow rate to within ±0.1%. While splitting off the top carrier fluid layer in other FFF systems causes large fluctuations in the detector flow rate and consequentially uneven fractions and poor repeatability, the Eclipse DCM actually improves detector flow uniformity while simultaneously enriching fractions. All Eclipse separation channels, including the semi-preparative channel, include a DCM port.

A complete FFF-MALS-DLS system

Separation of exosomes by an Eclipse FFF system is the basis for extended characterization that includes both online and offline analyses. Online characterization includes size, structure and charge, and identification of accompanying macromolecules by molar mass and size; further offline analysis might comprise genetic sequencing, mass-spec proteomics and lipidomics, imaging by cryo-EM and more. Hence the Eclipse naturally pairs with a DAWN™ MALS instrument with embedded WyattQELS™ DLS module, an Optilab™ differential refractive index (dRI) detector and online UV/Vis absorbance detector. These online detectors provide a wealth of information.

Both MALS and DLS provide measures of size, i.e. rms radius R_g (or geometric radius R_{geom}) and hydrodynamic radius R_h, respectively. These technologies are complementary: MALS determines R_g from 10 nm to 500 nm, and is about 20x more sensitive than DLS; DLS determines R_h from 0.5 nm to 300 nm. The combination of R_g and R_h is indicative of shape and structure. MALS can also calculate the particle concentration in each eluting fraction, which is quite helpful in comparing samples and deciding which fractions should undergo further analysis.

Combining MALS with the concentration signal from a UV or dRI detector yields molar mass. This analysis is primarily useful for proteins, nucleic acids and other macromolecules, though it can also be applied to the exosomes themselves. Data acquisition and analysis for online MALS and DLS are accomplished by ASTRA™ software. ASTRA is launched automatically by VISION RUN to begin data acquisition, synchronized with the FFF run.

In addition, the UV spectrum may be helpful in estimating the content of the vesicles. VISION RUN can acquire sampled or complete spectra from an Agilent 1260 MWD or DAD.

MALS complements DLS for serum analysis

Figure 5 presents the analysis of exosomes in serum, highlighting the importance of combining MALS and DLS for this complex sample. The top graph overlays DLS measurements of size with the light scattering fractogram; early-eluting peaks are readily measured by DLS. The measured sizes indicate serum albumin and lipoproteins, and these identifications are confirmed by molar mass calculations (not shown). The exosomes themselves are, in this case (where the DCM was not employed), at too low a concentration for DLS size determination.

The lower graph in Figure 5 overlays size determined by MALS with the same fractogram. Here the smaller proteins are below the size limit for MALS (which does not prevent analysis of molar mass by MALS). However, the larger lipoprotein peak and the exosomes are readily quantified across their entire size range.

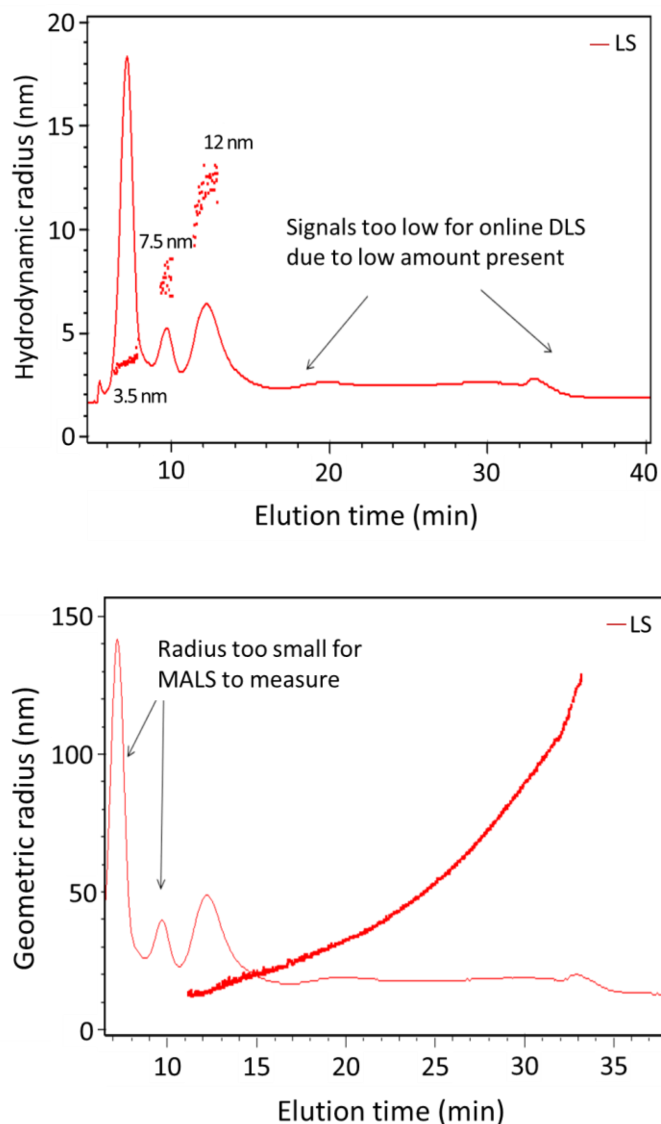

Figure 5: Separation and characterization of exosomes by FFF-MALS-DLS. Top: size determination by DLS succeeds for the protein peaks but the exosome signal is too weak for DLS. Bottom: MALS measures the size of the exosomes and larger lipoprotein, but the smaller proteins are outside the size limits for MALS (their molar mass can still be measured).

Measuring charge and zeta potential

The Mobility™ system combines with an Eclipse to measure the charge and zeta potential of each eluting fraction. It replaces the standard asymmetric-flow FFF channel with an electrical/asymmetric-flow FFF (EAF4) channel that incorporates platinum electrodes, one at the top of the channel and one underneath the frit, in order to shift the elution time according to particle charge (Figure 6). The shift in retention time with applied electric field is analyzed to calculate the zeta potential and charge of each eluting population. Typically this requires two to three runs, each at a different applied field, beginning with $E_{applied} = 0$. EAF4 analysis does not make use of MALS or DLS; rather size is estimated from retention time. The entire analysis is carried out in VISION DESIGN.

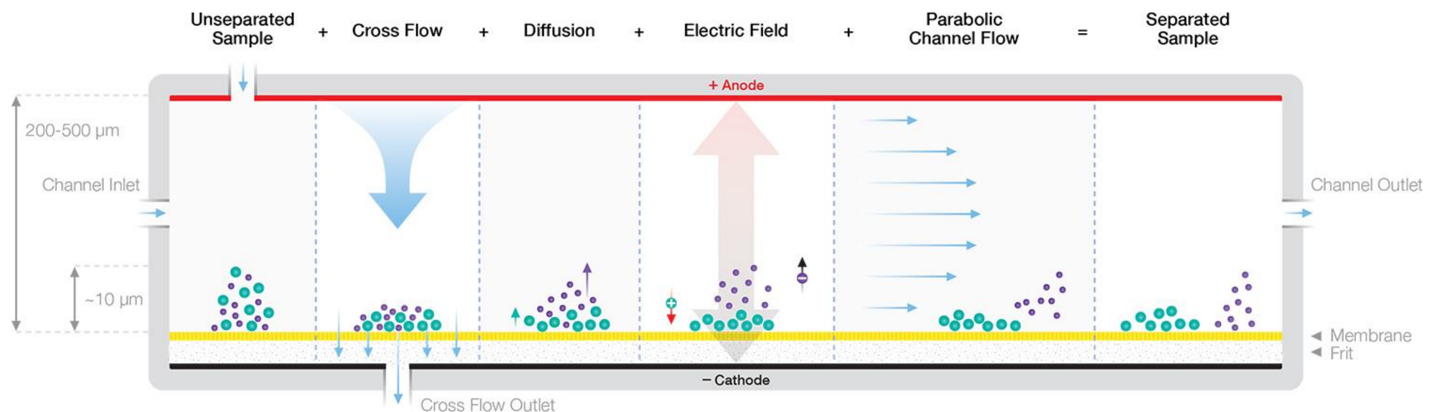

Figure 6. Mobility EAF4 channel, incorporating top and bottom electrodes. The applied electric field shifts the average height of particles in the channel according to their charge, which subsequentially shifts the elution time. The shift in elution time with applied field is analyzed to determine zeta potential and charge of each population.

New biology

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" discovered that all exosomes do not have the same essential nature. Applying FFF-MALS-DLS for isolation and sizing, plus extensive additional offline characterization and testing, they 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. Furthermore, these groupings are conserved across most cell lines, indicating that they are essentially independent classifications of bionanoparticle with correspondingly distinct origins and function. The significance of these findings for cancer research are discussed in Scientists Discover New Nanoparticle, Dubbed Exomeres. The full experimental protocol is described in a protocol exchange paper.[4]

While the Zhang paper represents a major breakthrough in exosome research, it is not the first instance of exosome studies to make use of the Eclipse/DAWN system. In fact, a similar 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 another potentially life-saving biomarker. Additional publications making use of FFF-MALS-DLS to characterize exosomes have been published.[6–8]

Conclusions and Outlook

FFF-MALS-DLS is a powerful platform for separating, characterizing and isolating exosomes and other bionanoparticles. In addition to light scattering, it supports additional online detectors such as UV/Vis absorption, fluorescence and even mass spectrometry for extended analyses. Size fractions obtained with the Eclipse system are

further enriched using DCM technology, while the Mobility EAF4 module can determine zeta potential and so provide full, detailed distributions of zeta potential vs. size.

The Eclipse/DAWN system is already used to great advantage in R&D of gene vectors, nanopharmaceuticals, vaccines and other bionanoparticles. Further adoption of the platform by scientists involved in extracellular vesicle studies is a promising avenue to enhancing their depth and productivity.

For more information on the technology and applications of FFF-MALS, please visit www.wyatt.com/FFF-MALS.

To learn more about Eclipse, click the button below.

Request information

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