

Purpose

Zeta potential (ZP), a measure of net charge in solution, is widely utilized to optimize formulations of nanoparticle drug delivery systems for colloidal stability.

Standard technologies for determining ZP such as Phase Analysis Light Scattering (PALS) typically provide a ZP value that is averaged over the entire ensemble. Nanotracking Analysis (NTA), a more recent development, can provide a low-resolution distribution of ZP vs. size.

However, a more general and high-resolution method for characterizing ZP and other properties of nanoparticles has been lacking.

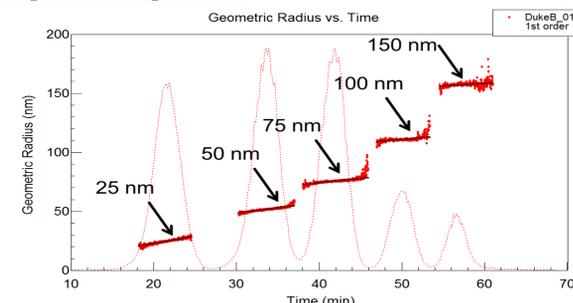
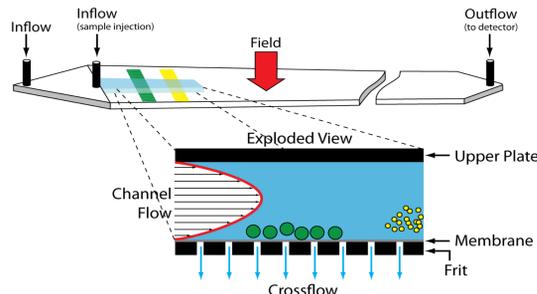
We demonstrate a novel technique, FFF-

PALS, which combines high-resolution, size-based separation of nanoparticles with downstream **MP-PALS** detection. FFF-PALS quantifies high-resolution distributions of nanoparticles by size and ZP with the potential for adding additional characterization techniques in-line such as spectroscopic, conformational or thermal analysis.

Field-Flow Fractionation for Protein, Polymer and Nanoparticle Separation

The Wyatt Eclipse Asymmetric-Flow Field-Flow Fractionation (AF4) system performs sized-based fractionation of macromolecules and nanoparticles from 1-1000 nm.

- Separates complex fluids containing molecules *and* colloids
- Separation power as good as or better than SEC, over a much larger range of sizes
- Resolution of size distributions much better than DLS or NTA: 1-2% vs. ~ 3x.



AF4 + light scattering analysis of polystyrene latex spheres, exhibiting excellent resolution plus the polydispersity of the standards within each size range.

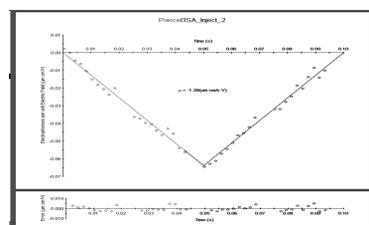
- No packed phase, so particles experience little shear or surface interaction.
- Separation power is easily tunable to optimize range and/or resolution.
- Fluid and sample handling provided by a standard HPLC system
- Downstream light scattering, UV, RI and other detectors perform characterization independently of elution time.

MP-PALS for Sensitive, Real-Time Zeta Potential

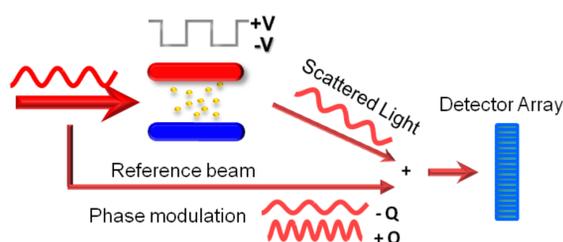
The Möbiuζ measures electrophoretic mobility of macromolecules and particles via phase analysis light scattering (PALS). Simultaneously it measures size via dynamic light scattering (DLS).

The combination of mobility + size permits calculation of the zeta potential, an indicator of nanoparticle stability in suspension.

The Möbiuζ incorporates a flow cell for connection to an autosampler. It may also be used in manual mode, either with a dip electrode or by injecting sample into the cell with a syringe.



A 'V-curve' showing the change in particle position over one cycle of positive and negative applied field.



FFF-PALS Realized

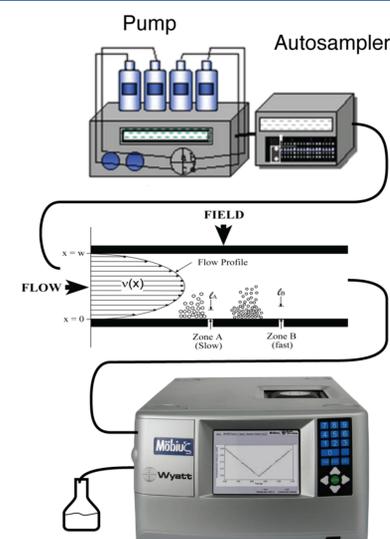
The Möbiuζ employs massively-parallel PALS which is sensitive enough to measure zeta potential *online*, during flow.

For validation against SEC, a Möbiuζ and Optilab T-REX were connected sequentially downstream of an Agilent HPLC autosampler and pump + Wyatt SEC column.

For testing with FFF, a Möbiuζ and Optilab T-REX were connected sequentially downstream of an Eclipse DualTec AF4 system using an Agilent HPLC autosampler and pump.

DYNAMICS software was used to acquire and analyze mobility, DLS, net molecular charge and zeta potential.

ASTRA software acquired RI data from the Optilab and converted the signals to concentration.

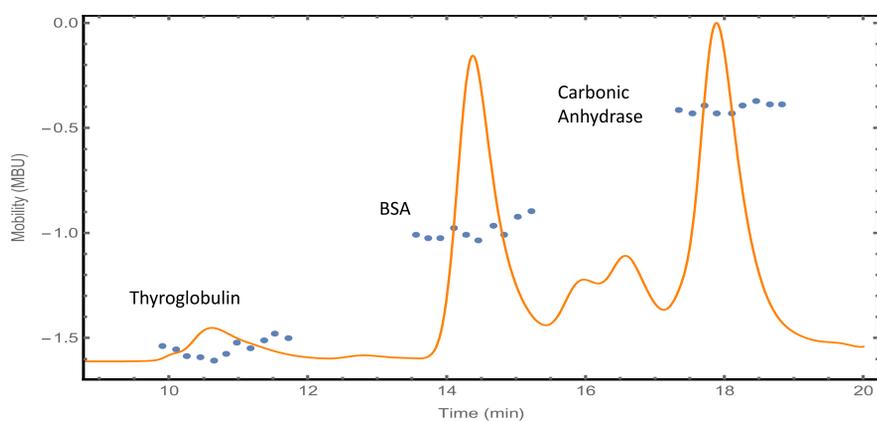


SEC - PALS for validation of online PALS with proteins

- Standard electrophoretic mobility measurements are carried out in 'batch' mode, without flow or fractionation.
- Validation was done using protein standards: BSA, thyroglobulin (THY) and carbonic anhydrase (CA)
- Online PALS using SEC was run with individual proteins and with a mixture that was separated on the column
- For BSA and THY, the online and batch mobility values are nearly identical within the quite reasonable

uncertainty limits.

- CA showed different values in batch and under SEC, but also different sizes. The size difference indicates that the unfractionated sample was heavily aggregated (hence the extra peaks in the chromatogram)
- We can conclude that the online mobility measurements are accurate and the dispersion in the Möbiuζ flow cell not excessive.



Condition	Thyroglobulin (MBU)	BSA (MBU)	Carbonic Anhydrase (MBU)
Batch	-1.48 ± 0.10*	-1.03 ± 0.08	-0.82 ± 0.05*
Online individual	-1.59 ± 0.10	-1.04 ± 0.09	-0.41 ± 0.07
Online mixed	-1.56 ± 0.06	-1.03 ± 0.09	-0.42 ± 0.09

* Showed high R_h (i.e. aggregates)

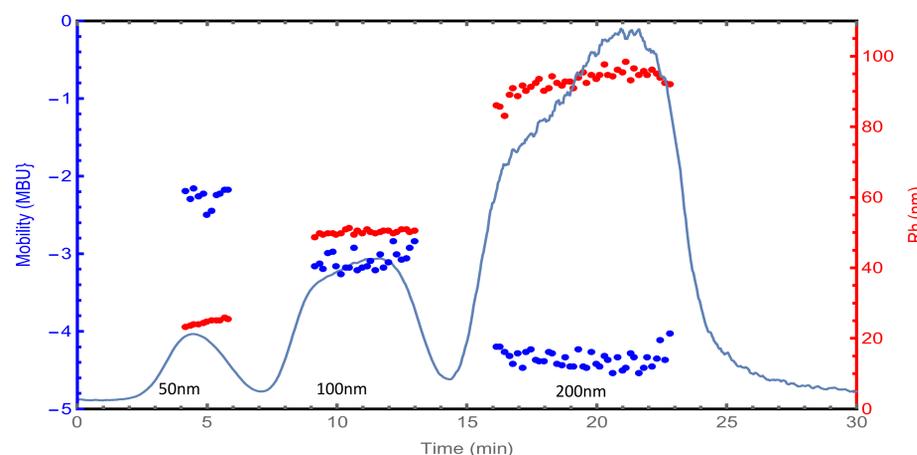
FFF-PALS with nanoparticle standards

Polystyrene latex spheres of 50, 100 and 200 nm size were injected onto the FFF-PALS system individually and in a mixture. The results were compared to batch measurements of the individual homogeneous samples.

The three nanoparticle sizes achieved near-baseline separation in the FFF-PALS setup despite dispersion in the Möbiuζ' relatively large flow cell. While the 100 nm and 200 nm particles were nearly homogeneous in size,

the 50 nm particles exhibited a distinct polydispersity spanning 80-100 nm.

Each particle size was associated with a distinct value of electrophoretic mobility. The values of size and mobility correlate well with those obtained in batch (unfractionated) measurements of the individual samples, except for the 50 nm particle mobility.



Condition	Size	50 nm	100 nm	200 nm
Batch		-3.3 ± 0.13	-3.6 ± 0.13	-4.6 ± 0.09
Online		-2.3 ± 0.1	-3.1 ± 0.11	-4.4 ± 0.09

The promise of FFF-PALS:

The FFF-PALS system is very promising for complete nanoparticle characterization. In addition to an MP-PALS detector for size and ZP, instruments such as UV/Vis absorption or fluorescence spectrometers, multi-angle light scattering for molar mass, size and conformation, and ICP-MS for elemental analysis may be added to the detector chain.

Flow-PALS is also useful for characterization of biomacromolecules and polymers. SEC-PALS of proteins is demonstrated here.

Until now, size-resolved zeta potential measurements have been limited to batch NTA analysis, which is only as good as NTA size resolution – on the order of 2.5x – 3.0x in radius.

FFF-PALS exhibits sensitive size-resolved zeta potential

measurements of nanoparticles, down to 1-2% in radius. FFF-PALS offers much higher resolution than possible with standard batch PALS or NTA, albeit with certain limitations:

- Size-based DLS in flow mode is limited to about 250 nm in radius, though this can be increased by slowing down the flow rate below 0.5 mL/min. Many types of nanoparticles may be sized by online multi-angle light scattering (MALS) up to 500 nm in radius and beyond.

Without size data, zeta potential cannot be calculated but electrophoretic mobility may still be measured.

- In principle AF4 can separate beyond 1 μm using the 'steric mode', relying on calibration of retention time to determine size and thus calculate zeta potential.

We have yet to explore the limits of sizes for mobility measurements, which will depend on the combination of mobility, size and flow rate.