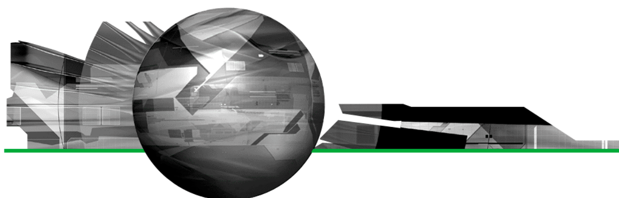

application note

Molecular weight measurements with the Zetasizer Nano system

Introduction

A common misconception within the light scattering market is that multi-angle instrumentation is essential for measuring the absolute molecular weight of macromolecules. This misconception is a consequence of the historical development of static light scattering techniques for the characterization of high molecular weight polymers. Samples of this type, that have a comparatively large size, exhibit scattering profiles that are non-isotropic, with measured intensities that are dependent upon the angle of observation. As the particle size decreases however, the sample scattering becomes isotropic and the angular dependence of the measured intensity is minimized. This application note addresses the theoretical limits of single angle static light scattering and highlights the use of the Zetasizer Nano system for measuring the molecular weight of small proteins and polymers.

Theory

The Rayleigh expression describing the intensity of light scattered from a particle in solution is given in Equation 1, where K is an optical constant defined in Equation 2, C is the particle concentration, R_θ is the Rayleigh ratio of scattered to incident light intensity, M is the weight average molecular weight, A_2 is the 2nd virial coefficient, $1/P(\theta)$ is an angle dependent term defined in Equation 3, R_g is the radius of gyration, λ_o is the vacuum wavelength of the incident light, θ is the scattering angle, N_A is Avogadro's number, $\tilde{n}_o$ is the solvent refractive index, and $d\tilde{n}/dC$ is the solvent and analyte dependent refractive index increment.[1]

$$\frac{KC}{R_\theta} = \left(\frac{1}{M} + 2A_2C\right)\frac{1}{P(\theta)} \quad (1)$$

$$K = \frac{2\pi^2}{\lambda_o^4 N_A}\left(\tilde{n}_o \frac{d\tilde{n}}{dC}\right)^2 \quad (2)$$

$$\frac{1}{P(\theta)} = 1 + \frac{16\pi^2 \tilde{n}_o^2 R_g^2}{3\lambda_o^2}\sin^2\left(\frac{\theta}{2}\right) \quad (3)$$

The Rayleigh ratio (R_θ) term in Equation 1 is the ratio of scattered to incident light intensity. Since the magnitude of the incident light interacting with the molecule is difficult to measure, the standard approach is to measure the scattering intensity of the analyte relative to that of a well-described reference with a known Rayleigh ratio. A common reference used in light scattering is toluene. The expression used to calculate the sample Rayleigh ratio from a toluene standard is given in Equation 4, where I_A is the residual scattering intensity of the analyte (= sample intensity – solvent intensity), I_T is the toluene scattering intensity, $\tilde{n}_o$ is the solvent refractive index, $\tilde{n}_T$ is the toluene refractive index, and R_T is the known Rayleigh ratio of toluene.

$$R_\theta = \frac{I_A \tilde{n}_o^2}{I_T \tilde{n}_T^2} R_T \quad (4)$$

The $1/P(\theta)$ term in Equation 1 embodies the angular dependence of the sample scattering intensity. The angular dependence arises from coherent interference between light scattered from different points within a particle or molecule (see Figure 1). This phenomenon is known as Mie scattering. When the particles in solution are much smaller than the wavelength of the incident light, Mie scattering is minimized. Under these conditions, $1/P(\theta)$ reduces to 1 and the angular dependence of the scattering intensity is lost. Figure 2 is the classic 'doughnut' plot which shows there is no change of intensity

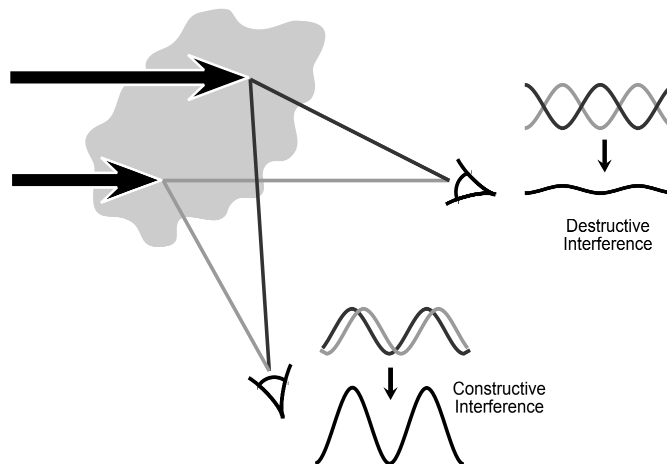

Figure 1: Schematic showing the effect of Mie scattering on the waveform and intensity measured at the APD.

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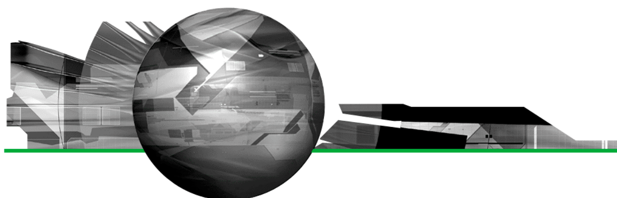

with angle in the horizontal plane. This type of scattering is known as Rayleigh scattering.

On the surface, it would appear then that the question of whether or not multi-angle light scattering instrumentation is essential for molecular weight measurements can be reduced to the following:

- If the particle is large, Mie scattering should be assumed and multi-angle instrumentation should be utilized.
- If the particle is small, Rayleigh scattering can be assumed and multi-angle instrumentation is redundant.

However, a quick review of the $1/P(\theta)$ term defined in Equation 3 indicates no point of discontinuity and hence no clear distinction between "large" and "small". As such, the gray area between large particle Mie scattering and small particle Rayleigh scattering can be problematic when considering the need for multi-angle instrumentation.

The particle size dependent expected error in single angle molecular weight measurements can be calculated using Equation 5.[2] This expression can then be used to calculate the upper size limit for a user defined acceptable error in the molecular weight measurement.

$$\%E_M = |P(\theta) - 1| * 100 \qquad (5)$$

Figure 3 shows a plot of the calculated %Error in molecular weight as a function of particle size for aqueous samples ($\tilde{n}_o$ = 1.333) characterized with a single angle 173° backscatter system incorporating a 633nm laser – instrumental parameters consistent with the Zetasizer Nano system. The solid lines in the figure indicate upper size limits of 39 and 57nm for acceptable molecular weight errors of 5 and 10% respectively. For globular proteins, a 39nm diameter corresponds to a mass on the order of 4,000 kilo Daltons. Typical proteins have molecular weights ranging from 10 to 1000kDa. Therefore, an upper molecular weight limit of circa 4,000kDa is sufficient to cover many protein applications.

For small particles in the Rayleigh region, Equation 1 can be reduced to the linear form shown in Equation 6.

$$\frac{KC}{R_\theta} = \frac{1}{M} + 2A_2C \qquad (6)$$

This form of the Rayleigh equation then can be used to generate a Debye plot, which is a linear fit of KC/R_θ vs. concentration, where the intercept is equal to the inverse of the molecular weight and the slope is twice the 2nd virial coefficient. An example of a Debye plot for a 1kDa MW latex standard in toluene is shown in Figure 4. From the intercept, the measured molecular weight is 1.08kDa. From the slope, the 2nd virial coefficient for this polymer/solvent pair is -0.0237×10^{-4} mL mol / g^2. The 2nd virial coefficient represents particle interaction strength and has been correlated with sample solubility.[3] The negative value measured in the

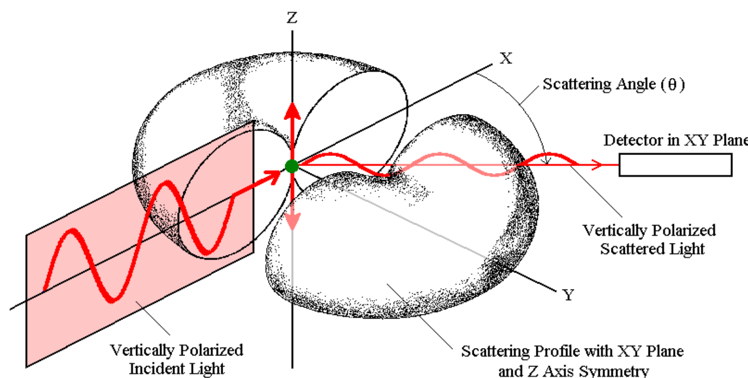

Figure 2: Schematic showing symmetrical scattering profile for small particles in the Rayleigh scattering region.

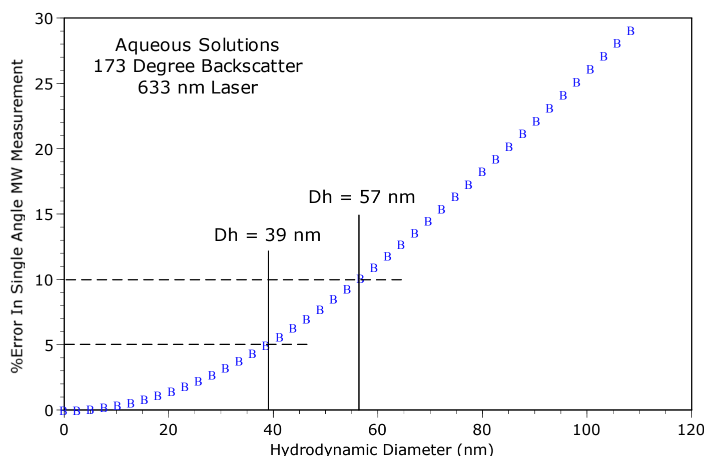

Figure 3: Expected error in molecular weight as a function of the hydrodynamic diameter, for aqueous globular proteins measured with a 173° backscatter system with a 633nm laser.

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example shown in Figure 4 indicates that the polymer has a slight preference toward aggregation as opposed to solvation.

Experimental

The solvent conditions and specific refractive index increment (dñ/dC) values for each of the samples cited herein are given in Table 1. The dñ/dC value is a function of the particle density. The 0.185 value used here for the protein samples is a good rule of thumb value for globular proteins in aqueous solution at 633nm and 25°C. For linear polymers, typical dñ/dC values are on the order of 0.1mL/g.

The dñ/dC values for the polymers used in this study were measured with an Optilab differential refractometer. It is noted here that standard refractometers generally lack the precision necessary for an accurate measurement of dñ/dC, and as such, it is always better to use a differential refractometer to measure dñ/dC.

Different sample concentrations were prepared by dilution of a high concentration stock solution. Protein samples were filtered with 100nm Anotop filters prior to measurement, with the sample concentration being determined, post filtration, by UV spectroscopy. The latex standard was prepared at the desired concentration, with no filtration.

The toluene reference and solvent were both double filtered with 20nm Anotop filters prior to measurement. Toluene and water are immiscible, so the sample cell was cleaned by rinsing with ethanol between the organic and aqueous phase measurements.

Data collection and calculations were managed using the Molecular Weight function in the DTS software for the Zetasizer Nano system, which compiles the static intensity measurements, generates a standard Debye plot (see example of software screen shot in Figure 4), and then calculates the molecular weight and 2nd virial coefficient. During the protein molecular weight measurements, dynamic light scattering size distribution data was collected simultaneously in order to:

1) Establish the sample modality (# of particle size families);
2) Verify that the particle size was within the Rayleigh scattering size range; upon dilution.
3) Insure that the sample was stable

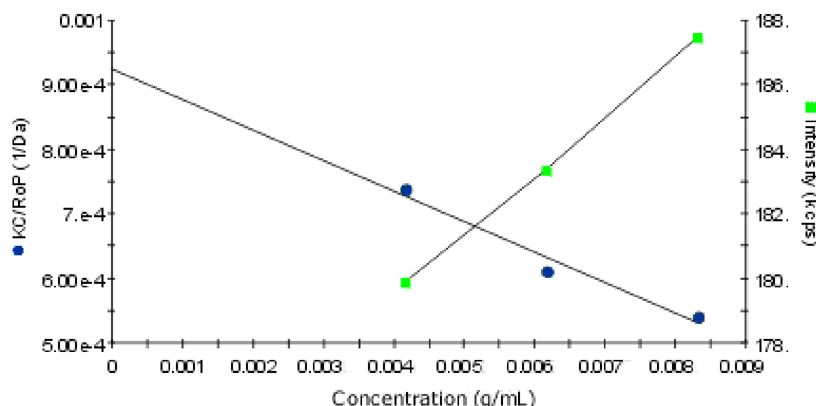


Figure 4: Example Debye plot for a 1kDa latex standard in toluene.

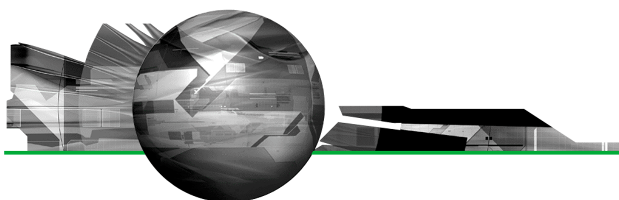
Results

Overlays of the Debye plots for all of the samples are shown in Figure 5. The light scattering results are compiled in Table 2, which includes a comparison of the measured to the reported molecular weight values.

As seen in Table 2, most of the molecular weight values measured with the Zetasizer Nano system are consistent with the values reported by alternate techniques. Two of the samples (BSA and the antibody fragment) however, show measured molecular weights that differ from the reported values by more than 5%, and it is instructive to examine these differences individually.

Sample	Solvent	dñ/dC (g/mL)
Bovine serum albumin	133 mM PBS, pH 7.5	0.185
Ribonuclease A	133 mM PBS, pH 7.5	0.185
Hen egg lysozyme	133 mM PBS, pH 7.5	0.185
Ovalbumin	100 mM NaCl, pH 6	0.185
Antibody fragment	10 mM NaCl	0.185
96 kDa Polystyrene	Toluene	0.105

Table 1: Solvents & dñ/dC values for molecular weight measurements.



Sample	A_2 (mL mol/g^2)	MW_M (kDa)	MW_R (kDa)	MW Diff	Technique	Diam (nm)	Pd
Polystyrene	1.880-3	95.6	96	0.4%	Multi-angle SLS	-	-
BSA	8.902-5	71.4	67.0	6.6%	Various	9.5	31%
Ribonuclease	7.426-4	14.9	14.7	1.4%	Various	4.4	19%
Lysozyme	-3.229-4	14.6	14.7	0.7%	Various	4.1	17%
Ab Fragment	-4.98E-3	20.7	18	15%	SEC	5.5	19%
Ovalbumin	6.784-4	46.8	45	4.0%	Various	6.2	22%

Table 2: Comparison of MW measured at a single angle, (MW_M) to reported (MW_R) molecular weight values, SEC is size exclusion chromatography, SLS is static light scattering, Diam is the hydrodynamic diameter and Pd is the % polydispersity or relative standard deviation of the size distribution from dynamic light scattering.

Discussion

The first point of interest in the data shown in Table 2 is the negligible difference between the molecular weight values measured by single and multi-angle techniques for the Polystyrene molecular weight standard. The consistency between these two values is direct experimental support for the theoretical prediction of isotropic scattering from particles much smaller than the wavelength of the incident light.

In contrast to the Polystyrene standard data, the measured molecular weight of BSA differs significantly from the reported value. The molecular weight of BSA measured on the Zetasizer Nano system is 71.4kDa, which is 6.6% larger than the known molecular weight of the 67kDa monomeric form of the protein. The molecular weight measured by the static light scattering technique used in the Nano system is a weight average molecular weight. In the absence of pre-measurement physical separation, such as that achieved in liquid chromatography, the weight average SLS molecular weight value will include contributions from all of the oligiomeric states of the protein in the solution. So the larger molecular weight measured for BSA suggests the presence of low order aggregates or oligiomers.

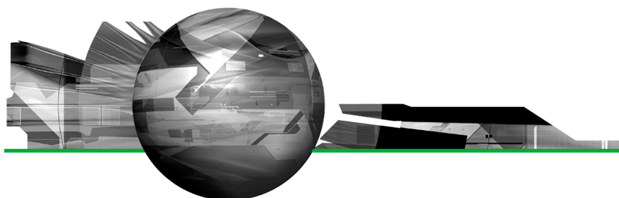
In common laboratory buffers, BSA is known to exist as a mix of monomer, dimer, and 8-mer. Support for the presence of these multi-mers can be seen in Figure 6, which shows the concentration dependent size distribution for BSA, along with an overlay of the size distribution measured for a 200nm nanosphere. In the dynamic light scattering (DLS) technique used to measure size distributions in the Zetasizer Nano system, baseline resolution is achieved for particles with circa 3X difference in size. Short of baseline resolution, the presence of oligiomers will lead to an increase in the polydispersity or the width of the peak in the size distribution. A good rule of thumb for polydispersity cut off for monomodal samples is ≤ 20%, similar to that observed for the 200 nm nanospheres shown in Figure 6. The 31% polydispersity measured for the BSA samples on the other hand, supports the suggestion that the BSA sample includes multi-mers.

The 15% difference between the measured MW value of 20.7kDa and the reported 18kDa value for the antibody fragment also merits discussion. The reported value was measured by size exclusion chromatography, which estimates the MW based upon comparison of the column elution time of the unknown sample to a calibration curve developed from standards with shape, composition, and charge density properties similar to those of the unknown. With regard to the antibody fragment cited in this study, it is unknown whether or not:

1) The fragment has a globular or random coil structure in solution
2) The calibration curve was appropriate for the sample
3) The SEC data indicated a narrow, wide, or multimodal distribution.

As noted in Table 2, the polydispersity for the antibody fragment is < 20%, which would suggest that the sample is monodisperse. This observation would tend to rule out any distortion in the SEC measured molecular weight value arising form wide or multimodal distributions.

A commonly used method for estimating molecular weight from light scattering data is to measure the hydrodynamic size by dynamic light scattering (DLS) and then estimate the molecular weight from a size vs. mass calibration curve - similar to the approach used in size exclusion



chromatography. Using this method, the 5.5nm hydrodynamic diameter of the antibody fragment would be consistent with a molecular weight of 36kDa if the fragment has a globular solution structure and 18kDa if it has a random coil solution structure. The 18kDa value for a random coil structure is identical to the reported SEC molecular weight, which lends credence to the SEC value and suggests that the solution structure is a random coil. If such is the case, the difference between the measured and reported molecular weight values in Table 2 for the antibody fragment is likely a consequence of an incorrect dñ/dC value. As discussed in the experimental section, dñ/dC is a function of the particle density, with a 0.185g/mL dñ/dC value being a good rule of thumb estimate for globular proteins. For a random coil polypeptide, one would expect a reduction in both dñ/dC and the subsequently calculated molecular weight derived from the static light scattering technique.

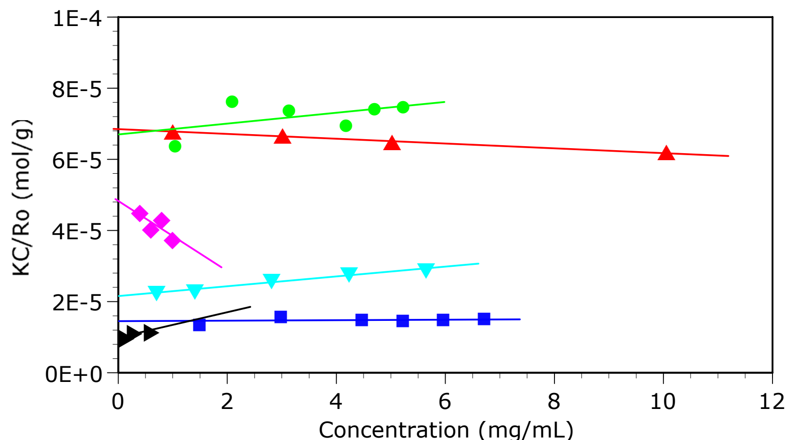


Figure 5: Debye plots for BSA (■), Ribonuclease (●), Lysozyme (▲), an antibody fragment (◆), Ovalbumin (▼), and a Polystyrene MW standard (▶).

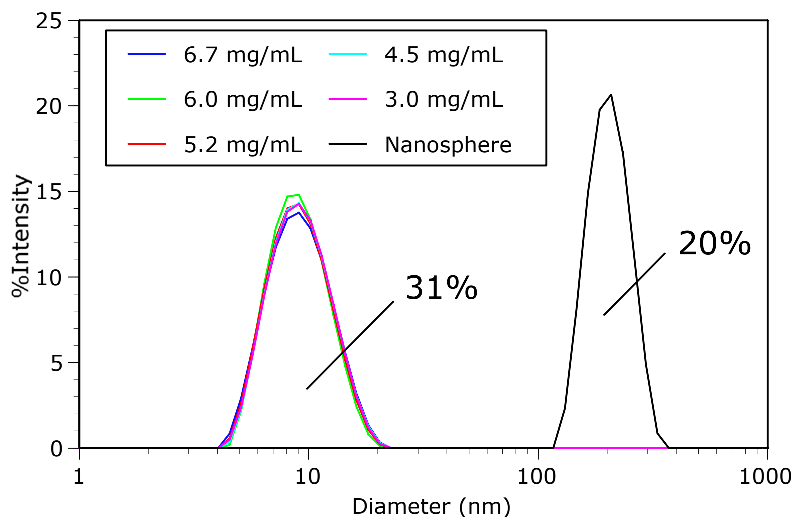


Figure 6: Concentration dependence of the measured particle size distribution for BSA overlaid with the measured size distribution of a 200nm nanosphere. The % polydispersity for each sample is noted.

Conclusion

In contrast to the commonly held perception that multi-angle light scattering instrumentation is essential for absolute molecular weight measurements, theory predicts that the angular dependence of the scattering intensity will be negligible when the particles are much smaller than wavelength of the incident light. From the theoretical considerations and experimental data presented here, it is apparent that accurate molecular weight determinations can be obtained from static light scattering measurements performed at one angle for globular macromolecules with molecular weights of up to circa 4,000kDa. This upper molecular weight limit for measurements at one angle is sufficient to cover most protein applications. While the above discussion and conclusions do not preclude the use of multi-angle instrumentation when working with smaller particles such as proteins and biopolymers, if molecular weight is the target, the addition of multiple angle data becomes redundant.

There is an additional advantage in using a combined dynamic and static light scattering instrument. Measurement of the molecule size using the DLS capability of the Zetasizer Nano system is so fast and simple, it can be used to determine whether the sample falls in the size range suitable for measurement of the molecular weight at a single angle.

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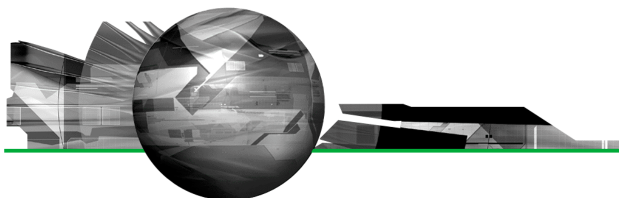

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2) Mattison, K; Kaszuba, M. Measuring Absolute Protein Molecular Weight: Is Multi-Angle Instrumentation Absolutely Essential? *American Biotechnology Laboratory* **2003**; *21(7)*, 28.

3) George, A; Wilson, W. Predicting protein crystallization from a dilute solution property, *Acta Crystallography* **1994**, *D50*, 361.

Zetasizer Nano System

The Zetasizer Nano system from Malvern Instruments is the only commercially available instrument to include the hardware and software for combined dynamic, static, and electrophoretic light scattering measurements, giving the researcher a wide range of sample properties, including the size, molecular weight, and zeta potential. The system was specifically designed to meet the low concentration and sample volume requirements typically associated with pharmaceutical and biomolecular applications, along with the high concentration requirements for colloidal applications. Satisfying this unique mix of requirements was accomplished via the integration of a backscatter optical system and the design of a novel cell chamber. As a consequence of these features, the Zetasizer Nano specifications for sample size and concentration exceed those for any other commercially available DLS instrument, with a size range of 0.6nm to 6µm, and a concentration range of 0.1mg/mL lysozyme to 40% w/v. In addition, temperature control between 2 and 90°C is a standard component of each instrument. As an added bonus, the Zetasizer hardware is self optimizing, and the software includes a unique "one click" measure, analyze, and report feature designed to minimize the new user learning curve.

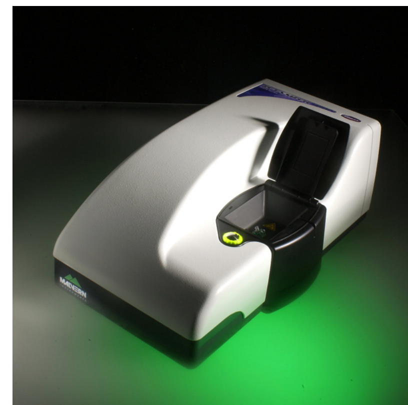

Figure 7: The Zetasizer Nano system from Malvern Instruments. The only commercially available system to include the hardware and software for combined static, dynamic, and electrophoretic light scattering.

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