Project:

Measuring Batch Mode DLS

Measuring the Size of Nanoparticles Using Batch Mode Dynamic Light Scattering

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1 Introduction

The sizing of particles in the nanometer range can be performed directly in aqueous solutions using batch-mode dynamic light scattering. This protocol provides procedures for sample preparation and the estimation of the average size and broadness of the distribution for diluted particles in the submicrometer range.

Dynamic light scattering (DLS) measures the hydrodynamic diameter (or radius) of particles in solution and is also known with different terms, such as photon correlation spectroscopy (PCS) or quasi-elastic light scattering (QELS).

This protocol deals with the measurement of the hydrodynamic diameter (and its distribution) of NPs dispersed in aqueous solutions, through the determination of their diffusion coefficient. Guidelines for making successful DLS measurements are provided, as well as a discussion of relevant standards for quality control and criteria for data analysis. This document describes the SOPs to be used with the DLS system produced by Malvern (Nano series). The protocol should be modified to be applicable to other systems.

2 Principle of the Method

When submicron NPs are dispersed in a liquid they are in constant random Brownian motion due to the interaction with the liquid molecules. At very low concentration the movement of particles depends by the liquid viscosity, temperature, and the size of particles. This relationship is expressed by the Stokes-Einstein equation used to calculate the hydrodynamic diameter, d_H from the measured diffusion coefficient.

$$d_H = kT / 3\pi\eta D$$

where k in the Boltzmann constant, T is the temperature, η is the viscosity of the liquid and D is the diffusion coefficient.

The instrument measures the autocorrelation function of the sample and then through a fitting procedure determines the diffusion coefficient of the sample.

$$G = \int_0^\infty I(t)I(t+\tau)\,dt = B + Ae^{-2q^2 D\tau}$$

With q (modulus of the scattering vector) = $4\pi n \sin(\theta/2) / \lambda_0$

Where τ is the delay time, B is a normalization factor, A an instrumental factor, D the translational diffusion coefficient, n refractive index of the dispersion medium, θ the scattering angle, λ_0 the wavelength of the laser in vacuum.
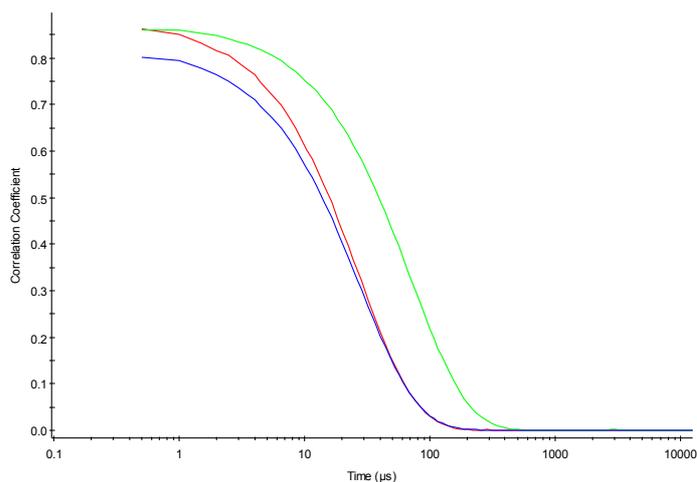
Figure 1: Autocorrelation function of gold NP of 50 nm (green), gold-NP of 20 nm (red) and silver-NP of 20nm (blue)

The cumulants analysis fits the autocorrelation function with a second order polynomial expansion in delay time τ (see ISO 22412:2008) giving the mean particle size (Z-average in Malvern terminology) and the polydispersivity index (PdI).

The two values (Z-average and PdI) are related to the intensity-weighted particle-size distribution. Assuming a Gaussian distribution of particle sizes the polydispersity index gives the relative variance of the hypothetical gaussian distribution:

σ = Z-average * √PdI

Another possible way of analyzing the data (i.e. the autocorrelation function) is the Laplace inversion to obtain the particle-size distribution. In this case, it is assumed that multiple diffusion coefficients are present in the sample and the autocorrelation function is fitted with a multiple exponential form. The size distribution is reported as a discrete set of diameters and corresponding intensity-weighted fractions. If the refractive indices of both the liquid and the particles are known it is possible to derive the volume and number-based distributions of the sample.

The Laplace inversion of the DLS autocorrelation function is an ill-posed inversion problem with several possible solutions, due to the presence of many variables. There are many different methods used for the data analysis, but no standardized algorithm and different methods of data analysis can give different particle-size distributions. The Malvern software uses a proprietary implementation of the Non-Negative Least Squares (NNLS) algorithm to derive the PSD.

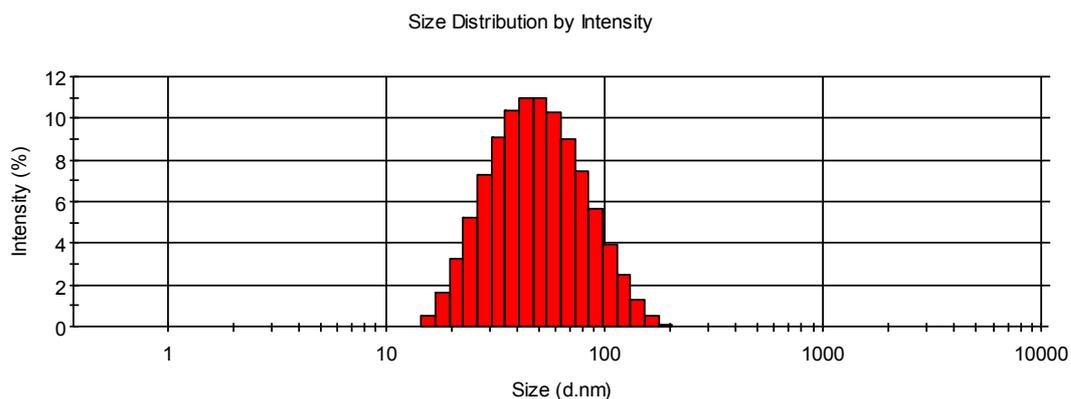Only the cumulants method is included in the International Standard.

Figure 2: Intensity-based particle size distribution of gold nanoparticle sample of 50nm

3 Applicability and Limitations

3.1 Nanoparticle concentration

NP concentration should be maintained in a range that allows both to obtain a good signal to noise ratio, while at the same time avoiding multiple scattering interferences. Using the automatic attenuation selection and the optimization of the positioning routines available in the Malvern instruments usually provides good results. Generally speaking, ideal concentrations are reached when instrument gives attenuation factors between 7 and 9. In some cases it may be necessary to dilute the samples (see later for procedure).

A good parameter to keep track of the concentration of the samples is the derived count rates, that can be found amongst the Malvern parameters.

3.2 Absorption in Strongly Absorbing samples

Recording the UV-VIS spectrum of the sample is always recommended. If the sample adsorbs strongly at the wavelength of the instrument's laser, then the results should be treated with care and possibly checked using a different size-measuring instrument or with a different laser.

3.3 Effects of Salt Concentration, Stabilizers, and pH

The hydrodynamic size derived from DLS can depend on the salt concentration of the suspending medium, pH and concentration of dispersant.

In deionized water the additional drag induced by the extension of the double-layer into adjacent bulk solution causes an apparent increase in size. This effect can be particularly relevant for small particles. Therefore a preliminary study of the effects of salt concentration could be useful in some situations.

In some cases NP are stabilized by the presence of dispersants (such as citrate for gold and silver NP, or surfactants) and their dispersions are stable only in a limited range of pH values. When performing sample dilutions to obtain the optimal concentration for DLS measurement it is

important to ensure that the pH and dispersant concentration are kept constant. The reduction of dispersant concentration and changes in pH can lead to NP aggregation and increase in polydispersivity of the sample.

3.4   Dust Contamination

The intensity of light scattered by nanosize particles is proportional to the sixth power of the particle diameter; thus larger dust particles will scatter much more light than smaller ones. Thus, the best practice to achieve good reproducibility is to eliminate dust prior to analysis. Cuvettes, sample vials, solvent bottles, etc., should remain closed as much as possible to minimize contamination. Dispersion media should be filtered to a 0.2 μm pore size. It is recommended that the dispersion medium should be periodically checked for background scattering; ensure that it is within the instrument guidelines and record its average count rate for future comparison.

If preliminary DLS results indicate the presence of contaminating large particles it may be necessary to remove large particles contaminants from the sample itself. This can be achieved either by centrifugation or by proper filtration.

3.5   Temperature control

In the Stokes-Einstein equation the hydrodynamic diameter is a function of diffusion coefficient, solvent viscosity and temperature. Therefore, during the measurement the temperature of the cuvette has to be stable. For this reason, an equilibration step of the temperature of the cuvette is introduced prior to the measurement.

4   Equipment and Reagents

4.1   Equipment
- ZetaSizer Nano ZS or similar equipment (Malvern Instruments)
- Disposable Cells

4.2   Standards for QC

It is advisable to select a standard in the same size ranges of the NPs to be analyzed. For example, two different polystyrene nanosphere size standards (NIST Traceable, distributed by thermoscientific), with diameters of 60 and 203 nn can be used, accordingly to the size range of the NPs you are planing to image (60 nm standard for NPs with average diameter<100-150 nm, 200 nm standard for NPs with a diameter >150 nm). For the preparation of NIST treaceble polystyrene spheres of 60 and 203 nn the preparation described in [1] should be followed:

- *Prior measurement, the standards needed to be diluted with suitable medium, such as NaCl 10 mM. Therefore, an aqueous solution of NaCl 10 mM should be prepared with ultrapurewater and filtered through a 0.22 μm filter (Millipore).*
- *The standards that are stored at 4∘C should let to warm up to reach the ambient temperature prior to their use. Then, they should be homogenized by gently inverting the dropper-tipped vial without shaking.*

- *After ensuring that the neck of the dropper-tippedvial is clear from encrusted nanoparticles and after discarding the firstdrop, 4 drops of the standard 60 nm should be added in a flask and then diluted into 20 mL of the NaCl solution 10 mM. The same procedure should be applied to prepare the appropriate dilution of standard 203 nm. In this case, 7 drops of standard 203 nm should be diluted with 2 mL of the NaCl solution 10 mM.*

NIST traceable Gold standards (e.g. RM-8012) can be an alternative. Refer to the certificate of the analysis of each standard for the specific protocol to be use to prepare the standard for DLS analysis and for the conditions chosen for DLS measurements.

4.3   Preparation of the sample

The optimum sample concentration is determined by the nature (light scattering properties) of the analyte NPs, and it has to be defined case by case. As a starting indication the optimal sample concentration is reached when the automatic attenuator factor in the Malvern instrument is between 7 and 9. It may be necessary to dilute the sample to reach the ideal concentration, ideally diluting them by serial 1:10 dilutions. In this case care should be taken to control the ionic strength, stabilizer concentration and pH of the dispersants. All these parameters should be documented and reported.

4.4   Preliminary analysis of the sample

- An UV-VIS spectra of the dispersion should be recorded between 400 and 800 nm to check the non-absorbance in the laser wavelength of the Malvern instrument (either 633 nm or 532 nm, depending on the equipment).
- A preliminary concentration study is suggested before the first analysis of unknown samples. To check if the dilution is in the right concentration during the measurement, be sure that the average counting rate and the attenuation factor are appropriate.
- pH should be measured and reported along with the size measurement (in the same conditions). DLS measurements are affected by small quantities of ions or organic impurities contained in the dispersion.

4.5   Measurement Cells

Use a new disposable cell for each measurement.

4.6   Measure procedure:

- As first step, remember to switch on the instrument 30 minutes before starting the measurement to warm up the laser.

- Clean the disposable cell once with filtered water and once with the filtered media used for the measurement.

- Loading Sample in the Measurement Cell
  - o Load sample in the cell using the minimum amount needed to reach a liquid level at least 2mm above the height of the laser beam. With disposable Mini cells for Malvern Zetasizer instruments this corresponds to around 500 uL of sample.

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Leave the software to automatically choose the measurement position into the cuvette.

- o Cap the cuvette to prevent dust contamination and solvent evaporation.
- o Control that no air bubbles are present on the optical window area.
- Temperature equilibration. Equilibration time prior to the measurement should be set at least at 5'. If the temperature of the sample differ ± 5°C from the temperature of the measurement (e.g. from RT to 37°C), leave the sample to equilibrate for more time (rate of 1°C/min).
- Perform 10 measurements per sample per temperature setting to establish measurement repeatability. Measurement duration should be set according to instrument manufacturer's recommendations, and will differ depending on particle size and scattering characteristics, as well as the optical characteristics of the instrument itself (e.g., detector sensitivity, scattering angle, etc.). A minimum duration (measurement time) of 60 s is recommended for nanosize particles.
- A faster way to proceed is to create appropriate SOP using the Malvern tools. Use the parameters in Table 1 to create the SOP

Table 1: SOP for DLS measurements

Parameter	Value
Equilibration time	> 5' (1°C/min)
Scattering Angle	173°
Number of Measurements	10
Number of Runs	12
Run duration	10 s
Delay between measurements	0 s
Volume inside the cuvette	According to the manufacturer's instructions
Positioning Method	Seek for optimum position
Automatic attenuation selection	yes
Analysis mode (Malvern)	General Purpose

- The data analysis requires the knowledge of some properties of the system. In particular, the viscosity and refractive index of the liquid phase for the cumulants method. The estimation of the volume and number-based particle size distribution requires also the refractive index of the particles.

Table 2: properties of different aqueous media

Aqueous Medium	Absolute viscosity (mPa . s)			Refractive Index
	20 °C	25 °C	37 °C	
pure water	1.002	0.890	0.692	1.332
10 mM NaCl	1.003	0.891	0.693	1.332
isotonic saline (154 mM NaCl)	1.020	0.908	0.710	1.334
phosphate buffered saline (PBS)	1.023	0.911	0.713	1.334

4.7   Reporting Data

- Critical information that should be reported includes: particle concentration (mass or volume based), sample preparation (dilutions …), dispersion medium composition, refractive index values for the particles and the dispersion medium, viscosity value for the medium, measurement temperature, filtration or other procedure used to remove extraneous particulates/dust prior to analysis (including pore size and filter type), cuvette type and size (path length), instrument make and model, scattering angle(s), laser wavelength.
- Parameters of the measurement worth reporting are the attenuation factor and the derived count rates.
- Report the size classes and limits used in the data analysis.
- Also report the measurement of the NIST traceable standard (at least z-ave and PdI) you have used for QC.

The following table can be used to report data, it includes the mean hydrodynamic diameter (Z-average), the polydispersivity index, along with their standard deviations based on 10 replicates. In addition the intensity-based particle size distribution, together with the relative plot, should be reported.

Sample	Cumulants Method		PSD analysis		
	Z-average ± SD	PDI ± SD	intensity-PSD (mean) ± SD	σ of peak 1	% of peak area
Gold-NP 20nm	18.7 ± 0.3	0.06 ± 0.01	20 ± 0.4	5.1	100 ± 4 %
PSD Plot					

5   Quality Control, Quality Assurance, Acceptance Criteria

5.1   Instrument verification

DLS measurements are based on first principles and hence no calibration is required. However, the instrument should be verified by running an appropriate quality control standard. For this reason is recommended to run a NIST traceable standard before starting each measurement session. ISO/DIS 22412 recommends that a polystyrene latex with narrow size distribution and average diameter as measured by DLS in the size range of 60-200 nm be used. The measured average diameter (z-average size) of the latex sample should be within 2 % of the stated size range (look the certificate of analysis). Additionally, the polydispersity index should be less than 0.1. Deviations beyond the above stated limits indicate that a problem may exist with the instrument performance, the measurement cell, or the water used to dilute the standard prior to measurement.

Certified gold-NP reference materials of 20 and 40nm are provided by NIST and can also be used to regularly verify the instrument.

To perform the instrument validation with these standards, follow the SOP reported in table 1. The number of measurement with the standards can be reduced to 5 (instead of 10), as indicated by NIST and ISO/DIS 22412.

Sample concentration. Check that the scattering intensity is within the acceptable range. For unknown samples, a preliminary concentration dependent study is advisable. The mean counting rate of scattering light reported during the measurement should ideally be between 100 and 500 k counts per seconds (kcps). The instrument attenuator value should be between 4

and 9. The quality of DLS measurements should be always checked by looking at the autocorrelation function (see EU_NCL_PCC001). Its value of the intercept of the autocorrelation function should be around 1 (>0.9). In reliable conditions (good concentration range), a series of repeated measurements of the same dispersions (e.g. 5- measurements of the same cuvette) should provide comparable z-average and PdI (no significant differences between the different replicas with cumulant analysis). Moreover, the derived count rate should be proportional to NP concentration (increased proportionally to NPs concentrations). If such conditions are satisfied, you have found a suitable concentration range for your DLS measurements.

Count rate plot. It reports the number of photons reported per second. The count rate plot should be stable over time (Fig. 3), and can be used to check dispersion stability during the measurement. If the dispersion is not stable, sample aggregation is possibly occurring.

*Figure 3: **Counts per second plot of a DLS measurement as a function of time.***

5.2   Acceptance criteria for comparison of size measurements
According to the ASTM and to the ISO standard guidelines [2,3], a maximum variation of 10% relative standard deviation in the reported z-averagenumerical value is acceptable when comparing data measured for a sample in the same conditions, if at least 3 measurements are compared. In addition, a few round robin reported in literature have demonstrated that DLS measurement are very sensitive to small number of impurities [4].

6   Health and Safety Warnings, Cautions and Waste Treatment
To minimize exposure; appropriate safety precautions and protective gear such as gloves, lab coat and goggles must be worn. After the measurement the samples should be discharged as appropriate for nanomaterials.

7   Abbreviations
DLS: dynamic light scattering

ISO: International Organization for Standardization

NIST: National Institute of Standards and Technology

NP: nanoparticles

PdI: polydispersity index

PSD: particle size distribution

SD: standard deviation

UV-VIS: UV-Visible spectroscopy

8 References

[1] F. Varenne, J. Botton, C. Merlet, M. Beck-Broichsitter ,F-X Legrand, C Vauthier Standardization and validation of a protocol of size measurements bydynamic light scattering for monodispersed stable nanomaterialcharacterization, Colloids and Surfaces A: Physicochem. Eng. Aspects 486 (2015) 124–138.

[2] ISO 13099-2:2012, Colloidal system- Methods for zeta potential determination-part 2: Optical methods, June 2012.

[3] ASTM E2865-12, Standard Guide for Measurement of Electrophoretic Mobility and Zeta Potential of Nanosized Biological Materials, ASTM International, West Conshohocken, PA, 2012, www.astm.org.

[4] G. Roebben, S. Ramirez-Garcia, V.A. Hackey, M. Roesslein, F. Klaessig, V. Kestens *et al.*, *J Nanopart Res*, 2011, *13*, 2687.

9 Annex

10 Related Documents

Document ID	Document Title
	Certificate of analysis of the standards
	Detailed protocol for sample preparation
EUNCL-PCC002	*Zeta potential*
EUNCL-PCC013	*Measurement of pH*