A common European approach to the regulatory testing of nanomaterials

Standard Operating Procedure

Title	***NRCWE SOP for measurement of hydrodynamic Size-Distribution and Dispersion Stability by Dynamic Light Scattering (DLS)***
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NRCWE SOP for measurement of hydrodynamic Size-Distribution and Dispersion Stability by Dynamic Light Scattering (DLS)

Key words: DLS, hydrodynamic diameter, average zeta-size, PDI, size-distribution, intensity, stability

Introduction

The purpose of this SOP (Standard Operation Procedure), is to give a basic introduction to dynamic light scattering method and the NANoREG procedure for measurement of the hydrodynamic size-distributions of stock dispersions and exposure media and their stabilities.

The current SOP is based on a previous SOP reported in NANOGENOTOX (Jensen et al., 2010).

Basic explanation of the DLS method

Dynamic Light Scattering (DLS), also called Photon Correlation Spectroscopy (PCS) or Quasi-Elastic Light Scattering (QELS), is a technique for characterization of colloidal systems based on the scattering of visible light resulting from the difference in refractive index between the dispersed colloids and the dispersion medium. The method may be applied for sizing particle suspended in a liquid in the range from about 0.6 nm to about 8 μm depending on the instrumentation, optical properties of material, medium and software.

The principle in DLS is measurement of fluctuations in laser light scattered by vibrating particles suspended in a liquid as function of time. The vibration is due to Brownian motion caused by collision with solvent molecules of the liquid. The Brownian motion varies as a function of particle size and causes variation in the intensity of scattered light as function of time. A correlator compares the signal as function of time, t_i, using the initial signal at t_0 as reference. The response is a relative signal comparability with different very short time intervals, *dt* (the so-called autocorrelation). As the particles move, the correlation between t_0 and subsequent *dt* signals decreases with time, from a perfect correlation (1) at t_0, to a complete decorrelation (0) at infinite time (order of milliseconds). In case of big particles, the signal changes slowly and the correlation persists for a long time, whereas small particles have high Brownian movement causing rapid de-correlation.

In fact a DLS instrument measures the velocity of Brownian motion, defined by the translational diffusion coefficient *D* of the particles. The particle size, or more precisely its hydrodynamic diameter d_H, is then estimated using the Stokes-Einstein equation assuming spherical shape:

$$d_H = \frac{kT}{3\pi\eta D}$$

k: Boltzmann's constant
D: translational diffusion coefficient
T: absolute temperature
η: viscosity

It should be noted that even if a particle is really spherical, the spherical DLS size is fundamentally different from the physical spherical size. The hydrodynamic size includes the double-layer with highly polarized water molecules around the physical particle. When the particle morphology is highly nonspherical, the hydrodynamic size should be understood as the equivalent hydrodynamic spherical size. Similar concept is used in aerodynamic measurement of airborne particles. Establishment of mean hydrodynamic size and size distributions (intensity, number, volume) is reached by DTS software algorithms, by fitting the correlation function (cf data treatment section).

Samples for DLS are measured in transparent cuvettes of glass or polymer. DLS measurements rely on the non-invasive backscatter (NIBS®) technology developed by Malvern Instruments, in which the signal is detected at 173° (Figure 1). The signal is treated by a digital correlator, and transmitted to the computer. The DTS software enables fitting of the correlation data; either by a single exponential, called the cumulant

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analysis (as defined by ISO 13321 Part 8) to obtain mean size (Z-average diameter) and polydispersity index (PDI), or by a multiple exponential known as the CONTIN method to obtain a distribution of particle sizes.

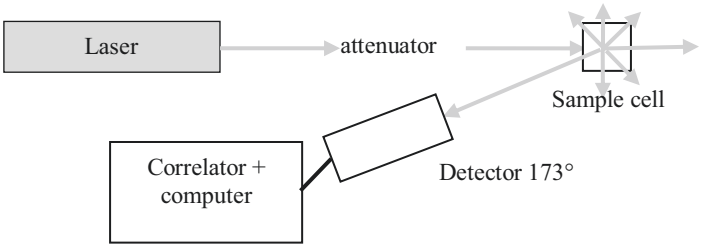

Figure 1: Simplified sketch of the optical configuration for DLS measurement on Zetasizer Nano ZS

Dispersions and Equipment

The following items are generally required for proper DLS analysis

- Personal protection equipments incl. gloves, lab-coats, glasses as required.
- Clean work environment with negligible dust and high-quality ventilation systems.
- Dispersion to be measured
- Zetasizer Nano ZS (Malvern Instruments), mounted with e.g., 633 nm laser diode
- Zetasizer software
- Viscometer (e.g, Malvern Inc., SV-10 Vibro Viscometer) *Optional for measurement of true viscosities*
- DLS cuvette of clear disposable polymer, glass cells or folded capillary zeta cells. In NANoREG, we recommend to use the 1 ml Malvern DLS cuvette.
- Pipettes and pipette tips
- Syringes and syringe filters
- Computer

Procedure

Samples

The NANoREG samples are produced using the different dispersion protocols selected for NANoREG and further specific measurement requirements for calibration protocols and exposure media (Tables 1 and 2).

Table 1: Characterization requirements in toxicological tests using dispersions

Element in the workflow	Recommendation (R) and Mandatory requirement (M); Optional (O)
Batch dispersion	Ten repeated measurements of hydrodynamic size (DLS) are made without pause in combination *In vitro* (M) and eco-tox (M).
Initial exposure medium	Ten consecutive measurements of hydrodynamic size (DLS) are made (if technically possible) without pause on the same sample. *In vitro* (M) and eco-tox (M)
Final exposure medium	Ten consecutive measurements of hydrodynamic size (DLS) are made (if technically possible) without pause on the same sample. *In vitro* (M) and eco-tox (M).

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Stability of dispersion during assay	Sedimentation rates can be assessed from tests using the DLS. By DLS one may analyze both agglomeration and sedimentation. *In vitro* (R) and eco-tox (R).

Table 2: Dispersion and probe-sonication calibration protocols.

Type of test	Protocol
Calibration of sonicators for *in vitro* and *In vitro* studies	Calorimetric method combined with adjustment using the NM-200 benchmark material NANOGENOTOX batch medium
***In vitro* studies**	NANOGENOTOX
***In vitro* studies**	NANOGENOTOX or ENPRA
Calibration of sonicators for ecotox studies	Calorimetric method combined with adjustment using the NM-200 benchmark material in water
Eco-toxicity studies	Either the NANoREG water or enhanced NOM* water ecotoxicological dispersion protocols for CNT and hydrophobic MNM.

* Natural Organic Matter

Malvern apparatus is designed to measure samples over a large concentration- and particle size-ranges. General specifications and limitations of sample properties (concentration range, size of nanoparticles, medium) can be found in the documentation from Malvern Instrument accessible on their website.

The critical point for all measurements is that it is necessary that the dispersion is reasonably stable within the time-frame of each measurement to obtain high-quality data.

General measurement

DLS measurements may be performed in disposable polystyrene cuvettes or re-usable glass cuvettes. It is recommended to repeat the measurements on the same samples 10 times to establish average diameters and intensity size distributions, which can be converted to number or volume size-distribution. The high number of repeated analysis are requested to enable better analysis of stability of dispersion and the size-distributions as well as omission of measurements with bad correlation data or abnormal solutions to the correlation function. In complex materials, such sudden “outliers” are sometimes observed, which can be due to actual variations or sudden disturbances due to fluctuations or sedimentation of larger particles. Such data must be carefully considered before use as they do not represent the general dispersion.

This procedure is the general approach for DLS measurement of the NM-200 probe calibration media, batch dispersions as well as initial and final exposure media.

1. Turn on the computer and then the DLS instrument
2. Allow the instrument to warm up according to the manufactor’s recommendation (ca. 30 minutes)
3. Upload the DTS software and the “Measurement” window for entering material specific data on dispersion mediums and test materials as well as specific analytical settings.
    - Select/enter the medium to retrieve its refractive index (R_i) and absorption values (R_a), dielectrical constant, and kinematic viscosity*. If a suitable medium is not entered, please

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enter your own medium. If you have no data, choose the default water and correct your data afterwards using the Edit function if needed.
- Select the test material to obtain its refractive index (R_i) and absorption values (R_a). If not existing and you have no data, a different material may be selected. If needed your data can be corrected afterwards using the Edit function**.
- Select measurement temperature (25°C for batch dispersions, NM-200 calibration dispersions, and ecotoxicological test media; 37°C for *in vitro* and *In vitro* exposure media). Use an equilibration time of 120 seconds.
- Select automatic for all measurement conditions; measurement position, laser attenuation, number of runs and sub-runs, respectively. Do NOT select extended measurement time for large particles.
- Select 10 repeated measurements with not pause between the measurements.
- Select the "General purpose" model for initial evaluation of data. This is the generic model for calculation of the average size and size-distribution.

4. Select a 1 mL sample cuvette and ensure that it does not contain dust, nor defects or scratches in the measurement area of the cuvette. Some producers have been found to deliver cuvettes with scratches or folding structure in the measurement area at one side of the cuvette. Dust may be cleaned out by rinsing the cuvette in dispersion medium.
5. Sample 1 mL of the dispersion and fill it into the 1 mL measurement cuvette using a pipette. For batch dispersion measurements the 1 mL sample is drawn 10 minutes after sonication allowing the dispersion to stabilize and harmonization of the sampling time.
6. Place the sample cuvette immediately thereafter in the DLS sample holder and start the analysis.

* If high-accuracy size-data are required, it is necessary to determine the viscosity of the tested dispersion. The measured dynamic viscosity can be determined and for data-correction after the DLS measurement has been completed (see Point 12 above), so there is no delay in the measurement process. Viscosity measurements can be made using e.g., the SV-10 Vibro Viscometer. Figure 2 below gives a theoretical example of the importance of a correct viscosity measurement if exact size-data are required.

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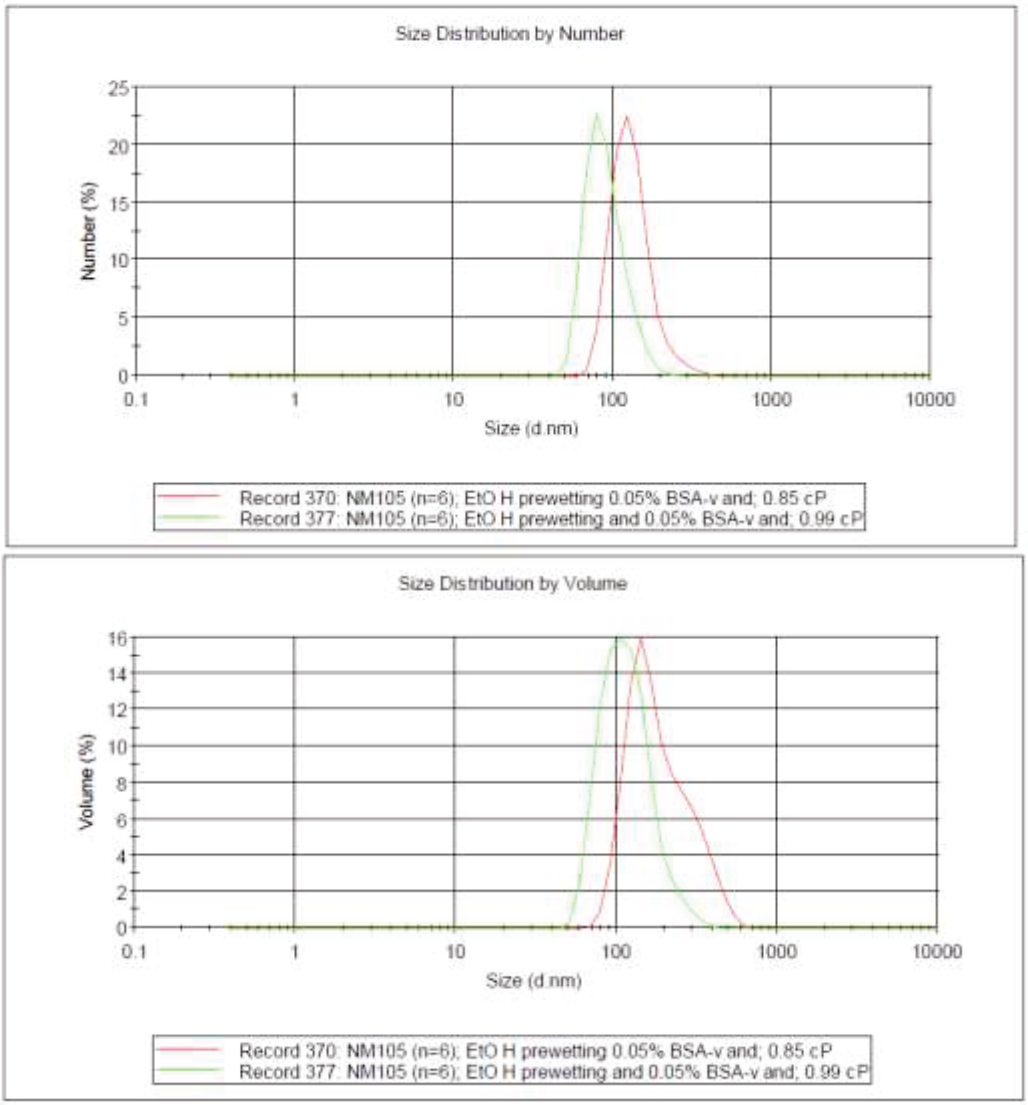

Figure 2: These graphs illustrate the importance of viscosity on the calculated number- and volume size-distributions of NM-105 dispersed according to the NANOGENOTOX dispersion protocol. Measurements were conducted at 37°C. The red line (record 370) is the result calculated using the standard viscosity of water (0.85 cP) and the green line are the same data calculated using the typical 0.99 cP viscosity of the dispersion medium at 25°C. The maximum peak values of the number size-distributions are 122.4 (141.8 volume) and 78.8 (105.7 volume) nm for the water and BSA-water dispersion viscosities, respectively.

** The optical parameters of the test material does not influence on the average zeta-sizes and PDI. However, if data are converted to volume or number size-distributions, it will normally affect shape and the peak positions of the calculated hydrodynamic size-distributions. The effect is particularly important in calculations of volume size-distributions. See example in Figure 3.

Measurement of dispersion stability

It is of high interest to gain further understanding of the dispersion behavior during testing. However, this is normally a very difficult task to perform in situ. One may get indirect information on both sedimentation and agglomeration behavior by using the DLS to measure the dispersion behavior in a cuvette over time.

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This procedure may be applied for assessment of the dispersion stability using a DLS cuvette to simulate the dispersion behavior in the exposure vial.

The measurement procedure consists of two parts.

Part 1: Conduct the sampling and measurement as listed above under point 1 to 6.

Part 2: After completion of the first 10 measurements using automatic optimization, the DTS software is now programmed to conduct measurements using the measurement position, laser attenuation, number of runs, and sub-runs, identified during the automatic optimization.

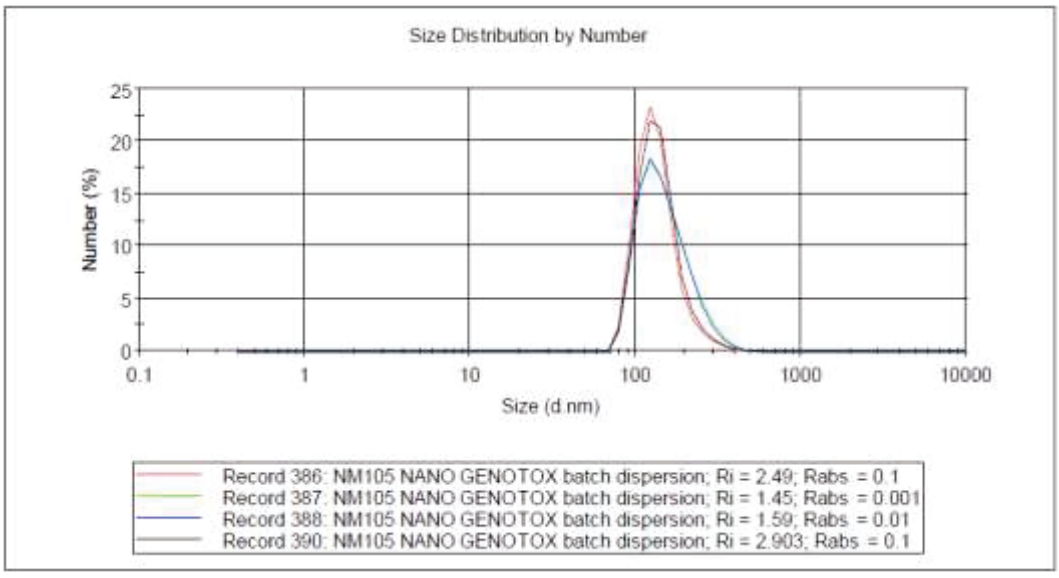
Set the number of measurements and pause to cover the measurement period and time-resolution of interest. A pause of 20 or 30 minutes between each measurement is usually sufficient to assess the dynamics in the dispersion.

Locking these measurement parameters enables comparison of fluctuations in intensity and average zeta-size as described in section 6.

Evaluation of DLS size-data

Consult the Malvern manual and homepage for advice on data-interpretation. Below a number of specific considerations are listed.

1. The average zeta-size may be immediately accepted if the DTS default Result quality says: “Good”.
2. If the analysis suggests that the sample has a bi- or multimodal size-distribution, the size-distribution result may be carefully evaluated by recalculation the data using the high-resolution algorithm selected under “Data processing” in the “Edit result…” menu.
   Remember; do not delete the original data measurement as this deletes the raw background data for all calculations.



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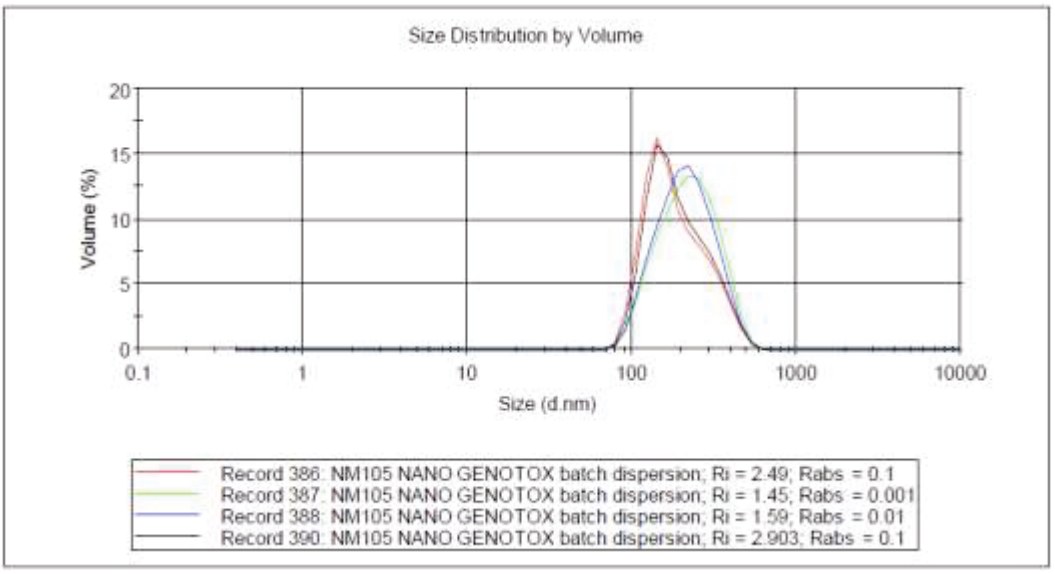

Figure 3: These graphs illustrate the importance of the Refractive (R_i) and Absorption indices (R_{abs}) on the calculated number- and volume size-distributions of NM-105 dispersed according to the NANOGENOTOX dispersion protocol. Measurements were conducted at 37℃ and calculated for medium viscosity of 0.99 cP. R_i = 2.49 is for anatase; R_i = 1.45 is for protein, R_i = 1.59 is for polystyrene latex and R_i = 2.903 is for rutile. The maximum peak values in the volume size-distributions are 141.8 nm for the anatase and rutile, 220.2 nm for the polystyrene latex, and between 220.2 and 255 for protein optical indices, respectively.

3. If the default Result quality says: "Refer to quality report", the sample should be further analyzed. Check the size-distribution report and the in-build Expert advice to investigate whether the result suffer from high polydispersity (PDI) due to polymodal distributions, presence of dust, cuvette errors, large particles, sedimentation, wall-deposition etc. Reliability in size-distribution data can be made by checking the fit in the Cumulant and Distribution fit window. If significant deviations from the fitting curves occur the size-distribution data are no longer reliable.
4. If the sample contains particles with large spread in size-distribution or the data failed under point 3, one may consider filtering the sample through different syringe filters to investigate presence of sub-µm and smaller nm-size particles. Dust, hair and particles agglomerates larger than the sizing range disturbs the measurements. In addition, small nm-size particles may not be fully resolved when coarser particles are present due to the large drop (10^6 per decade) in scattered light intensity with size.
5. If parameters such as refractive indexes, absorption coefficient or viscosity were wrong or unknown at the measurement time, the correction can be made afterwards using the command Edit (right click on the measurement) in DTS software.

The optical parameters for many materials can be found at internet resources such as http://webmineral.com/. Table 3 lists some examples of optical refractive and absorption indices used by NRCWE.

Table 3: Optical values used at NRCWE for some typical nanomaterials.

	Rutile	Anatase	Synthetic Amorphous Silica	ZnO	CNT
R_i	2.903	2.49	1.544	2.02	2.02
R_{abs}	0.10	0.10	0.20	0.40	2.00

Evaluation of particle behaviour in dispersions

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If the procedure in section 4.3 was followed, the particle behavior in the dispersion under investigation may be assessed by analyzing the evolution in relative scattered light intensity and average zeta-size / size-distribution as function of time. This section describes the procedure to enable analysis of relative intensity and average zeta-size (Z_{ave}).

1. Export the DLS measurements of interest to enable working with the data in a spread sheet. Key data for the calculations are: Date and time; Derived count rate; Average Zeta-size; and PDI. Additional data may be useful or used in specific assessments and can be exported as well
2. Open the exported text file in a spreadsheet using e.g., MS Excel and save it in a suitable file-format to avoid losing your work saving the file as .txt.
3. Insert a column for calculation of time (hours, minutes, seconds as you prefer); a second column for the "relative scattered light intensity", LOG (I/Io); and a third column for the ratio between the relative change in hydrodynamic zeta-size (Zave), LOG (Z/Zo).
4. Calculate the time between each between t_i and t_{i-1} measurement in the column called e.g., Time [hours]. One procedure is:

$$n(t_i\text{-}t_{i-1}\text{ minutes})/60 + (60 - t_{i-1}\text{ seconds} + t_i\text{ seconds})/3600$$

5. Calculate the "relative scattered light intensity" in the LOG (I/Io) column using the derived count rate data. Select the first measurement at t_o (or alternatively an average of the first stable measurements; $t_{o,ave}$) as the reference value for the intensity for the full dispersion. The LOG(I/Io) value is then the LOG to the derived count rate at t_i divided by the reference derived count rate at t_o or $t_{o,ave}$.
6. Calculate the "relative change in hydrodynamic size" in the LOG(Z/Zo) column. Select the first measurement at t_o (or alternatively an average of the first stable measurements; $t_{o,ave}$) as the reference value for the Zave for the full dispersion. The LOG(Z/Zo) value is then the LOG to Z_{ave} at t_i divided by the reference value Z_{ave} at t_o or $t_{o,ave}$.
7. Plot LOG(I/Io) and LOG(Z/Zo) or Z_{ave} as function of time and the LOG(Z/Zo) versus LOG(I/Io) to compare the relative importance of the two parameters as needed. See example in Figure 4 below.
8. Evaluate the data, considering that different changes in LOG(I/Io) and LOG(Z/Zo) may occur depending on the dispersion behavior (see Figure below). For example, I/Io may increase due to accumulation of particles at the measurement position (ca. 3 mm above the bottom of the vial) or growth in particle size due to agglomeration/aggregation. In some case several processes are important at the same time.
9. If data allows, establish a regression curve for Time versus LOG(I/Io) to calculate the apparent 25% (LOG(I/Io) = -0.25), 50% (LOG(I/Io) = -0.5) and 75% (LOG(I/Io) = -0.75) sedimentation times (see Figure 5).

Comments on the use of DLS data

DLS is very suitable for size and stability analysis of particles in liquid dispersions. However, great care should be taken in interpretation of data; especially when the sample contains both μm- and small nm-size particles and particles with non-spherical morphologies, such as dispersed fibers.

For better accuracy in determinations of size-distributions, it is important to obtain true values of the optical properties and viscosity of the dispersion liquid.

References

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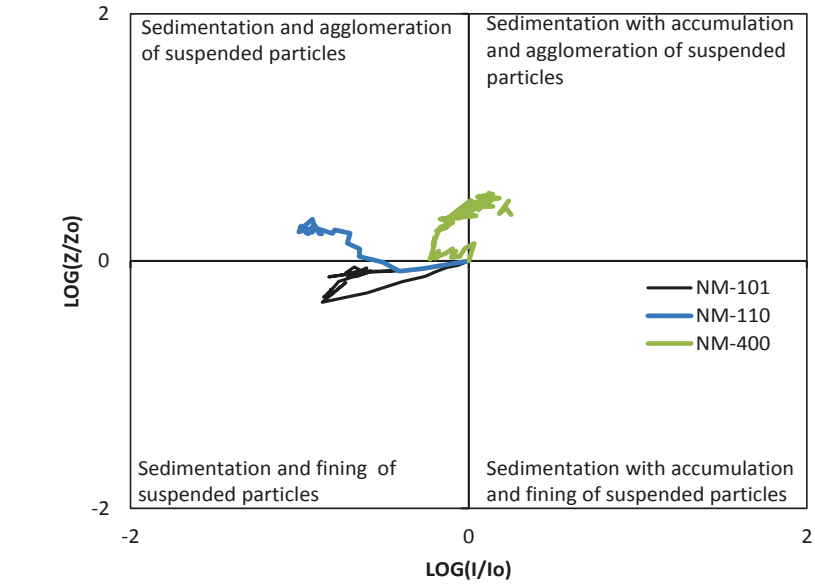

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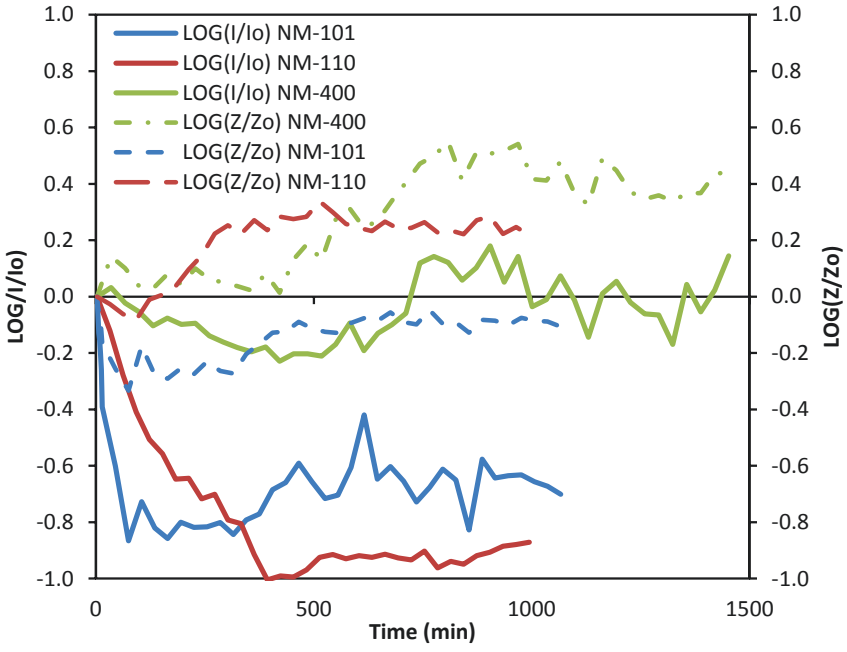

b)

Figure 4: A) Plot of LOG (Z/Zo) versus LOG (I/Io) temporal variations for three different MNM dispersions in a cell medium. The dispersions show different behaviour. B) Plot of the temporal evolution in LOG (I/Io) for the three dispersions in Figure A. The plots show very fast sedimentation and deposition of NM-101 and slower sedimentation of NM-110. Slot to moderate deposition occurs in the medium with NM-400 ending with accumulation of NM-400 close to the bottom at ca. 10 hours.

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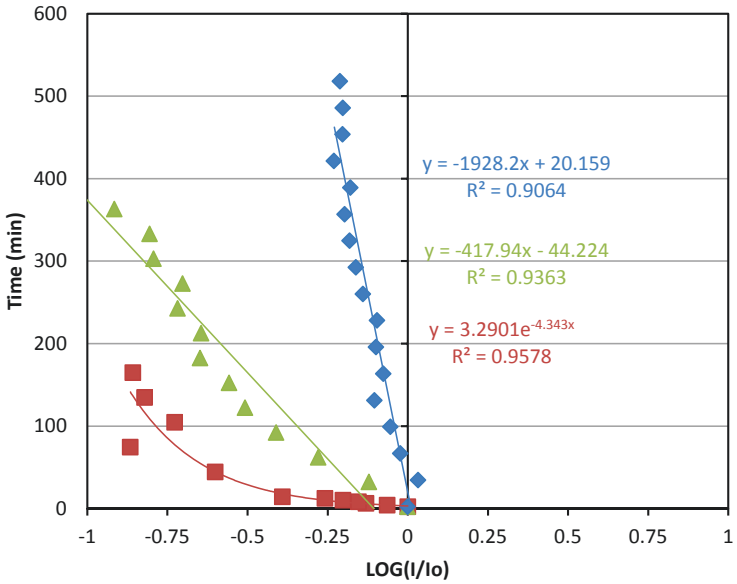

Figure 5: A) Plot of Time versus LOG (Z/Zo) for data assessment to establish regression curves for calculation of dispersed MNM sedimentation times. Data are only plotted in the time-frame during which there is a systematic decrease in LOG (I/Io) and sedimentation is predominant.

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