SCIENCE-BASED RISK GOVERNANCE
OF NANO-TECHNOLOGY

RISK GONE

Consolidated pre-validated guidance document on hydrodynamic diameter and size distribution determination

DELIVERABLE 4.2

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Abstract

The objective of Task 4.1 of RiskGONE project is to expand the scientific background for supporting the generation of guidance documents for the physico-chemical characterization of engineered nanoparticles (ENMs). Different techniques are used to characterize the physicochemical properties of ENMs and it is necessary to obtain consolidated standard operating protocols (SOPs) that can be later transferred to Risk Governance Council (RGC) and to regulatory agencies, such as OECD, for their implementation into official documents, recommendations and guidelines. For the characterization of the hydrodynamic diameter and the size distribution specifically, the already existing OECD test guideline (TG110 – Particle Size Distribution/Fibre Length and Diameter Distributions) has been not updated by modern techniques such as dynamic light scattering (DLS). RiskGONE also contributed to the ongoing international efforts under the MALTA initiative and NANOHARMONY, the Gov4Nano project and the OECD for the establishment of a guideline for the determination of size distribution of ENMs. The data contained in this deliverable are at least partially based on the experience and experimental results that are part of the WNT Project 1.4: Draft TG on Particle Size and Size Distribution of Manufactured Nanomaterials (PSD), which is supported by the MALTA initiative, NANOHARMONY and Gov4Nano. The goal of this deliverable is to provide a consolidated SOP for the determination of the hydrodynamic diameter and size distribution of ENMs in water and in biological media. The document resulted from the Round Robin (RR) exercises that were organized with RiskGONE partners involved in Task 4.1 partners to demonstrate the validity and the reproducibility of the proposed guidance document.

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TABLE OF CONTENTS

Document History 2
Abstract 3
TABLE OF CONTENTS 4
List of Abbreviations 5
1. Technical & Scientifical progress 6
1.1 Introduction 6
1.2 Principles of the method 6
1.3 Applicability and limitations 7
1.4 Materials 8
1.4.1 Reagents 8
1.4.2 Materials and Equipment 8
1.5 Procedure 8
1.7 Quality control and quality assurance 11
1.8 Safety warnings 11
2. Deviations from Description of Action – impact/how you cope with them 11
References 11

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List of Abbreviations

CLS - Centrifugal Liquid Sedimentation

DLS - Dynamic Light Scattering

DMEM - Dulbecco's Modified Eagle Medium

EDL - Electrical Double Layer

EM - Electron Microscopy

ENMs - Engineered Nanomaterials

PCS - Photon Correlation Spectroscopy

PDI - Polydispersity Index

SOP - Standard Operating Procedure

TG - Test Guideline

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1. Technical & Scientifical progress

1.1 Introduction

Several different methods can be used for measurements of particle size and size distribution of engineered nanomaterials (ENMs), such as electron microscopy (EM), centrifugal liquid sedimentation (CLS), dynamic light scattering (DLS) and others. Each method has each own size range of applicability and its owns advantages and limitations. From a regulatory point of view, the reference Guidance document for the measurement of the hydrodynamic diameter and the size distribution is the OECD TG110 [1], which was published in 1981. However, this TG is only adapted for large particles, typically larger than 1-2 um and it is not adapted for nanosized particles (below 100 nm). The technique described by the TG110 and also chosen by RiskGONE is the DLS (also known as photon correlation spectroscopy - PCS) that is very convenient for the characterization of the hydrodynamic diameter of nanomaterials [2]. Additionally, DLS is an easy and accessible method to characterize ENMs both in academia and industry, and several commercial instruments are available on the market and there exist ISO standard [3], even though was not considered in the TG 110.

The objective of this document is to provide a consolidated SOP for the characterization of the hydrodynamic diameter and the size distribution of ENMs using dynamic light scattering and showing the evidence that the proposed method is adapted and suitable for ENMs of different size, shape and composition.

1.2 Principles of the method

When a diluted particle dispersion is illuminated by a light source (e. g. laser), particles will scatter light in all directions. The scatterers can be anything with a refractive index different than the one of the medium into which the scatter is dispersed and with a stable behavior over the duration of the experiment. The scatterers are typically dispersed solid particles (e. g., metal oxides, latex particles) or soft particles (e. g., proteins and micelles). These particles present Brownian motion, i.e. movement that arises from random thermal motion of the medium's molecules, which causes fluctuations on the intensity of the scattered light that can be mathematically related to the diffusion coefficient of the particle (D_τ). When the movement of particles over time is monitored, information on the hydrodynamic size (R_h) of the particles can be obtained since large particles diffuse more slowly than small particles. The relationship between R_h of a particle and its D_τ is given by the Stokes-Einstein equation (equation 1).

$$D_\tau = \frac{k_B T}{6\pi\eta R_h} \qquad 1$$

where k_B is the Boltzmann coefficient (1.380 x 10^{-23} kg.m^2.s^{-2}.K^{-1}), T is an absolute temperature, and η is the viscosity of the medium [4,5].

In a DLS instrument, the fluctuations of the scattered light are recorded and analysed in correlation delay time domain. The motion of dispersed particles is described by an intensity autocorrelation function that can be expressed as an integral over the product of intensities at time t and delayed time ($t + \tau$) as presented by equation 2.

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

where τ is the delay time between two points, B is the baseline ($\sim$1), β is the coherence factor that depends on the detector area, optical alignment, and scattering properties of the particles, q is the Bragg wave vector, which is calculated as $q = \frac{4\pi n}{\lambda} \sin(\theta/2)$, where n is the medium refractive index, λ is the light wavelength, and θ is the scattering angle.

Modern instruments are supplied with software packages and computational tools that perform data analysis, using various different algorithms (e.g. cumulants, NNLS, CONTIN, etc.) to primarily evaluate hydrodynamic diameter (Z-average) and size distribution (given by the polydispersity index, PDI) of particles [4,5]. The Z-average size is the most important and stable output provided by the technique. The value of this parameter is the one that must be considered when providing data for quality control purposes.

1.3 Applicability and limitations

As described by the manufactures, the determination of hydrodynamic diameter by DLS is limited to spherical particles in the size range from 0.3 nm to 10 µm, depending on the instrument that is available, the nature of the ENMs analysed and of the dispersion medium.

ENMs must be dispersed in a liquid medium prior of the measurement. One assumption that is necessary while performing a DLS measurement is that dispersed particles are able to move freely in the dispersion medium, with the only restriction to their movement coming from the interaction with the dispersant molecules. The concentration of particles in the sample should be adjusted so that the scattering of the samples is much stronger than the scattering from the medium's molecules. The particle count rate should be between 10^4-10^6 s^{-1}. Too high concentration will cause multiple scattering and particle-particle interactions that can affect the diffusion coefficient, complicating the data analysis. On the other hand, too low concentrations will cause particle number fluctuations which invalidate the autocorrelation function, producing too weak scattering and then reducing the measurement efficiency. In principle, one should measure the autocorrelation function obtained from a dispersion as dilute as possible, if there is enough scattering.

The DLS method determines the *equivalent spherical hydrodynamic diameter*, which includes the electrical double layer (EDL) around the particle surface in solution and any other species linked to the particle surface. The electrical double layer's thickness will depend on the ionic strength of the dispersant. Application of DLS to non-spherical particles and high-aspect-ratio nanoparticles is not recommended because the evaluation of the data is based on a spherical approximation.

The DLS method is not capable of distinguish between primary particles and agglomerates and/or aggregates. If the measurement meets quality criteria a size (Z-average) will be reported regardless the identity of the particles.

The absorption of the incident light by the samples can lead to local heating that can affect particles movement, leading to changes in size determination. Hence, in order to be comparable, measurements are requested to carry out at the same temperature. On the other hand, it is always

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recommended to record a UV-Vis spectrum of the sample. If the sample absorbs at the incident wavelength, a different technique to measure the particle size should be used, as DLS would not be adequate in these conditions [4,5].

In summary, the comparison of the Z-average values measured to different samples it's allowed only if 1) the particle shape could be approximate to a sphere; 2) the particle size distribution is monomodal (i.e. only one peak), 3) the same dispersant is used, 4) measurements are carried out at the same temperature.

The polydispersity index is dimensionless value. For a Malvern instrument, for example, this value is scaled such that values smaller than 0.05 are rarely seen other than with highly monodisperse standards. Values greater than 0.7 indicate that the sample has a very broad size distribution and is probably not suitable to be analysed with the DLS technique. The various size distribution algorithms work with data that falls between these two extremes. The calculations for these parameters are described in the ISO standard documents 13321:1996 E and ISO 22412:2008.

Conversion of intensity-weighted distributions to number-weighted and volume-weighted distributions is possible, but not recommended because it can lead to some deviations.

Most of the equipment can provide reports about the quality of the measurements (quality criteria). In general, the measurement doesn't reach the desired quality if the samples precipitate, agglomerate or aggregate during the measurement.

1.4 Materials

1.4.1 Reagents

- Stock solutions of ENMs selected within the RiskGONE project.
- Ultrapure water (resistivity <18.8 MΩ.cm, for example, Millipore or Sigma-Aldrich) or other dispersant of choice (such as DMEM + 10% vol/vol FBS, PBS, etc).

1.4.2 Materials and Equipment

- ZetaSizer Nano ZS (Malvern Instruments) or similar equipment
- Disposable measurement cells (e.g., disposable polystyrene cells, minimum volume 1 mL)
- 1 mL syringe
- 0.22 µm Nylon filters
- Pipette (to transfer variable volumes - from 100 µL to 1 mL)
- Laboratory vortex mixer, with speed range 300-3500 rpm, touch mode

1.5 Procedure

1. Turn on the equipment 30 minutes before use to warm up the laser and start the software.
2. Preparation of the sample:

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a. From stock solution, disperse the sample in filtered ultrapure water at 100 μg/mL, vortex for 30 seconds. The dispersant must be filtered using a nylon filter 0.22 μm before use. Never use the first few drops from the syringe. In case there are any residual dust particles in the filter that may contaminate the dispersant.

Comment 1: To know how to prepare stock solution, please check the ***"Consolidated pre-validated guidance document on the dispersability of ENMs"*** published by the RiskGONE project.

Comment 2: Please, be aware if the sample suffers any sedimentation, agglomeration or aggregation in the time between preparation and measurement. If so, the sample is not suitable for DLS size determination.

Comment 3: The optimum concentration of the sample is determined by the optical light scattering) properties of the ENMs, and it has to be defined on a case basis[4]. As a starting step, use a concentration equal to 100 μg/mL and check if the outputs meet the quality criteria established for your instrument (see "Data report" section below). If not, adjust the concentration according to the recommendations written on the user manual of your equipment.

b. Cleaning: rinse the disposable cell three times with the filtered dispersant (through 0.22 mm nylon filter) and then store it in a dust-free environment prior to use.

c. Loading the sample: Load 1 mL of sample in the cell. The cell should be filled slowly to avoid air bubbles from being created. Cap the cuvette to ensure greater sample thermal stability -and to prevent dust introduction. Check there are no bubbles on the optical window area. Clean the external walls of the cuvette using a precision wipe. Never use disposable cuvettes more than once since they are easily scratched leading to measurement errors.

d. Put the cell loaded with the sample in the cell holder.

3. Measurement

a. Equilibrate the temperature of the sample prior the measurement for at least 2 minutes (rate = 1°C/min). If needed, let it equilibrate for longer time. Perform 3 measurements per sample at 25ºC to gain measurement repeatability. Follow instrument manufacturer's recommendations to fix the measurement duration.

b. If possible, create a p internal protocol to your instrument with the tools available from the software. Use the parameters given in **Error! Reference source not found.** to create it. Additional information will be further required specific for each ENM and dispersants.

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Table 1. Internal protocol for DLS measurements using a ZetaSizer Nano ZS (Malvern Instruments).

Parameter	Value
Equilibration time	120 s (1°C/min)
Scattering angle	173°
Laser wavelength	633 nm
Number of measurements	3
Number of runs	Automatic
Run duration	Automatic
Delay between measurements	0 s
Positioning method	Seek for optimum position
Extend duration for large particles	No
Automatic attenuation selection	Yes
Analysis mode	General Purpose

4. Data report
    a. Check if the measurement meets quality criteria for the ZetaSizer Nano ZS equipment:
        i. The count rate is useful for monitoring the sample quality. The scattering intensity should be within the acceptable range. The mean counting rate of the scattering light reported during the measurement should be between 100 and 500 k counts per second (kcps). The count rate plot should be stable over time. The presence of dust, agglomeration or sedimentation cause great variations in the count rate plot. The instrument attenuator should be between 4 and 9.
        ii. Check the autocorrelation function (defined by equation 2 and given by the equipment). The value of the intercept should be around 1 (>0.9)[6].
    b. In order to perform the analysis, the viscosity and the refractive index of the liquid phase for the cumulants method should be known. The refractive index of ENMs is also required to obtain the volume and the number-based particle size distribution. If the viscosity of the media is not included in the database of the instrument's software, it is possible to measure it using inexpensive glass U-tube viscometers or more expensive instruments such as rotational and falling-ball viscometers [7]. Be sure to use an instrument capable of measuring in the 0.5 and 2.0 cSt range; most

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dispersants have viscosities between 0.60 and 1.25 cSt (e. g. water viscosity is 0.890 at 25°C).

c. The following parameters should be recorded: Z-average value (also referred as cumulants mean), polydispersity index (PDI), intensity-weighed particle size distribution, % of the peak area and standard deviations.

1.7 Quality control and quality assurance

Although no calibration is required to DLS measurements, the performance of the instrument should be verified by using a standard quality control. According to ISO 22412 [3], polystyrene latex particles with narrow size distribution and average diameter (as measured by DLS) in the size range of 60-200 nm can be used. The measured average diameter (Z-average size) of the latex sample should be within 2% of the stated size range and the polydispersity index should be less than 0.1. Use the protocol reported in Table 1 to perform the instrument validation. In addition, it is important to check if all the measurements are carried out under operational qualification of the instrument. Please, check the quality report given by the equipment for each measurement.

1.8 Safety warnings

To minimize the human exposure to the ENMs, handle the samples with care. Use appropriate protective gear, such as lab coat, gloves, googles and masks. Further information on handling the specific ENMs and the safe handling of the used equipment is described in the material's data sheets and user manuals developed by the manufacturers, respectively.

2. Deviations from Description of Action – impact/how you cope with them

No major deviation to report until now.

References

[1] OECD. OECD TG110-Particle Size Distribution/Fibre Length and Diameter Distributions 1981.

[2] Varenne F, Botton J, Merlet C, Beck-Broichsitter M, Legrand FX, Vauthier C. Standardization and validation of a protocol of size measurements by dynamic light scattering for monodispersed stable nanomaterial characterization. Colloid Surf A 2015;486:124–38. https://doi.org/10.1016/j.colsurfa.2015.08.043.

[3] ISO. ISO 22412:2017. Particle size analysis — Dynamic light scattering (DLS) 2017.

[4] Xu R. Particle characterization: Light scattering methods. Particle Technology Series. Kluwer Academic Publishers; 2002.

[5] Stetefeld J, McKenna SA, Patel TR. Dynamic light scattering: a practical guide and applications in biomedical sciences. Biophys Rev 2016:409–27. https://doi.org/10.1007/s12551-016-0218-6.

[6] Calzolai L. Measuring Batch Mode DLS (EUNCL-PCC-01) 2016.

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[7] DeLoid GM, Cohen JM, Pyrgiotakis G, Demokritou P. Preparation, characterization, and in vitro dosimetry of dispersed, engineered nanomaterials. Nat Protoc 2017;12:355–71. https://doi.org/10.1038/nprot.2016.172.

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