SCIENCE-BASED RISK GOVERNANCE
OF NANO-TECHNOLOGY

RISK GONE

Consolidated pre-validated guidance document on nanoparticle counting

DELIVERABLE 4.6

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0.1	19-01-2022	Sebastien Cambier (LIST)	First Draft sent to WPL and collaborators involved in the task	Sebastien Cambier (LIST)
0.2	24-01-2022	Elisa Moschini (LIST)	Second Draft sent to PMB and EAB	Elisa Moschini (LIST)
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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 procedures (SOPs) that can be later transferred to regulatory agencies, such as OECD, for their implementation into official documents, recommendations and guidelines. For the specific determination of the nanoparticle number concentration, an ISO technical report providing guidance on the measurement of number concentration (ISO, TR 24672 Nanotechnologies - Guidance on the measurement of nanoparticle number concentration) is under development in the ISO TC 229, the technical committee on nanotechnologies. RiskGONE also contributed to the ongoing international efforts under the MALTA initiative, NANOHARMONY and Gov4Nano projects for the establishment of a guideline for the determination of number concentration of ENMs. The data contained in this deliverable is at least partially based on the experience and experimental results gained within 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 number concentration of ENMs in water-based media. The document resulted from the first Round Robin (RR) exercise organized with the RiskGONE partners involved in WP4 Task 4.1 to demonstrate the validity and the reproducibility of the proposed guidance document.

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List of Abbreviations

SAXS – X-ray scattering

DLS – dynamic light scattering

CLS – centrifugal liquid sedimentation

spICP-MS – single particle inductively coupled plasma mass spectrometry

PTA – particle tracking analysis

DMAS – differential mobility analysing system

CPC – condensation particle counter

RPS – resistive pulse sensing

ISO– international organization for standardization

ENMs – engineered nanomaterials

SOP – standard operating procedure

UV-Vis – ultraviolet and visible light

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1. Technical & Scientifical progress

1.1 Introduction

Several different methods can be used for the measurement of the particle number concentration such as the small angle X-ray scattering (SAXS) [1], the dynamic light scattering (DLS) [2, 3] and the centrifugal liquid sedimentation (CLS) [4, 5]. Other techniques relying on particle-by-particle analysis are also available such as the single particle inductively coupled plasma mass spectrometry (spICP-MS) [6], the particle tracking analysis (PTA) [7], the differential mobility analysis system (DMAS) with integrated condensation particle counter (CPC) [8], the resistive pulse sensing (RPS) [9], etc. Each method has its own protocol describing how to perform both particle size analysis and particle number concentration determination besides to list advantages and limitations of the technique.

From a regulatory point of view, the reference Guidance Document for the measurement of the particle number concentration is the ISO, TR 24672 [10], which is still under development. For the purposes of RiskGONE project the selected technique has been the PTA (also known as Particle Tracking Analysis) that is also very convenient for the characterization of the hydrodynamic diameter of nanomaterials [11]. Additionally, PTA is an easy and accessible method to characterize engineered nanomaterials (ENMs) both in academia and industry, and several commercial instruments are available on the market.

The objectives of this document are providing a consolidated standard operating procedure (SOP) for the determination of the particle number concentration of ENMs using the particle tracking analysis and showing the evidence that the proposed method is suitable for ENMs of different size, shape and composition.

1.2 Principle of the method

When a diluted particle dispersion is illuminated by a light source (e. g. laser), particles will scatter light in all the directions. The scatterers can be anything behaving as localized non-uniformities with a refractive index different than the one of the media into which thy are 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 a Brownian motion (i. e. movement that arises from random thermal motion of the medium's molecules), which causes fluctuations of the intensity of the scattered light that can be mathematically related to the diffusion coefficient of the particles (D_τ). When the movement of the particles over time is monitored, information on the hydrodynamic size (R_h) of the particles can be derived since large particles diffuse more slowly rather than small particles. The relationship between R_h of a particle and D_τ is given by the Stokes-Einstein equation (equation Eq.1).

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

where k_B is the Boltzmann coefficient (1.380 x 10^{-23} kg.m^2.s^{-2}.K^{-1}), *T* is the absolute temperature and η is the viscosity of the medium [12, 13].

In a PTA instrument, the measurement is based on measuring the diffusion movement of particles in a suspension by using laser illumination, thus allowing particle identification and localization by imaging

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of the scattered light. The light scattered by the particles is then recorded with a light sensitive CCD or CMOS camera. This camera is arranged at a 90° angle to the irradiation plane allowing the detection and tracking of the Brownian motion of particles with size ranging between 10 and 1000 nm. This makes possible the tracking of the single particles identified during a certain amount of time thus allowing simultaneously the determination of both size distribution and concentration of those particles. Using the PTA, the fluctuation of every single particle is registered in two dimensions. This two-dimensional mean square displacement is then combined with the Stokes-Einstein relationship to determine the particle diameter d through the following equation:

$$d = \frac{4k_B T}{3\pi\eta t} \cdot \frac{4t}{\langle x, y \rangle^2} = \frac{16k_B T}{3\pi\eta \langle x, y \rangle^2} \qquad \text{Eq.2}$$

Particle tracking analyzers are supplied with software packages and computational tools performing data analysis to primarily evaluate hydrodynamic diameter (Z-average) and size distribution of the particles. In addition to the Z-average size parameter, a second output is provided by the technique. This second output is the particle concentration.

1.3 Applicability and limitations

As described by the manufactures, the use of PTA is limited to particles ranging from 10 nm to 1 µm, depending on the instrument, on the available software, on the nature of the ENMs analysed and of the dispersion medium.

ENMs must be dispersed in a liquid medium prior of the measurement. The PTA technique assumes that while performing the measurement, the particles can move freely in the dispersion media, with the only restriction to their movement coming from the interactions with the dispersant molecules. The concentration of particles in the sample should be adjusted, therefore the number of particles by frame will allow a good acquisition. The particle count rate should be between 10^6-10^9 particles by frame. In one hand too high concentrations are known to cause too much scattering and will prevent the software from working properly as it won't be possible to distinguish individual particles from each other. On the other hand, too low concentrations don't contain enough particles to ensure high accuracy and efficiency of the measurement. The PTA method does not allow to distinguish between primary particles and agglomerates and/or aggregates. If the measurement meets quality criteria the size (as Z-average) and the particle number concentration will be reported regardless the identity of the particles.

Another important parameter to check while performing the measurement is the temperature. The emission of the laser on the sample over time could lead to an increase of temperature inside the chamber that may affect the particle movement, thus leading to changes during the measurements even if the temperature is monitored during the acquisitions. Hence, measurements are requested to be carried out at the same temperature in order to be comparable and it's always recommended to record a UV-Vis spectrum of the sample.

In conclusion, the determination of the particle concentration from different samples is allowed and reliable only if the following conditions are valid:

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1) the particles are able to stay in suspension

2) the particle concentration in the analysed suspensions is between 10^6-10^9 particles by frame

3) the same dispersant is used

4) measurements are carried out at the same temperature

Most of the available instruments can provide quality reports about the analyses performed. In general, quality criteria are not met if particle agglomeration, aggregation or precipitation, during the measurement is observed.

1.4 Materials

1.4.1 Reagents

- Stock suspensions of the ENMs selected within the RiskGONE project (prepared by following the provided SOP ***"Consolidated pre-validated guidance document on the dispersibility of ENMs"*** (D4.3) already submitted by the RiskGONE project).
- Filtered ultrapure water (resistivity <18.8 MΩ.cm, for example, Millipore or Sigma-Aldrich) or other continuous phase dispersant of choice such as 70% ethanol (avoid cell culture media due to strong interference).

1.4.2 Materials and Equipment

- NanoSight (Malvern Instruments) or similar equipment
- Eppendorf tubes (minimum volume 1.5 mL)
- Pipette and tips (to transfer variable volumes - from 100 µL to 1 mL)
- 0.22 µm Nylon filters
- Laboratory vortex mixer with speed range 300-3500 rpm, touch mode

1.5 Procedure

1. Turn on the equipment (NanoSight & Computer) 30 minutes before use to warm up the laser and start the software.
2. Clean the instrument's flow cell and tubing with ultrapure water prior measurement to ensure minimum background particle counts. Check that the measurement cell is clean.
3. Sample preparation:
    a. Dilute the ENM stock suspensions in filtered ultrapure water at the concentration of 100 □g/mL and vortex for 30 seconds.

Comment 1: The dispersant must be filtered using a 0.22 µm nylon filter before use. Never use the first few drops from the syringe since they might contain residual dust particles coming from the filter which may contaminate the dispersant.

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Comment 2: check the sample for sedimentation, agglomeration or aggregation occurring between the preparation and the measurement steps. If any of these phenomena is observed the sample is not suitable for PTA.

Comment 3: The optimum concentration of the sample for the recording is determined by the number of particles visible in the frame during the acquisition, and it must be defined on a case-by-case basis. As starting step use a concentration equal to 100 □g/mL and check if the outputs meet the established quality criteria (see "Data report" section below 5.a). If not, adjust the concentration according to the recommendations written on the user manual of your equipment.

b. Prime the NanoSight: preload the system with filtered ultrapure water accordingly to the recommendations written on the user manual of your equipment while being very careful of not having any air bubbles in the system.

c. Loading the sample: load 1 mL of sample in the 1.5 mL Eppendorf tube. The Eppendorf tube should be filled slowly to avoid air bubbles from being created. Check if there are no bubbles.

d. Place the tube on the sample holder following the instrument guidelines in order to have a proper loading of the sample.

4. Measurement

a. Open and run NTA3.2 and select "Start Camera". In the meantime, ensure that the temperature for your system is being read appropriately. Then move to the desired 'Scatter' position for recording. It is extremely important to record all videos at the same position to achieve the most accurate results acquisition.

b. Check that the system and the filtered ultrapure water are clean. For this purpose, select camera level "16" and if more than 3 particles in the field of view are visible clean again the system according to the recommendations written on the user manual and replace the filtered ultrapure water.

c. Load the sample according to the recommendations written on the user manual.

d. Adjust the camera settings, "level" and "focus". Ensure that all the particles inside the sample are still visible. Ensure that the camera settings allow to visualise all the particles present in the sample. Too high values for the camera parameter "level" can lead to unwanted scatter and this is signalled by a red pseudocolor dot in the middle of the particle. Ideally, no more than 10-15% of the particles on the screen should be red in the centre. Adjust the parameter "focus" by using the FOCUS slider available in the 'Hardware' tab in order to improve the visualization of the particles on the whole screen and to get sharp images (see below examples of different quality "focus" – middle image, ideal; left, not good; right, very bad).

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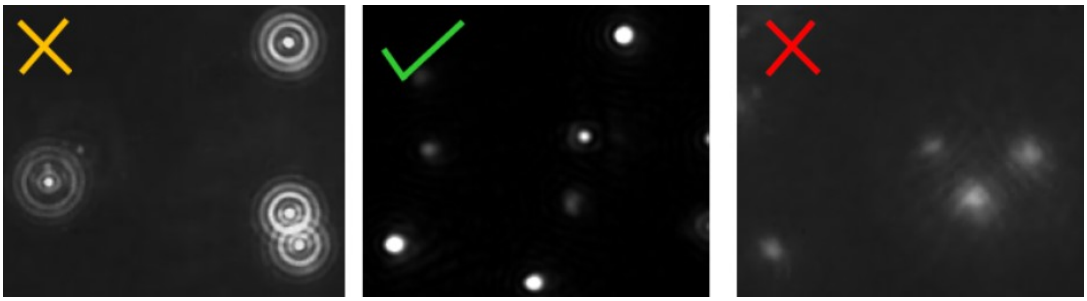

e. Create a measurement protocol: make sure that the Number of Captures = 3 and that the Capture Duration = 60 sec. Make sure that the 'Process After Capture' box is not selected. Then start the measurement. A full measurement protocol is reported in **Error! Reference source not found.**. Additional information will be further required specifically for each ENMs and dispersants.

f. When all the samples are measured switch off the instrument according to the recommendations written on the user manual.

Table 1. Internal protocol for PTA - particle concentration measurement using a NanoSight NS500 (Malvern Instruments).

Parameter	Script
Equilibration of the temperature	SETTEMP 25
Start of the camera interface	CAMERASETTINGSMSG
Start the loop of acquisition	REPEATSTART
Pump a little volume	PUMPADV
5 sec stabilization	DELAY 5
60 sec of recording	CAPTURE 60
1 sec break	DELAY 1
Restart the loop 2 times	REPEAT 2
Switch off temperature control	TEMPERATURECONTROLOFF
Start the data processing	PROCESSSINGLESETTING
Open the export window	EXPORTRESULTS

5. Data report

a. Check if the measurement meets quality criteria for the NanoSight equipment:

i. The count rate is in the right range.

ii. The scattering intensity of the particles during the acquisition remains stable.

iii. The particles are mainly remaining sharp during the 60 sec acquisition and within the 3 acquisitions.

iv. Absence of a movement of all particles going in the same direction.

b. Data analysis: load in the files corresponding to the sample. Select the 3 recordings and process the files with the correct settings for the "Detection Threshold". The

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detection threshold value should allow to select as many particles as possible, within the following provisions:

i the number of Particles in the Frame should be below 200.

ii be careful that the red crosses in the image are not identifying real particles but only fake signals. The number of red crosses should be lower than 10 during the analysis of the record.

iii the selected detection threshold should be between 2 and 20.

c. The following parameters should be reported: Mean and Mode values of the hydrodynamic diameter, SD (corresponding to the measure of the width (spread) of the particle size distribution), D10, D50, D90 (as intercepts for 10%, 50% and 90% of the cumulative mass and concentration reported as Particle/mL or Particles/frame).

1.7 Quality control and quality assurance

Although no calibration is specifically required to perform PTA measurements, the performance of the instrument should be verified by using a standard quality control helping to ensure intra and interlaboratory data comparability. Unfortunately, no standards for particle concentration calibration using the described method are available.

To perform the instrument validation for the purposes of this project use the protocol reported in Table 1. In addition, it is important to check if all the measurements are carried out under the operational qualification of the instrument. Thus, verify the quality report given by the instrument after 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 safe handling of the selected ENMs and the used equipment is described in the material's data sheets and in the 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.

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