SCIENCE-BASED RISK GOVERNANCE
OF NANO-TECHNOLOGY

RISK GONE

Consolidated pre-validated guidance document on the dispersibility of ENMs

DELIVERABLE 4.3

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Abstract

The objective of Task 4.1 of RiskGONE project is to provide guidance documents for the techniques used to characterize the physicochemical properties of engineered nanomaterials (ENMs), that will be later available to regulatory agencies, such as OECD. The goal of this deliverable is to provide a consolidated pre-validated guidance document on the dispersibility of of the applied ENMs in the RiskGONE project. Round Robin (RR) exercises were organized with task partners to demonstrate the validity and reproducibility of the proposed guidance document.

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List of abbreviations

DLS - dynamic light scattering

DSE(cr) – (critical) delivered sonication energy

EDL - electrical double layer

ENMs - engineered nanomaterials

GDs - guidance documents

OECD - Organization for Economic Co-operation and Development

RR – Round Robin

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1. Technical & Scientifical progress

1.1 Introduction

ENMs suspended in liquids may form agglomerates. Not only the extent of agglomerate formation, and the size distribution and stability of agglomerates, are determined by the intrinsic properties of the primary ENM particles (i.e., composition, size, shape, surface chemistry) and the extrinsic properties of the fluid (i.e., ionic strength, pH, protein and other biomolecular types, and content) (Pyrgiotakis, 2013) but the degree of agglomeration and the stability of the resulting size distributions are also highly dependent on the methods used to disperse the ENMs in the liquid (Cohen, 2013). Agglomeration reduces the total number of particles, as well as the total surface area of the suspended ENM available for interaction with cells. So, a reproducible protocol for achieving well-dispersed ENM dispersions is a primary requirement for obtaining comparable toxicity data.

A reproducible dispersion preparation includes two key components: (i) calorimetric calibration of the sonication equipment for accurate knowing the delivered sonication energy (DSE), and (ii) determination of the material-specific critical delivered sonication energy (DSE_{cr}) required to achieve a suspension with the smallest possible agglomerates that are minimally polydisperse and maximally stable over time (24h). To generate dispersions in media for use in experiments, the material is first sonicated in deionized water with a delivered sonication energy (J/mL) equal to DSE_{cr}, and subsequently diluted in cell culture media to the desired initial concentration for application to cells.

1.2 Principles of the method

During the process of sonication, sound waves propagate through the liquid medium in alternating high- and low-pressure cycles at frequencies in the 20–40 kHz range. Microscopic vapor bubbles are formed (low-pression cycle) in a process known as cavitation. The bubbles then collapse during the high-pressure cycle (compression) producing a localized shock wave that releases tremendous mechanical and thermal energy. In direct sonication, the probe is in contact with the suspension, reducing the physical barriers to wave propagation and therefore delivering a higher effective energy output into the suspension. For direct sonication, the following equation relates acoustic vibrational energy to probe and medium parameters (Taurozzi et al. 2011):

$$P = \frac{1}{2}\rho c A^2 (2\pi f)^2 a$$

where **P** is the acoustic power (W) of the ultrasound source, **a** is the 'emission area' (m^2), which is the surface area of the emitting ultrasound source, **r** is the liquid density (kg m^{-3}), **A** is the amplitude (m) of oscillation of the ultrasound probe, **c** is the speed of the acoustic wave in the liquid medium (m s^{-1}) and **f** is the vibration frequency (Hz).

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The net fragmentation effect from applying ultrasonic energy to a suspension is dependent on the total amount of energy delivered to the sonicated medium. However, not all the produced cavitational energy is effectively utilized in disrupting particle clusters. The delivered energy is consumed or dissipated by several mechanisms, including thermal losses, ultrasonic degassing, and chemical reactions, such as the formation of radical species. Only a portion of the delivered energy is used in breaking particle-particle bonds to produce smaller particle aggregates, agglomerates, and primary particles. Moreover, an excessive energy input can potentially result in agglomerate formation or re-agglomeration of previously fragmented clusters.

A critical parameter for the reproducible preparation of sonicated ENM suspensions is the delivered sonication power. As previously mentioned, only a portion of the total output power is effectively consumed in particle disruption. The efficiency of the energy received by the sonicated suspension is heavily instrument dependent. Because of this, the sonication equipment must be calibrated before use to ensure that the exact delivered sonication energy to ENMs is known and reported for any experiment. For this purpose, the calorimetric method has been proposed to standardize sonochemistry studies and used to calculate the amount of acoustic energy delivered to a liquid medium subject to direct sonication. This will also ensure that sonicators from different manufacturers or models can be used to deliver the specific sonication energy of interest.

The delivered acoustic power P (J/s) of the sonicator probe is calculated as:

$$P = \frac{dT}{dt} MC_p$$

Where **dT/dt** = slope of temperature (K) vs. time (s), **M** = mass of water (g), and $\mathbf{C_p}$ = specific heat capacity of water (4.186 J/g°K)

Once the probe sonicator is calibrated the determination of the energy DSE to a suspension can easily be calculated by measuring the increase of temperature at increasing sonication times (different intervals). DSE is expressed in Watts/mL (*DSE = P x t/V)* where **P** is the delivered acoustic power determined in the calibration step, **t** is time in seconds, and **V** is the volume of the suspension in milliliters.

DSE value allows the reproducibility of the sonication conditions between different systems and can be used to calculate the **material-specific $\mathbf{DSE_{cr}}$**. DSE_{cr} represents the minimum energy that is needed to obtain the smallest stable particle dispersion in the given medium solution. A dispersion is considered stable when the hydrodynamic diameter does not change significantly in the 24 hours following the sonication (DeLoid GM, 2017). Thus, the DSE_{cr} (J/mL) for a specific ENM can be calculated by plotting cumulative DSE (*x* axis) vs. mean hydrodynamic diameter (measured using dynamic light scattering (DLS) or other size characterization) (*y* axis) as the cumulative DSE at which further sonication does not further reduce the mean hydrodynamic diameter by more than 5% (slope approaches zero).

Finally, given the DSE_{cr} for a specific ENM, knowing the P (acoustic power) of a generic sonicator, it is possible to calculate the sonication time for a known volume of ENM dispersion, using the expression described below:

$$\text{Sonication time (s)} = (DSE_{cr}/P) \times \text{Dispersion volume (mL)}$$

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1.3 Applicability and limitations

As the hydrodynamic sizing of the ENM dispersions is carried out by DLS, the applicability of this protocol is limited to particles in the size range from 0.3 nm to 10 µm, depending on the available instrument.

One assumption in DLS measurement of a dilute colloidal dispersion is that particles move freely, the only restriction coming from the dispersant molecules. The concentration of sample should be adjusted so that the scattering of the samples is much stronger than the scattering from the medium's molecules. The 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 measurement efficiency. In principle, one should measure the autocorrelation function from 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 finds quality criteria, a size (Z-average) will be reported regardless the identity of particles.

The Z-average value only will be comparable with other techniques if the sample is monomodal (i.e. only one peak), spherical and monodisperse (i.e. no width to the distribution), and using the same dispersant. In addition, the Z-average size can only be used to compare results with samples measured in the same dispersant, using the same technique. 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 for the DLS technique. The various size distribution algorithms work with data that falls between these two extremes. The calculations for these parameters are defined in the ISO standard document 13321:1996 E and ISO 22412:2008.

1.4 Materials

1.4.1 Reagents

- ENM dispersions of RiskGONE project
- Ultrapure water (for example, Invitrogen)

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1.4.2 Materials and Equipment

- Spatula
- Pipettes
- High precision laboratory scale or analytical balance
- Probe sonicator (e.g., 3 mm diameter)
- Digital thermometer with measurement accuracy better than ±0.1°C or a thermometer associated with the sonicator
- Small 3-prong dual adjust clamp
- Sound enclosure for sonicator set up
- 15 mL and 50 mL conical polypropylene or polystyrene centrifuge tubes
- Laboratory vortex mixer, with speed range 300-3500 rpm, touch mode
- All materials required for measuring particle size using dynamic light scattering or other technique (for example, disposable cells and pipettes).

1.5 Procedure

DeLoid' protocol with small modifications has been followed (DeLoid GM, 2017)

1.5.1 Sonicator calorimetric calibration

1.- Fill a 50-mL Falcon tube with 40 mL distilled water.

2.- Place it and secure it on the table of the sonicator with a three-pronged dual adjust clamp checking that the probe will be centred and immerse in the water for 1/3 of the water level (from the top).

3.- Insert a thermometer probe into the water fixing it with a small 3-prong dual adjust clamp.

4.- Turn the sonicator power on in continuous mode at 100% amplitude. In the case of very powerful sonicators, adjust the amplitude (e.g., to 50%) in order to avoid too fast temperature rise.
Remember that the selected settings should be used while generating the future stock NP suspensions.

5.- Record the temperature every 30 s until the temperature stabilizes (~30–50 min), and then turn the sonicator power off.

6.- Repeat the process (from Step 1 to 5) two other times in order to obtain three measurements.

7.- Calculating the acoustic power P (W = J/s) provided by the sonicator based on the following formula: P = (dT/dt) M x Cp where dT/dt is the slope of temperature (°C) vs. time (s), M is the mass of water (40 g for 40 mL deionized water), and Cp is the specific heat of water (4.186 J/g °C)

8.- The calibration curve is linear over the first 10 min of sonication, which is the time point used to make the DSE calculation. For example, for the CSIC´s 3 mm probe sonicator, the calculated P = ACOUSTIC POWER is equal to 9.6 W (J/s) for 40 mL of water using the sonicator with 100% amplitude in continuous mode.

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1.5.2 Determination of DSE_{cr} and optimal sonication time for ENMs dispersions

1.- Prepare 1 mg/mL dispersions from the ENM and vortex the suspension at high speed for 30 s. Prior to sonication, take 100 µL dilute them at 100 µg/mL in ultrapure water. This will constitute the t_0 of the mean of the hydrodynamic diameter.

2.- Sonicate the 1 mg/mL ENM dispersion for increasing time using 100% of power and maximum amplitude (or the same power settings used during calibration).

3.- Vortex 30 s high speed and take 100 µL, thus dilute them at 100 µg/mL in ultrapure water.

4.- Measure the hydrodynamic diameter on the 100 µg/mL dispersion using DLS. Monitor the evolution of the hydrodynamic diameter of the dispersed particles over time (typically every 1-2 minutes) along 10 minutes (linearity range of the calibration curve) repeating steps, from 2 to 4.

5.- Repeat steps 2 and 4 until the mean hydrodynamic diameter does not further decreases by more than 5% between steps.

6.- Plot cumulative DSE (x axis) vs. mean hydrodynamic diameter (y axis).

7.- Identify the DSE_{cr} (J/mL) as the cumulative DSE at which further sonication does not further reduce the mean hydrodynamic diameter by more than 5% (slope approaches zero).

8.- Repeat hydrodynamic diameter measurement after 24 hours. (First mix the suspension either by high speed vortexing or pipetting up and down several time).

9.- Determine the critical DSE (DSE_{cr}) required to achieve a suspension with the smallest as possible agglomerates that are minimally polydisperse and maximally stable over the time (24 h).

10.- Optimal sonication time calculus for delivering the DSE_{cr}

$$t_{cr} = (DSE_{cr} / P) \times V$$

where "$\mathbf{t_{cr}}$" is the sonication time, $\mathbf{DSE_{cr}}$ is the Critical Delivered Sonication Energy, "**P**" is the acoustic power proper of your sonicator (as calculated using the equation shown in the sonicator calorimetric calibration) and "**V**" is the volume in mL of your ENM dispersion.

It must be noted that the solid bridges that hold the primary particles together in the dry powder aggregates are often too strong to be broken via sonication or other moderate dispersion processes. Therefore, in some cases, it can be unrealistic to expect sonicated powders to break down to their reported, nominal primary particle size. As a result, powders will be effectively de-agglomerated to aggregates of several primary particles (i.e., 'primary aggregates'), rather than isolated primary particles. These aggregates should be thought of as the effective primary size for the ENM (Taurozzi et al. 2011).

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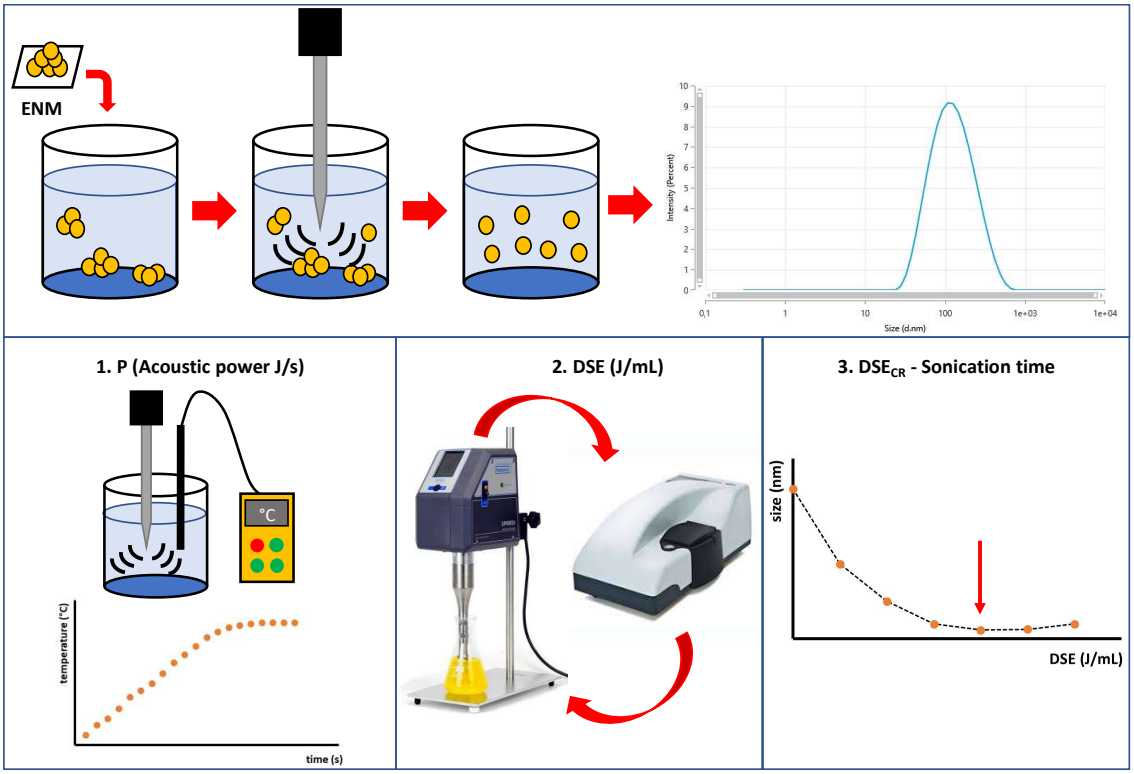
Scheme 1: Procedure for obtaining a well-dispersed ENM solution



1.6 Quality control and quality assurance

Although no calibration is required to DLS measurements, the instrument should be verified by using a quality control standard. According to ISO 22412 (ISO, 2017), a polystyrene latex 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. In addition, it is important to check if all the measurements are carried out under operational qualification of the instrument.

1.7 Safety warnings

Samples must be carefully handled to minimize exposure to the nanomaterial. Use appropriate protective gear, such as lab coat, gloves, googles and masks. Further information on handling the nanomaterials and the safe handling of the used equipment are described in materials data sheet and user manuals developed by manufacturers, respectively. After the measurements, please dispose of the dispersions in a suitable container for nanomaterials.

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

No major deviation to report until now.

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3. Conclusion and next steps

Nanoparticles will typically destabilize and form agglomerates when introduced to test media, due to charge screening by electrolytes or interaction with medium components. Particle agglomeration upon introduction to relevant media complicates both the determination of the delivered nanoparticle dose and the discrimination of size-specific particle effects, since the target (cell or animal model) is simultaneously exposed to both nanoscale particles and microscale agglomerates of the test material. While a wide variety of agents have been studied for nanoparticle stabilization in biological media, proteins are an especially attractive option due to their biocompatibility and the fact that proteins can mimic the actual corona coating that particles spontaneously acquire when introduced to biological matrices (Taurozzi et al. 2013). So on, once ENMs dispersions produced by sonication in sterile ultrapure water are optimized, a post-sonication step could be added to the procedure, by the introduction of proteins as the stabilizing agent for finally preparation of dispersions in the relevant test media by direct introduction of the protein stabilized ENM aqueous concentrate.

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