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RISK GONE

Consolidated pre-validated guidance document on effective density

DELIVERABLE 4.7

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Abstract

The major obstacle to the development of cost-effective toxicological screening methods for engineered materials (ENMs) is the need for accurate in vitro dosimetry, which relies on the effective density and diameter of formed agglomerates in cell culture media. The objective of this deliverable is to provide a consolidated pre-validated guidance document for effective density measurements of the applied ENMs during in vitro experiments. To verify the applicability of the proposed guidance document, two Round Robin (RR) exercises were organized with task partners (results available on ANNEX I).

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List of Abbreviations

AUC - analytical ultracentrifugation

CNTs - carbon nanotubes

DLS - Dynamic Light Scattering

DMEM - Dulbecco's Modified Eagle Medium

DoA - Description of Action

DSE - delivered sonication energy

ENMs - Engineered Nanomaterials

FBS - fetal bovine serum

HEPA - High Efficiency Particulate Air

ICP-MS - inductively coupled plasma mass spectrometry

JRC – Joint research center

MWCNT – Multiwall carbon nanotubes

PCV - packed cell volume

RR – Round Robin

SF - stacking factor

TG - Test Guideline

VCM - volumetric centrifugation method

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1. Technical progress

1.1 Introduction

In a typical *in vitro* cellular assay, engineered nanomaterials (ENMs) are normally dispersed in cell culture medium, and the resulting suspension is dispensed onto cells in multiwell cell culture plates. Cellular responses are measured following a post-exposure incubation over a range of doses to establish a dose-response relationship. The nominal dose is related either as total particle mass, particle number or particle surface area. ENMs are in most cases in suspension as agglomerates consisting of multiple protein-coated primary ENM particles and trapped intra-agglomerate fluid (DeLoid et al., 2014). If agglomeration occurs, the total number of particles and the total surface area of suspended ENMs become smaller, thus the biological effects observed in cellular toxicity studies might differ from those induced by single primary particles. In addition, agglomeration also determines hydrodynamic particle size and effective density, i. e., key properties that determine the fate and the transport of particles in suspension. The effective density of the agglomerate unit is often lower than the primary particles due to the lower density of entrapped media and proteins. This parameter can be empirically estimated using a theoretical fractal-based model for agglomeration or it can be measured by analytical ultracentrifugation (AUC) technique. Although AUC technique provides direct measurement of the effective density, it requires the use of high expensive equipment not available in many laboratories. In this context, volumetric centrifugation method (VCM) has gained lately more attention as it allows high-throughput measurement of effective density using less expensive materials (Cohen et al., 2015; DeLoid et al., 2014; 2017).

For this reason the main purpose of the following work is to provide a consolidated pre-validated guidance document on "Determination of the effective density of engineered nanomaterials (ENMs)" based on the volumetric centrifugation method. The protocol described here follows the methodology already presented in DeLoid et al. (2017), which includes (i) preparation of stable ENMs dispersions by sonication, (ii) colloidal characterization of suspended ENMs, and (iii) measurement of the effective density of the engineered nanomaterials using VCM.

1.2 Principles of the method

Volumetric centrifugation method (DeLoid et al., 2014) is low-cost method to determine ENM effective density by measuring the volume of the pellet obtained by low speed, benchtop centrifugation of engineered nanomaterial suspensions in a packed cell volume (PCV) tube. Based on this technique, a sample of ENM suspension is centrifuged in a PCV tube to produce a pellet, consisting of both packed agglomerates and intra-agglomerate media, into the capillary. The volume of the pellet can be easily measured by using a reader measuring device for PCV tubes. In an ideal situation, assuming the perfect stacking of ENM agglomerates (i. e., with no intervening space), the total volume of the agglomerate in a sample of ENM suspension (V_{agg}) is equal to the volume of the pellet as measured after centrifugation(V_{pellet}). However, in a real situation, part of the medium can be easily trapped within the empty spaces between agglomerated particles, leading to a lower effective density of agglomerates in comparison to the one of the primary particles. The volume of the medium trapped within agglomerates (V_{media}) can be calculated as

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$$V_{media} = V_{agg} - V_{ENM}, \quad (1)$$

where V_{ENM} can be then calculated knowing the mass of the ENM in suspension and the material density.

The effective density of the agglomerates, ρ_{EV}, can be calculated as a volume-weighted average of ENM density, ρ_{ENM}, and media density, ρ_{media}, as:

$$\rho_{EV} = \frac{(\rho_{media} V_{media}) + (\rho_{ENM} V_{ENM})}{V_{agg}} \quad (2)$$

Nevertheless, the stacking of agglomerates is not perfect, and the media remains between stacked agglomerates. Therefore, V_{agg} has to be considered as a fraction of the pellet and it can be calculated by using the stacking factor (SF) as follows:

$$V_{agg} = V_{pellet} \times SF. \quad (3)$$

The substitution of equation 3 in equation 1 and 2 and the replacement of V_{ENM} with the equivalent expression considering the ENM density, ρ_{ENM}, and the ENM mass, M_{ENM}, yields:

$$\rho_{EV} = \rho_{media} + \left[\left(\frac{M_{ENM}}{V_{pellet} SF}\right) + \left(1 - \frac{\rho_{media}}{\rho_{ENM}}\right)\right]. \quad (4)$$

For soluble materials (e. g., Ag, ZnO), the mass of the original ENM samples that is solubilized in the medium (M_{ENMsol}) must be subtracted to avoid overestimation of the effective density of these nanomaterials (equation 5). M_{ENMsol} can be determined by analyzing the supernatant (e. g. by inductively coupled plasma mass spectrometry, ICP-MS) of an ENM suspension after centrifugation (DeLoid et al., 2014; 2017).

$$\rho_{EV} = \rho_{media} + \left[\left(\frac{M_{ENM} - M_{ENMsol}}{V_{pellet} SF}\right) + \left(1 - \frac{\rho_{media}}{\rho_{ENM}}\right)\right] \quad (5)$$

Values for SF may range from 0.634 for random stacking, to the theoretical maximum of 0.74 for ordered stacking, in case of uniform spheres. For the roughly spherical agglomerating particles, SF values can be approximated to the theoretical value for random close stacking, whereas for non-agglomerating ENMs, SF values can be considered close to the theoretical value for ordered stacking (DeLoid et al., 2014).

1.3 Applicability and limitations

The determination of the effective density by VCM is limited to relatively low-aspect-ratio ENMs, such as metals, metal oxides, and different carbon-based ENMs as well as incidental nanoparticles (e.g., resulting from natural combustion processes) and particles released by nano-enabled products.

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However, this methodology is not recommended for high-aspect-ratio ENMs, such as carbon nanotubes (CNTs) or other 2D materials such as graphene. This is due mainly to two reasons: (i) the hydrophobicity of these materials makes difficult the dispersion in aqueous phase without using a surfactant or a dispersing agent; (ii) the sedimentation and the diffusion equations assumes that particles and agglomerates can be approximated as spheres with a given hydrodynamic diameter (DeLoid et al., 2014; 2017).

1.4 Materials

1.4.1 Reagents

- Low-aspect-ratio ENMs from those selected under RiskGONE project: TiO_2 Sigma-Aldrich (ERM00000062), TiO_2 JRC (ERM00000064); ZnO (identifiers ERM00000063), CuO (ERM00000088), WC-Co (ERM00000089).
- Sterile deionized water (resistivity 18 MΩ cm)
- Cell culture medium of choice (for example, RPMI 1640 (Thermo Fisher Scientific, cat no. 11875093) or DMEM (Thermo Fisher Scientific, cat no.11995065) supplemented with 10% (vol/vol) fetal bovine serum [FBS] (Thermo Fisher Scientific, cat no. 16000044)

1.4.2 Materials and Equipment

- Spatula
- Pipette
- High precision laboratory scale or analytical balance
- Sonicator with a minimum power rating of 250 W is recommended (according to DeLoid et al., (2017)) to achieve an adequate particle dispersion and to allow reasonable sonication times
- Sonicator Probe (e. g. 3, 7 or 13 mm probe diameter, approximately length 100 mm)
- 600 mL borosilicate glass beaker, low form (height 125 mm and 90 in diameter) with spout
- Digital thermometer with measurement accuracy better than ±0.1°C or digital probe 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
- TPP Packed Cell Volume (PCV) tubes without graduations (10.5 mm x 43 mm, TPP Techno Plastic Products, cat. no. 87007) and caps (13.5 mm, TPP Techno Plastic Products, cat. no. 87008)

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- Easy read measuring device for PCV tubes (TPP Techno Plastic Products, cat. no. 87010)
- Laboratory centrifuge with swinging bucket rotor (rotor must be swinging bucket style, not fixed angle)
- Microtube size bucket adapter (12 mm in diameter)
- Dynamic Light Scattering analyzer
- All the materials required to measure Particle size (hydrodynamic diameter) by using DLS technique. Refer to RiskGONE protocol for Particle Size determination by DLS-RR3.

1.5 Procedure

This protocol is divided in two parts. The first part gives the guidelines to prepare a stable ENM suspension by sonication while the second part gives the guidelines to measure the ENM effective density by VCM method.

1.5.1 Part 1: ENM dispersion preparation

In order to ensure a good ENM dispersion, first at all the sonicator must be calibrated to determine the exact delivered sonication energy (*DSE*) or acoustic power. *DSE* value needs to calculate the critical DSE (DSE_{cr}) for each ENM, meant as the energy per unit volume of ENM suspension (mL) required to achieve the smallest possible agglomerates while producing the most stable suspension over time. Finally, by knowing the DSE_{cr} proper for each ENM and the volume of the ENM suspension, it is possible to calculate the critical sonication time, meant as the time needed to produce a stable dispersion.

Sonicator calorimetric calibration

1. Fill a 600 mL cylindrical borosilicate beaker with 500 mL of water.
2. Using an analytical balance, weigh 40.00 g deionized water in a 50 ml conical centrifuge tube.
3. Place and secure the tube with a three-pronged clamp in the center of the beaker.
4. Immerse the sonicator probe approximately 2.5 cm below the liquid surface in the center of the tube.
5. Insert the thermometer probe into the water in the tube (without touching the tube walls).
6. Turn the thermometer on. Measure the temperature with an uncertainty smaller than 0.1°C.
7. Turn the sonicator power “on,” select and record the power settings (for example, 100% amplitude, continuous mode).
8. Record the temperature values every 10-30 seconds until the temperature stabilizes (reaching a plateau). Then turn the sonicator power “off”.
9. Repeat Steps 2-8 other two times to generate a total of three data sets. Use a new beaker and a new tube each time you perform a new calibration.

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10. Calculate the delivered acoustic power P(W=J/s) as

$$P = \left(\frac{dT}{dt}\right) M C_p \qquad 6$$

where $\frac{dT}{dt}$ is the slope of temperature (K) vs. time (s), *M* is the mass of the water (≈ 40 g), and C_p is the specific heat of water (4.186 J/g K).

11. Calculate the average acoustic power (considering the three measurements) for the specific sonicator and record the value as well as the sonicator settings used.

Determination of DSE_{cr}

The determination of DSE_{cr} is needed to prepare stable dispersions from powder ENMs. Considering the materials selected within RiskGONE project, the suspensions of the following ENM were considered for the sonication step: TiO_2 Sigma-Aldrich (ERM00000062), TiO_2 JRC (ERM00000064), CuO (ERM00000088); WC-Co (ERM00000089).

As the selected ZnO from Sigma-Aldrich (ERM00000063), is already provided in suspension form the preparation of samples from this ENM did not require the sonication step, replaced by simple vortexing during 30s.

12. Weigh approximately 5 mg of nanoparticle powder into a 15 ml conical centrifuge tube. In the case of dispersions, consider the solid content of the dispersion to calculate the amount of dispersion needed in order to handle 5 mg of nanomaterial.
13. Add deionized water to achieve a final volume of 10 ml (concentration of 0.5 mg/ml).
14. Vortex suspension at high speed for 30 seconds.
15. Remove 1 ml of the suspension, measure the mean hydrodynamic diameter using DLS and return the sample to the tube.
16. Adjust the sonicator power settings to those used during the calibration step and turn the sonicator power "on".
17. Sonicate the suspension during 30-60 s.
18. Calculate the DSE (J/mL) for each sonication step as:

$$DSE = \frac{P \times t}{V} \qquad 7$$

where *P* is the delivered acoustic power determined in the calibration steps (steps 1-11), *t* is time in seconds, and *V* is the volume of the suspension in millilitres.

19. Remove 1 ml of the suspension, measure the mean hydrodynamic diameter using DLS and return the sample to the tube.
20. Repeat steps 17-19 until the mean hydrodynamic diameter decreases by <5% between steps.

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21. Plot cumulative DSE (x axis) vs. mean hydrodynamic diameter (y axis).
22. Identify 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).
23. Remove 100 µl sample of suspension, dilute to 100 µg/ml in cell culture media of choice, and measure the mean hydrodynamic diameter of the sample by DLS.
24. Repeat the size measurement of the ENM suspension in cell culture media at 24 hours from the preparation. Because to have a stable ENM dispersion is essential to ensure a proper determination of effective density, check if the mean size changes substantially (by more than 30%) after 24 hours. If so, it may be advisable to repeat Steps 12-24 with additional sonication time until the 24 hours post-sonication suspension mean size has a deviation of less than that 30% from the initial value.

Preparation of suspensions for characterization and use in in vitro experiments

25. Weigh the amount of nanoparticle powder required for the experiment into a 15 ml conical centrifuge tube
26. Add deionized water to achieve a final concentration of 0.5 mg/ml.
27. Vortex suspension at high speed for 30 seconds.
28. Select the sonicator power settings based on those set for the calibration step and $DSE_{cr.}$ measurement.
29. Calculate the time t (s) required for sonication as

$$t = \frac{V \times DSE_{cr}}{P} \qquad 8$$

where V is the volume of suspension (mL) and P is the delivered power (W or J/s) determined by the calibration step.

30. Sonicate the suspension for the calculated time.
31. Vortex the suspensions at high speed for 30 s.
32. Dilute to the final desired concentration in the medium or fluid of choice for P-CHEM characterization or *in vitro* studies.

1.5.2 Part 2: Determination of the ENM Effective Density (ρ_{EV})

33. Dilute the ENM water suspension as described above in the medium of choice to make ~4mL of suspension at 100 µg/ml.
34. Transfer 1 mL of suspension into each of three PCV tubes and cap the tubes.
35. Centrifuge the tubes at room temperature for 1 hour at 3000 × *g*.

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36. Use the "Easy read" measuring device to measure the volume of the pellet collected in the bottom of the capillary in each PCV tubes. Insert the PCV tube into the hole on top of the sliding holder so that it stays on the ramp on the back of the ruler. The holder contains a lens to magnify the capillary and the ruler graduations. Slide the tube and the holder along the ramp until the top edge of the pellet is aligned with the top of the ruler. Position your line of sight so that the horizontal cross-hair is aligned with the top edge of the ruler and the vertical line of the cross-hair is aligned with the capillary center. If the components are not properly aligned parallax error will result thus affecting the measurement.
37. Determine the density of the medium, ρ_{media} (g/cm^3). This can be performed either by i) picnometry method, ii) by weighing a known volume of medium in a tared vessel.
38. Calculate the effective density, ρ_{EV}, for each pellet (after having measured the volume) using equations 4 or 5, depending on the ENM solubility. In addition, the application of a theoretical SF value of 0.634 offers a reasonable approximation, since 90% ENMs (by volume) present in the market are include agglomerating metal and metal oxide ENMs.
39. Calculate the mean ρ_{EV} from the three individual measures.

1.6 Quality control and quality assurance

With regard to ENMs dispersions preparation, be sure that the dispersions obtained are homogeneous (not visible sedimentation). Although no calibration is required for the DLS analyser, the instrument should be verified at the beginning of the experiment by using a standard quality control. According to ISO 22412 (ISO, 2017), a polystyrene latex particles with narrow size distribution and average diameter (as measured by DLS in the size range of 60-200 nm) are recommended. The measured average diameter (Z-average size) of the latex particles should be within 2% of the stated size range, and the polydispersity index should be lower than 0.1.

To measure the Particle size (hydrodynamic diameter) and distribution of those particles within the instrument validation step, refer to the protocol prepared within RiskGONE project "Particle size determination by DLS".

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.

1.7 Safety warnings

To minimize the exposure to ENMs, handle the samples with care. Use appropriate protective gear, such as lab coat, gloves, googles and masks. Weighing steps must be carry on under a specific flow hood equipped with appropriate HEPA filters. Further information on safe handling of ENMs and laboratory equipment are described in the Material data sheets and on the "User manuals" developed by the specific manufacturers.

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

No major deviations except the low number of participants joining the task 4.1 – Characterization of ENMs – Effective density of ENMs. According to the Description of Action (DoA), 4 participants were suggested to join each RR in order to proceed with test guidelines (TGs) submission. However, only CID and LIST have been involved in the RR1 for this task, which could hinder the proposal of a pre-validated test guideline because of lack of data points. To solve this issue other partners have been invited to join the RRs exercises for the "ENM Effective Density determination". So far only KU Leuven accepted to join this task for the second RR exercise.

Thus, the protocol described in this document has to be considered as a tool to verify the opportunity to apply the described procedure within RiskGONE project. The protocol might be further improved and updated after the 3rd and 4th RR. To ensure that there will at least 3 partners in coming RRs, there might be call for external partner to join next RR.

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ANNEX I: RR1-RR2 experimental results

According to the protocol proposed, only low-aspect-ratio ENMs can be characterized, which limited the use of this methodology for high-aspect ratio ENMs, such as Ag nanowires and MWCNTs. The colloidal characterization and effective density in DMEM + 10% (vol/vol) FBS for low-aspect-ratio ENMs characterized in the first and second Round Robin (RR) exercises within RiskGONE project are reported in Table 1. The density of the medium, the total mass of the ENMs, and the SF used in the calculation of the Effective density are listed below:

ρ_{media} = 1.0084 g.cm^{-3}

M_{ENM} = 1.0 x 10^{-4} g

M_{ENMsol} = 0

A Hielscher sonicator (UP400S model, 400 W) equipped with 3- or 7-mm sonication probe was used to de-agglomerate the ENMs (provided in powder form) and to produce stable dispersions. Using 100% amplitude and continuous mode as sonicator power settings, the delivered power was 5.5 ± 0.4 J/s and 18.9 ± 2.9 J/S for the 3- and 7-mm probes, respectively.

The Effective density values have been quite reproducible for TiO_2 JRC (ERM00000064) and ZnO (ERM00000063) within the two RR exercises. All the ρ_{EV} values were smaller than the density values of the pristine material. Only for TiO_2 Sigma-Aldrich (ERM00000062) a higher Effective density value was calculated in RR2 compared to RR1.

Some inconsistencies were also observed in the determination of the DSE_{cr} needed to produce stable TiO_2 dispersions. For TiO_2 JRC (ERM00000064), the sonication at higher *DSE* induced a decrease in the hydrodynamic diameter of the final dispersion in water, but this didn't affect the final ρ_{EV} values. The opposite situation was observed for TiO_2 Sigma-Aldrich (ERM00000062). The use of a higher *DSE* not only resulted in a larger particle size (as measured by DLS), but also implicated the production of stable dispersions in presence of cell culture medium (DMEM + 10% FBS). Therefore, different ρ_{EV} values were determined for this ENM during Round Robin 1 and 2.

According to DeLoid's protocol (DeLoid et al., 2017), the reason for a decrease in the ρ_{EV} value may be attributed to the presence of proteins or trapped intra-agglomerated fluid within ENMs agglomerates formed upon the contact with culture media (DMEM + 10% vol/vol FBS).

To verify this hypothesis, ENM particle size analysis was performed on particle dispersions prepared in DMEM + 10% vol/vol FBS. If agglomerates are formed, an increase on particle size should be observed by DLS analysis. Nevertheless, results showed that for TiO_2 JRC particles (ERM00000064) a reduction of the particle size was registered after dispersion in DMEM/FBS. In RR1, for example, a mean hydrodynamic diameter of 196.0 ± 5.6 nm (PdI = 0.212) in water and 69.7 ± 0.8 nm (PdI = 0.175) in DMEM/FBS medium was observed for fresh TiO_2 JRC dispersions. Values were 169.1 ± 4.1 nm in water and 132.6 ± 0.8 nm in DMEM/FBS for RR2. The particle size measured in cell culture medium didn't change significantly during the next 24h.

These results indicate that proteins present in the medium may contribute to the colloidal stabilization of the dispersions. However, for dispersions prepared using ERM00000063, ERM00000088, and ERM00000089, none of the measurements performed met the quality criteria making DLS not a

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suitable technique to characterise the particles under the current experimental conditions. This is likely due to a large polydispersity (> 0.7) recorded in the samples or to the presence of large particles that made impossible a correct measurement by DLS technique.

Table 1. Characterization of ENMs used in the RiskGONE project for evaluation of effective density protocol.

ENM code	Sample name	DSE_{cr} (J/mL)	RR	Z-average size water/Z average DMEM+FBS	$\rho EV_{pristine\ material}$ (g/cm^3)	ρEV (g/cm^3)
ERM00000064	TiO_2 JRC (a990484)	453.6	#2	169.1±4 .1/132.6 ± 0.8	3.9	1.83±0.00
		340.2	#1	196.0 ± 5.6 / 69.7 ± 0.8		1.85±0.00
ERM00000062	TiO_2 Sigma (MKCK4358)	453.6	#2	224.2 ± 0.2 / 91.5 ± 2.6	3.9	2.73±0.01
		340.2	#1	161.3±4.5/ NA		1.85±0.00
ERM00000063	ZnO Sigma (MKCJ4155)	-	#2	614.4 ± 10.0/NA	5.61	2.33±0.04
		-	#1	627.6 ± 18.6/NA		2.26±0.02
ERM00000089	WC-Co (5561HW)	167.5	#2	289.9 ± 5.1 / NA	15.63	2.42±0.05
ERM00000088	CuO (PL-Cu)	418.8	#2	170.3 ± 2.1 / NA	6.31	2.48±0.15
NA: Result not acceptable because it does not meet quality criteria. DLS instrument is not able to measure hydrodynamic diameter of samples with polydispersity values larger than 0.7 and/or with a significant amount of large particles in the dispersion.						

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