SCIENCE-BASED RISK GOVERNANCE
OF NANO-TECHNOLOGY

RISK GONE

Harmonized standard operating procedure (SOP) for the resuspension of engineered nanomaterials (ENM) in biological media and *in vitro* dosimetry

DELIVERABLE 4.9

Due date of Deliverable:	30.06.2021
Actual Submission Date:	29.06.2021
Responsible partner:	IMI, Zagreb, HR
Report Author(s):	Barbara Pem, IMI; Ivana Vinković Vrček, IMI
Reviewed by:	RiskGONE WP4 Partners, NILU, EAB
Nature:	R (Document, SOP)
Dissemination Level:	CO (Confidential, restricted under conditions set out in Model Grant Agreement)

Call:	H2020-NMBP-13-2018
Topic:	Risk Governance of nanotechnology
Project Type:	Research & Innovation Action (RIA)
Name of Lead Beneficiary:	NILU, Norway
Project Start Date:	1 January 2019
Project Duration:	50-Months

Document History

Version	*Date*	*Authors/ who took action*	*Comment*	*Modifications made by*
0.1	03.05.2021	Barbara Pem (IMI)	First Draft, sent to WP4 partners	Ivana Vinković Vrček (IMI)
0.1	21.05.2021	Juliana de Souza (CID)	Review by WP4 partners	Jesus Martinez de la Fuente (CSIC), Andreas Tsoumanis (NovaMechanics)
0.1	26.05.2021	Mihaela R Cimpan (UiB)	Review by WP4 partners	
0.1	06.06.2021	Elisa Moschini (LIST)	Review by WP4 partners	
0.2	09.06.2021	Barbara Pem (IMI)	Second Draft based on review by WP4 partners	Ivana Vinković Vrček (IMI), Juliana de Souza (CID), Maria Dusinska (NILU)
0.3	14.06.2021	Ivana Vinković Vrček (IMI)	Sent to EAB members	
0.4	23.06.2021	EAB, M. Dusinska (NILU)	Final corrections	Ivana Vinković Vrček (IMI)
1.0	29.06.2021	PMO (NILU)	Submitted to Commission	

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 814425.

Abstract

As hazard characterization and risk assessment are an integral part of the lab-to-market innovation path of engineered nanomaterials (ENM), they should be based on the set of information that include physico-chemical characteristics and biological properties of ENM, mechanism of their interactions with biological systems, decisive *in vitro*/*in vivo* dosimetry and finally their mode of action. The aim of this document is to provide a standard operating procedure (SOP) for the accurate determination of the applied ENM dose during *in vitro* experiments. The SOP is developed under Task 4.3.

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List of abbreviation

ADS-RM = Agglomeration-diffusion-sedimentation-reaction model

AUC = Analytical Ultracentrifugation

BET = Brunauer–Emmett–Teller method

CNTs = carbon nanotube

CS = Centrifugal Sedimentation

DC = Disc Centrifugation

DG = Distorted Grid

DLS = Dynamic Light Scattering

DSMC = Direct Simulation MonteCarlo

EC-OC = Elemental Carbon–Organic Carbon Analysis

ELS = Electrophoretic Light Scattering

ENM = Engineered Nanomaterials

ENTM = Environmental Nano Testing Media

FTIR = Fourier Transform Infrared Spectroscopy

ICP-MS = Inductive Coupled Plasma Mass Spectroscopy

ISDD = In vitro Sedimentation, Diffusion and Dosimetry

LC-MS = Liquid Chromatography–tandem Mass Spectrometry

NTA = Nanoparticle Tracking Analysis

PDA = Phase Doppler Anemometry

SAXS = Small Angle X-ray Scattering

SEM = Scanning Electron Microscopy

SOP = standard operating procedure

SPM = Scanning Probe Microscopy

TEM = Transmission Electron Microscopy

TEM(SEM)-EDS = TEM(SEM)- Electron Dispersive Spectroscopy

TEM-SAD = TEM–Selected Area Diffraction

TGA = Thermo-Gravitational Analysis

TRPS = Tunable Resistive Pulse Sensing

VCM = Volume Centrifugation Method

XPS = X-Ray Photoelectron Spectroscopy

XRD = X-Ray Diffraction

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1. Technical and scientific progress

Introduction and background

As hazard characterization and risk assessment are an integral part of the lab-to-market innovation path of engineered nanomaterials (ENM), they should follow a multimethod tiered approach. This approach covers whole path of ENM development from the early phases of research to the final product. It starts from characterisation of physico-chemical and biological properties of ENM (Tier 1) to evaluation of their interaction with biological systems and decisive *in vitro*/*in vivo* dosimetry (Tier 2) and finally to identification of their mode of action (Tier 3).

Minimum physical and chemical parameters for characterizing ENM for hazard and exposure assessment as recommended by Prosafe Task Force (Steinhäuser and Sayre, 2017) include:

- Intrinsic, medium independent, properties including shape, density, crystal structure, hydrophobicity, primary particle size distribution, specific surface area, chemical composition, impurities
- Extrinsic, medium dependent properties including effective density, dustiness, hydrodynamic particle size distribution, zeta potential, aggregation rate, surface affinity, persistence such as solubility, UV-stability, thermal stability.

Despite excellent progress attained towards the identification of properties necessary for reliable risk assessment, there are still limits of using existing data for read-across analyses (Gao and Lowry, 2018). A significant obstacle for ENM characterization is the need to create stable dispersions not only in the optimal solvent, but also in *in vitro* media. The establishment of reliable and reproducible dispersion protocols for ENM is lacking, which is a main bottleneck of *in vitro* testing development. Moreover, additional bottleneck of reliable ENM characterisation is represented by the need to provide specific dispersion protocols for every ENM type.

The composition and properties of media may significantly affect stability, transformation, uptake, transport and biological effects of ENM (Geitner *et al.*, 2020). Thus, reliable hazard identification should follow a harmonizing approach. An expert workshop “Environmental Nano Testing Media (ENTM) Harmonization” (convened in 2016) brought recommendations for relevant and consistently characterized medium types in which ENM characterization and testing should be carried out. To enable comparability

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of datasets provided by different laboratories and expert groups, key media characteristics were proposed (Geitner *et al.*, 2020):

- primary characteristics (absolutely required), including pH, ionic strength and ionic content, addition of serum (percentage, source)
- secondary characteristics, such as CO_2 level, dissolved oxygen and organic carbon concentrations, addition of growth factors, etc., required when possible and/or including characteristics pertinent to the specific measurement requirements.

Colloidal properties of ENM in particular govern their behaviour during standard *in vitro*/*in vivo* toxicity testing, particularly influencing the amount of particles delivered to cells, tissues, organs. The ENM properties relevant for hazard identification studies and the established techniques for their evaluation are listed in Table 1.

Table 1. Properties relevant for hazard identification and risk assessment of ENM and the established methods for their determination.

General property	Specific property	Methods
Physical	Size	TEM, SEM, BET, XRD-Rietveld analysis
	Size distribution	TEM, SEM, sieving, Coulter method, PDA, SAXS
	Shape	TEM, SEM, SPM
	Density	Pycnometry
	Surface area	BET
	Porosity	BET/alkaline homologous series
	Charge	Faraday pail device
	Crystallinity	XRD, TEM-SAD
Chemical	Composition	ICP-MS, TEM-EDS, SEM-EDS, TGA, EC-OC, Raman spectroscopy, FTIR
	Surface chemistry	FTIR, XPS, RAMAN
	Molecular weight	ICP-MS, AAS
	Hydrophobicity	Dye adsorption; octanol–water affinity
Colloidal	Size distribution	DLS, TRPS, DC, TEM, SEM, NTA, CS
	Polydispersity	DLS, NTA, TRPS

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	Effective density	AUC, VCM
	Dissolution	ICP-MS, AAS
	pH	pH-metry
	Corona formation	DLS, ELS, LC-MS/MS
	Specific conductance	DLS
	Zeta potential	ELS, TRPS

AUC = Analytical Ultracentrifugation, BET = Brunauer–Emmett–Teller method, CS = Centrifugal Sedimentation, DC = Disc Centrifugation, DLS = Dynamic Light Scattering, EC-OC = Elemental Carbon–Organic Carbon Analysis, ELS = Electrophoretic Light Scattering, FTIR = Fourier Transform Infrared Spectroscopy, ICP-MS = Inductive Coupled Plasma Mass Spectroscopy, LC-MS = Liquid Chromatography–tandem Mass Spectrometry, NTA = Nanoparticle Tracking Analysis, PDA = Phase Doppler Anemometry, SAXS = Small Angle X-ray Scattering, SEM = Scanning Electron Microscopy, SPM = Scanning Probe Microscopy, TEM = Transmission Electron Microscopy, TEM(SEM)-EDS = TEM(SEM)- Electron Dispersive Spectroscopy , TEM-SAD = TEM–Selected Area Diffraction, TGA = Thermo-Gravitational Analysis, TRPS = Tunable Resistive Pulse Sensing, VCM = Volume Centrifugation Method, XPS = X-Ray Photoelectron Spectroscopy, XRD = X-Ray Diffraction

As part of the Tier 2 phase, decisive *in vitro* dosimetry represents one of the most critical challenges for risk and hazard assessment and it is directly related to stability, transformation, uptake, transport and biological effects of ENM. Recently, an integrated *in vitro* dosimetry approach has been proposed to comprise dispersion preparation, dispersion characterization and numerical fate and transport modelling of ENM to derive delivered dose metrics (DeLoid *et al.*, 2017).

ENM toxicity testing using *in vitro* assays requires the ENM to be dispersed in cell culture media, and applied to multiwell cell culture plates. There are numerous techniques and protocols for dispersing ENM in aqueous media that should be harmonized (Hartmann *et al.*, 2015). Various endpoints are measured during *in vitro* testing following the exposure, commonly lasting for 24-48 h, and the dose-response relationship is commonly reported (DeLoid *et al.*, 2017). However, the effective dose is not necessarily equal to the administered dose, since the cells seeded in the plate wells will only react to the ENMs that reach the bottom of the plate. Therefore, for the correct reporting of the ENM dosage regimen, the administered dose should be adjusted (Liu *et al.*, 2015).

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The factors that influence ENM diffusion and sedimentation are primarily size and effective density (including both primary particles and agglomerates). If those properties are known, the exposure dose can be calculated using mathematical models (Böhmert *et al.*, 2018). There have been a few models published so far, using different approaches. All of them calculate the diffusion and sedimentation using the data on aforementioned properties, but some also consider aggregation and/or dissolution. The *In vitro* Sedimentation, Diffusion and Dosimetry (ISDD) model (Hinderliter *et al.*, 2010) and distorted grid (DG) model (DeLoid *et al.*, 2017) require ENM size and effective density as input data, and are differential-equation based. Others, such as the stochastic direct simulation MonteCarlo (DSMC) model (Liu *et al.*, 2011) or Agglomeration-diffusion-sedimentation-reaction model (ADS-RM) (Mukherjee *et al.*, 2014), utilize stochastic (Monte Carlo) approaches. However, not all models are publicly available, or require licenced software to perform.

Main objective of this document is to develop a SOP for the accurate determination of the applied dose *in vitro* submerged experiment based on the harmonised resuspension protocol.

This SOP contains the procedure for resuspension of ENM in different biological media and instructions for ISDD and DG models, which are both relatively simple to implement and publicly available. The DG model has been considered more accurate, however it requires more experimental data and the access to the licenced software, thus making it less accessible to some laboratories. The researchers are advised to select the method appropriate for their desired outcome and level of availability. The objective of this document is to provide guidance for the preparation of ENM suspensions in biological media for *in vitro* testing, and to evaluate suspension properties for accurate *in vitro* dosimetry calculations.

2. Principles of the method

The *in vitro* dosimetry models presented here rely on experimental data on ENM characteristics in media to compute the delivered dose metrics as a function of the exposure time. The implemented methodology has been experimentally validated, as described in detail in original publications (Hinderliter *et al.*, 2010; DeLoid *et al.*, 2017). The ISDD model applies well established, long-used principles of diffusional and

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gravitational transport of particles in viscous media to calculate the movement of particles from the media to the bottom of a vessel where cells reside. The net rate of transport downward toward the bottom of the vessel is calculated within a single partial differential equation, which is solved numerically to calculate the fraction of material transported from media to the bottom of the vessel. Particle transport to cells is calculated by simultaneous solution of Stokes Law (sedimentation) and the Stokes-Einstein equation (diffusion). The ISDD model made it possible to calculate the "per well bottom surface area deposited mass", surface area, and number of particles, as well as the fraction of total suspended material deposited as a function of time. The DG model expanded on this, providing deposition, as well as concentration metrics as a function of time, both at the bottom of the well and as a function of position in the well. DG model also allows simultaneous simulation of all particles sizes in the distribution of a polydisperse suspension, modelling of soluble materials and modelling of variable binding kinetics ('stickiness') at the bottom of the well, based on a user-defined dissociation constant, K_D. Since both models focus on two essential parameters for dosimetry calculations, size and effective density, instructions are provided as to measuring those parameters. ENM size can be measured through multiple means, but this SOP calls upon the previous SOP for ENM size (hydrodynamic diameter) determination by DLS (in water), agreed on by RiskGone partners. A previous protocol for effective density measurements, based on DeLoid et al., has been in use by RiskGone partners. A known volume of suspension of a known ENM concentration is loaded into a PCV tube and centrifuged to collect the agglomerates in the capillary section of the tube. From the measured volume of the pellet and known volume of ENM, the effective density can be calculated as a weighted average of media and ENM.

3. Applicability and Limitations

All applicability criteria and limitations apply as stated in the RiskGone SOP "Measurement of hydrodynamic diameter and size distribution using dynamic light scattering (DLS)" for i) preparation of ENM dispersions in water; and for ii) size determination by DLS.

This document assumes the ENM are dispersible in cell culture media and of sufficient stability to allow *in vitro* experiment completion.

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The proposed methodology is not suited to certain ENM types (such as high-aspect-ratio carbon nanotubes (CNTs) or other 2D ENM such as graphene), since their suspensions might require special dispersants, and the mathematical model assumes their shape can be adequately approximated as a sphere. Modifications of the protocol may be required for ENMs that are soluble in the medium of choice, those that form large agglomerates, or those that are buoyant.

The ISDD model may be utilized in two ways. The diameter method requires only the measurement of ENM and agglomerate size (by DLS), while the density method also requires the measurement of effective density by volumetric centrifugation. If the volumetric centrifugation measurements are unavailable, the diameter method may be used, however this reduces the quality of the final result. Certain parameters in both models, if unknown, may be ignored and their value left as default.

Particle settling must not generate turbulence and dynamic agglomeration or other particle interactions are not accounted for in the model. The model may not be appropriate to apply where advection occurs in the cell culture system or where there has been significant advective or mechanical mixing over the course of the experiment. Formulated for spheres or particles that can be adequately described as spheres, ISDD should not be used for fibres without additional modification and testing. The model also does not account for the uptake of particles by cells, which is cell specific, or for the dynamic in vitro models like microfluidics.

4. Materials

4.1. Reagents

- ENM (e. g., TiO_2 and ZnO ENM)
- Ultrapure water (resistivity 18 MΩ cm, for example Millipore or Sigma) or other dispersant of choice (such as HEPES, PBS, etc.)
- Cell culture medium of choice (for example, RPMI 1640, or DMEM, supplemented with 10% (vol/vol) FBS)

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4.2. Materials and Equipment

For re-suspension in biological media

- 15 mL conical polypropylene or polystyrene centrifuge tubes, or appropriate glass vessels
- Pipettes and tips
- Laboratory vortex mixer, with speed range 300-3500 rpm, touch mode

For effective density measurements

- Packed Cell Volume (PCV) tubes without graduations, with caps ((TPP Techno Plastic Products, Trasadingen, Switzerland) or similar
- Easy-read measuring device for PCV tubes (TPP Techno Plastic Products, Trasadingen, Switzerland) or similar
- Laboratory centrifuge
- Swinging-bucket rotor (Rotor must be swinging-bucket style, not fixed-angle style)
- Microtube-size bucket adaptor

For dosimetry calculations

- PC running Windows 7 or 10
- ISDD GUI (Windows executable) available at https://nanodose.pnnl.gov/default.aspx?topic=ISDD
- DG web-based tool available at http://enaloscloud.novamechanics.com/riskgone/InVitroDosimetry/

4.3. SOPs

- RiskGone SOP “Measurement of hydrodynamic diameter and size distribution using dynamic light scattering (DLS)”
- RiskGone SOP “Protocol for DLS and Z potential measurements”
- RiskGONE SOP “Measurement of effective density of ENM using volumetric centrifugation method (VCM)”

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5. Procedure

5.1. Re-suspension in biological media

This protocol requires the primary dispersion to be performed according to the SOP "Measurement of hydrodynamic diameter and size distribution using dynamic light scattering (DLS)". Sonication of the samples should not be performed in the cell culture media to avoid generation of reactive oxygen species by sonolysis and/or denaturation of proteins.

Use clean, sterile pipette tips and sterile procedures.

It is recommended to work as fast as possible to avoid ENM sedimentation and/or physico-chemical alteration. Use the dispersion as soon as possible (within 30 to 60 min) and agitate (vortex) it immediately before its use.

1. Prepare the ENM suspension according to the aforementioned SOP to a desired concentration (10x higher than the desired concentration in the medium).

2. Pipette 9 mL of the desired medium into a 15-mL conical polypropylene tube.

3. Vortex the primary ENM suspension for 30 s immediately before the dilution with the medium.

4. Using a calibrated pipette, transfer 1 mL of the suspension to the tube containing the medium, and rinse the tip.

5. Vortex the suspension for 30 s to ensure adequate mixing.

5.2. ENM characterization for dosimetry assessment

Determination of the ENM size

The ENM size may be measured by one of the standard techniques for hydrodynamic diameter evaluation (DLS, NTA). This protocol recommends the size evaluation be performed according to the SOP "Measurement of hydrodynamic diameter and size distribution using dynamic light scattering (DLS)". The use of phenol red-free medium may be preferable for DLS measurements.

Determination of ENM effective density

Volumetric centrifugation method (VCM) is a simple and inexpensive technique for effective density measurements developed by DeLoid et al.(DeLoid *et al.*, 2017) *The*

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protocol is adapted from this paper. The alternative technique is analytical ultracentrifugation (AUC), which is less accessible. Either technique can be used.

1. Prepare the ENM suspension in the medium of choice, as described previously, to obtain approx. 4 mL of the suspension at 100 µg/mL.

2. Transfer 1 ml of suspension to each of three PCV tubes, and cap the tubes.

3. Centrifuge the tubes at room temperature (22 °C) for 1 h at 3,000 *g*.

4. Use the 'easy-read' measuring device to measure the volume of the pellet collected at the bottom of the capillary in each PCV tube.

The device resembles a thick ruler. The front face is etched along the top with graduations at 0.025-µl intervals. Insert the PCV tube into the hole on top of the sliding holder so that it rests on the ramp at the back of the ruler. The holder contains a lens to magnify the capillary and ruler graduations. Slide the tube and holder along the ramp until the top edge of the pellet is aligned with the top edge of the ruler. Position your line of sight so that the horizontal crosshair is aligned with the top edge of the ruler, and the vertical line of the crosshair is aligned with the capillary centre. If not properly aligned, parallax error will result in measurement error.

5. Calculate density of the medium, ρ_{media} (g/cm^3), by weighing a known volume of medium in a tared vessel, or by subtracting the mass of a pre-weighed vessel from the mass of the vessel with the medium and dividing by the volume.

6. Calculate the effective density, ρ_{EV}, for each measured pellet volume using the following equation:

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

ρ_{media} = the density of the medium (g/cm^3)

M_{ENM} = total mass of ENM (g) in the dispensed volume (1 mL) of suspension (theoretically calculated)

M_{ENMsol} = mass of dissolved ENM (g) in the dispensed volume (1 mL) of suspension (for insoluble materials, input 0; for soluble materials, determine ENM dissolution using an appropriate method, e.g. ultrafiltration + ICPMS)

V_{pellet} = measured pellet size (cm^3) (convert from measured volume in mm^3 by dividing by 1000)

ρ_{ENM} = the density of the bulk ENM (g/cm^3) (data from literature sources)

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SF = stacking factor (portion of the pellet composed of agglomerates; for ENM that form spherical agglomerates, input 0.634; for ENM that do not agglomerate, input 0.74)

7. Calculate the mean ρ_{EV} from the three individual measures.

5.3. *In vitro* dosimetry calculation

The two models presented here are both publicly available and require size and effective density as inputs. The ISDD model only inputs a single value for ENM size (the average hydrodynamic diameter), while the DG model allows for the input of the entire size distribution.

*The ISDD model can be accessed through the Pacific Northwest National Laboratory website (*https://nanodose.pnnl.gov/default.aspx?topic=ISDD*). It is publicly available as a Windows executable, that does not require MATLAB. Go to the website, click the software download option, and follow the instructions to install and run the GUI.*

Original DG model is available in a form of a MATLAB script (requires MATLAB and some knowledge of running scripts). It can be downloaded from the Supplementary section of the article by DeLoid et al.(DeLoid *et al.*, 2017) *(*https://www.nature.com/articles/nprot.2016.172#Sec26*). It was adapted to a web-based tool for RIskGone members by the NovaMechanics team (*http://enaloscloud.novamechanics.com/riskgone/InVitroDosimetry/*).*

The ISDD model operating procedure

1. Run the ISDD GUI

2. Select Diameter mode or Density mode. Use Diameter mode if you only performed DLS measurements. Use Density mode if you also performed effective density measurements.

3. Input parameters from Table 2:

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Table 2. Input parameters for the ISDD model.

Parameter	**Units**	**Meaning**
Simulation time	hours	Set to the duration of ENM exposure
No. of time points shown in results	N/A	Set to the desired value (*e.g.* 24 time points for 24h simulation will show the dosimetry results after every hour of exposure)
No. of grid points	N/A	Default
Boundary conditions	N/A	Default
Primary particle diameter	nm	Enter experimental value in water
Primary particle density	g/cm^3	Enter raw material density
Particle concentration	mg/L	Enter concentration applied to each well
Agglomerate diameter	nm	Enter experimental value in cell culture medium
Agglomerate density	g/cm^3	Enter experimental value
Fractal dimension	N/A	Default
Packing factor	N/A	Default
Dish depth	m	Enter the height of medium above the bottom of the well (ca. 3 mm for 100 µL in a typical 96-well plate, 300 µL in 48-well, 1.1 mL in 12-well and 2.8 mL in 6-well)
Volume	mL	Enter total volume in well
Temperature	K	Enter incubation temperature
Viscosity	$N \cdot s/m^2$	Enter medium viscosity (set to value used for DLS)
Density	g/cm^3	Enter medium density

4. The results will be written to an Excel file in the form of columns: time (h), fraction deposited, particle number deposited, surface area deposited (cm^2) and mass deposited (µg). All outputs represent the amount of NPs reaching the bottom of the well in certain time periods.

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The DG model operating procedure

1. Visit http://enaloscloud.novamechanics.com/riskgone/InVitroDosimetry/.

2. Enter the required properties from Table 3.

Table 3. Input parameters for the DG model.

Category	Parameter	Units	Meaning
Particle parameters	Material		ENM type (choose from a drop-down list, or add new)
	Density	g/cm^3	Raw material density (use provided value, or enter own)
	Effective density	g/cm^3	Experimental value (use provided, or enter own)
	Particle size distributions by volume	nm vs. fractions	Upload data in .csv format of ENM diameters and corresponding fractions from volume distributions (see example)
Solvent parameters	Density	g/cm^3	Enter medium density (recommended 0.9995 g/cm^3)
	Viscosity	Pa·s	Set to value used for DLS (recommended 0.00081 Pa·s)
	Temperature	°C	Enter incubation temperature (recommended 37°C)
Simulation parameters	Suspension column height	mm	Enter the height of medium above the bottom of the well (ca. 3 mm for 100 µL in a typical 96-well plate, 300 µL in 48-well, 1.1 mL in 12-well and 2.8 mL in 6-well)
	Height of subcompartment	mm	Default (0.005)
	Initial total concentration of material	mg/cm^3	Enter concentration applied to each well
	Centrifugation		Enter 1 for gravity
	Total time of simulation	hours	Set to the duration of ENM exposure
	Total interval for simulation	s	Default (0.5s)
Output parameters	Output time interval	min	Set to the desired value
	Output compartment height	mm	Set to the desired value (recommended 0.0005 mm)

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	Write output for…		Choose data to be written to output file. Option “all data”
Advanced parameters	Sedimentation coefficient, ks		Enter value (if known)
	Diffusion coefficient, kd		Enter value (if known)
	Initial dissolution fraction (optional)	N/A	Enter fraction of ENM dissolved at the start of the incubation (experimental value)
	Dissolution rate type (optional)	N/A	Select first option if no further dissolution is expected; select second if dissolution is expected to continue at constant rate; select third if dissolution is not linear
	Dissolution rate (optional)	N/A	If dissolution rate type is set to constant, enter fraction of total ENM dissolved per 1h (experimental value)
	Dissolution times (optional)	hours	If dissolution rate type is set specified curve, upload data in .csv format specifying timepoints and dissolution fractions (see example)
	Dissolution fractions (optional)	N/A	If dissolution rate type is set to 1, enter fraction of total ENM dissolved per each time point (experimental value)
	Bottom sticking coefficient	N/A	Enter particle–cell adsorption (set to 1 if adsorption to the bottom of the well is expected; set to 0 if not)
	Adsorption dissociation constant (optional)	mol/L	If stickiness is set to 1, enter the dissociation constant (recommended 1×10^{-9} M for highly-adhesive ENMS, 1×10^{-8} M for low-adhesive ENMs; or experimental value)

3. Click continue and wait for the calculation to process.

4. Download results in .xls format.

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The output Excel file contains multiple sheets. In the sheet named 'Bot Summary', the dose metrics in Table 4 are given for each time point at the bottom of the well (i.e., within the bottom compartment, representing the cell microenvironment, the height of which is defined by the value of the parameter "Output compartment height" selected for the simulation—typically 0.01 mm or 10 µm). In addition to the Bot Summary sheet, the output file contains one sheet for each of the parameters below, in which the value of the parameter over time is given at the centre z position of each compartment (defined by the "Output compartment height" value selected when running the simulation).

Table 4. Excel output dose metrics for the DG model.

Parameter	**Units**	**Meaning**
Mp vol^{-1}	mg/cm^3	Mass of ENM per unit volume, not including dissolved ENM
Mp+Diss vol^{-1}	mg/cm^3	Mass of ENM per unit volume, including dissolved ENM
Frx Mp	N/A	Fraction of administered ENM deposited (within compartment, e.g., bottom compartment, cell microenvironment)
Mp $area^{-1}$	mg/cm^2	Mass of ENM per unit area of well bottom, not including dissolved ENM
Mp+Diss $area^{-1}$	mg/cm^2	Mass of ENM per unit area of well bottom, including dissolved ENM
Np vol^{-1}	cm^{-3}	Number of particles per unit volume
Np $area^{-2}$	cm^{-2}	Number of particles per unit area of well bottom
SAp vol^{-1}	cm^2/cm^3	ENM surface area per unit volume
SAp $area^{-1}$	cm^2/cm^2	ENM surface area per unit area of well bottom
DissC vol^{-1}	mg/cm^3	Mass of dissolved ENM per unit volume
% Floor occ.	%	Percentage of well bottom occupied by adsorbed particles (reported only for sticky = 1)
Mbound $area^{-1}$	mg/cm^2	Mass of ENM bound to bottom per area of well bottom (reported only for sticky = 1)

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6. Quality control and quality assurance

Check if all the measurements are carried out under operational qualification of the instrument. For ENM dispersion in media, ensure the suspension is homogenous before sampling. For ENM size measurement in media, use phenol red- free medium if possible. For effective density measurements, make sure there is sufficient material in suspension to form a visible pellet. Small particles may require much longer to reach the bottom of the capillary. Consider adjusting the method as needed. For dosimetry calculations, make sure to correctly convert values to appropriate units. Use the most advanced *in silico* model available to you, and experimentally measure as many parameters as possible. At the end of simulations, check that all output values are sensible and applicable to your system.

7. Safety warnings

To minimize exposure to the ENM, handle the samples with care. Use appropriate protective gear, such as lab coat, gloves, googles and masks. Further information on handling the ENM 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.

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

No major deviation to report until now. The minor deviation in the performance of the RiskGONE web app following this SOP does not have a major impact on the D4.9. In this respect, main objective has been achieved and the points to strengthen have been identified.

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 814425.

References

Böhmert, L. *et al.* (2018) "In vitro nanoparticle dosimetry for adherent growing cell monolayers covering bottom and lateral walls," *Particle and Fibre Toxicology*, 15(1), p. 42. doi: 10.1186/s12989-018-0278-9.

DeLoid, G. M. *et al.* (2017) "Preparation, characterization, and in vitro dosimetry of dispersed, engineered ENMss," *Nature Protocols*, 12(2), pp. 355–371. doi: 10.1038/nprot.2016.172.

Gao, X. and Lowry, G. V. (2018) "Progress towards standardized and validated characterizations for measuring physicochemical properties of manufactured nanomaterials relevant to nano health and safety risks," *NanoImpact*, 9, pp. 14–30. doi: 10.1016/j.impact.2017.09.002.

Geitner, N. K. *et al.* (2020) "Harmonizing across environmental nanomaterial testing media for increased comparability of nanomaterial datasets," *Environmental Science: Nano*, 7(1), pp. 13–36. doi: 10.1039/C9EN00448C.

Hartmann, N. B. *et al.* (2015) "Techniques and Protocols for Dispersing Nanoparticle Powders in Aqueous Media—Is there a Rationale for Harmonization?," *Journal of Toxicology and Environmental Health, Part B*, 18(6), pp. 299–326. doi: 10.1080/10937404.2015.1074969.

Hinderliter, P. M. *et al.* (2010) "ISDD: A computational model of particle sedimentation, diffusion and target cell dosimetry for in vitro toxicity studies," *Particle and Fibre Toxicology*, 7(1), p. 36. doi: 10.1186/1743-8977-7-36.

Liu, H. H. *et al.* (2011) "Analysis of Nanoparticle Agglomeration in Aqueous Suspensions via Constant-Number Monte Carlo Simulation," *Environmental Science & Technology*, 45(21), pp. 9284–9292. doi: 10.1021/es202134p.

Liu, R. *et al.* (2015) "Evaluation of Toxicity Ranking for Metal Oxide Nanoparticles via an in Vitro Dosimetry Model," *ACS Nano*, 9(9), pp. 9303–9313. doi: 10.1021/acsnano.5b04420.

Mukherjee, D. *et al.* (2014) "Modeling physicochemical interactions affecting in vitro cellular dosimetry of engineered nanomaterials: application to nanosilver," *Journal of Nanoparticle Research*, 16(10), p. 2616. doi: 10.1007/s11051-014-2616-7.

Steinhäuser, K. G. and Sayre, P. G. (2017) "Reliability of methods and data for regulatory assessment of nanomaterial risks," *NanoImpact*, 7, pp. 66–74. doi: 10.1016/j.impact.2017.06.001.

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 814425.

www.riskgone.eu | riskgone@nilu.no

Zagreb, 29 06 2021

The publication reflects only the author's view and the European Commission is not responsible for any use that may be made of the information it contains.

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 814425.