IIT SOP for characterizing MNM fate in biological media and digestive fluids by multi-technique based method

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Objective

The aim of this guideline is to suggest a multi-technique based approach to trace the MNM fate (in terms of size and dissolution changes) using biological media (e.g., DMEM or RPMI) or simulated human digestive fluids by following a range of SOPs which have been developed in previous EU project (Nanogenotox) and in NanoREG.

Scope

The motivation is to apply a multi-technique based approach to improve the quality of the information and exploit the complementarity of the techniques to gain a more precise scenario of the evolving nature of the MNM properties in such complex fluids. To define a pattern of physical descriptors (size, surface charge, dissolution) in conditions, which must be meaningful for the in vitro test. This may impact on effective dose of MNM and facilitate the identification of secondary products of biotransformation that may influence the in vitro assay. The method may be applied to all MNM, which are measurable by DLS, TEM and especially to metal containing nanoparticles for which the elemental analysis may be possible by UF-ICPAES.

Principles

Define the concentration and time of exposure of MNM to synthetic biological fluids (e.g., biological media or digestive fluids). These values should be meaningful the in vitro test (for instance 24 hours of incubation for cell culture or 2 hours for gastric digestion). Harmonize the dispersion protocol for all the technique/method employed. Apply for basic measurements the already established SOPs for MNM dispersion, probe sonication, TEM analysis, etc. so to obtain more confident data in principle more suitable for comparison with data in complex matrixes.

Techniques required

- DLS
- Zeta pot
- TEM
- UF/ICPAES

List of SOP/protocols implemented or used for the multi-technique based method

1. Nanogenotox dispersion protocol (http://www.nanogenotox.eu/files/PDF/Deliverables/nanogenotox%20deliverable%203_wp4_%20dispersion%20protocol.pdf.)
2. NANoREG “SOP for probe-sonicator calibration of delivered acoustic power and de-agglomeration efficiency for *in vitro* and *In vitro* toxicological testing
3. NANoREG “SOP for measurement of hydrodynamic Size-Distribution and Dispersion Stability by Dynamic Light Scattering (DLS)”.
4. NanoREG Protocol(s) for size-distribution analysis of primary NM particles in air, powders, and liquids (D2.10)

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5. NanoREG Preparation of digestive matrixes (part of WP5, D5.2)
6. NANoREG Protocol for measurement of hydrodynamic Size-Distribution and Dispersion Stability by Dynamic Light Scattering (DLS). The SOP for DLS is adapted for complex juices and the method presented below
7. NANoREG Protocol for measurement of zeta potential of MNM in complex juices (reported below)

Materials and methods required

Dispersion of MNM and calibration of probe sonicator

The MNM where dispersed according to the Nanogenotox dispersion protocol, which includes sonication of the MNM using a probe sonicator. The sonicator used was a Sonics Vibracell VC750, equipped with a 13mm probe. Before starting the experimental session, the probe sonicator was calibrated by using a calorimetric procedure, according to the NANoREG "SOP for probe-sonicator calibration of delivered acoustic power and de-agglomeration efficiency for *in vitro* and *In vitro* toxicological testing", to the aim of ensuring inter-laboratory harmonization of the dispersion conditions. As resulting from the calorimetric calibration, the sonication parameters, necessary to deliver the same acoustic power as that used in the Nanogenotox protocol were 20% amplitude and 8'30'' duration of sonication. These calculated parameters were confirmed by testing the de-agglomeration efficiency on reference material NM200, and analysing the dispersion efficiency by DLS, according to the "SOP for measurement of hydrodynamic Size-Distribution and Dispersion Stability by Dynamic Light Scattering (DLS)".

DLS analysis and zeta potential characterization protocols

Malvern Zetasizer Nano was used to measure the size and charge profiles of MNM dispersed in DMEM and RPMI, and in the synthetic digestive juices (i.e.: saliva, gastric and intestinal juices).

For biological media, size profiles of MNM were monitored at time zero (t0), and after 24 and 48 hours, whereas sizes in the synthetic digestive juices were checked according to the timing of the employed *in vitro* digestion model, namely after 0, 5, 125 and 245 minutes for CTRL, saliva, stomach and intestine, respectively.

For the DLS analysis, about 50 µL of each sample from testing batch was diluted to 1 mL in MilliQ water and analysed using disposable polystyrene cuvettes at R.T. (10 repeated measurements). The optical parameters of the instrument were set for the selected MNM, as reported in the Table 1:

Table 23: R_i and R_{abs} indices

	NM-300K	NM-200	NM-110
Ri	0.180	1.544	2.020
Rabs	0.010	0.020	0.040

Charge profiles of MNM were tested at t0 of incubation in biological media and digestive juices. Each MNM testing batch was pelleted at maximum centrifugation speed (10 minutes, R.T.) and then re-suspended in an equal volume with MilliQ water. Subsequently, samples were taken by a syringe and injected slowly in a disposable folded capillary cell (Malvern DTS 1070). The zeta potential was monitored 10 times for each sample, at R.T.

TEM analysis: evaluation of MNM size and morphology

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The MNM size and morphology in cell culture medium and synthetic digestive juices were evaluated by TEM. The images were recorded by a JEOL JEM 1011 microscope operating at an accelerating voltage of 100 kV. TEM samples were prepared by dropping a dilute solution of MNM in water on carbon-coated copper grids (Formvar/Carbon 300 Mesh Cu).

The performance of the TEM was evaluated using a calibration sample.

TEM analysis evaluation criteria: From each TEM specimen, micrographs of ten regions on the grid are recorded and analysed. At least 500 particles have been analysed per specimen.

Quantification of MNM dissolution by UF/ICP-AES

UF-ICPAES was used to quantify the amount of dissolved MNM in form of ions. This method consisted of a preliminary UF in which particulate component of testing batch was treated to separate the complex organic part from the soluble saline fraction through the use of Amikon 3K cut-off filters. The 3K cut-off filters were centrifuged at 4000g for 40 minutes at R.T. Subsequently, the filtrated soluble fraction were dissolved o.n. in 0.5 mL of concentrated HNO3 and diluted to 5 mL with MilliQ water. The resulting solution was then directly analysed by ICPAES.

To measure the amount of dissolved nanomaterial into both DMEM and RPMI, MNM were spiked at the final concentration of 6.8 µg/mL and incubated in 0.5 mL of biological medium, for 0, 24 and 48 hours.

Dissolution into digestive juices was observed, using two starting concentration (47.5 µg/mL and 950 µg/mL) and after the incubation for 0, 5, 125, 245 minutes in the saliva, stomach and intestine, respectively. Experiments were carried out in triplicate.

The performance of the ICP-AES was evaluated before each measurement using a Standard Tuning Solution to optimize the sensitivity of the instrument.

ICP-AES analysis evaluation criteria: calibration standards were used to construct a multipoint standard curve covering the range of analyte concentrations possibly present in the samples. Data that fall in this concentration range were considered valid.

Terms and definitions

Dissolution

Dissolution or solvation is the process of dissolving a solid substance into a solvent to yield a solution (http://sweet.jpl.nasa.gov/2.3/procChemical.owl#Dissolution).

Nanomaterial

A natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50 % or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm - 100 nm (EU, 2011. Available at: http://ec.europa.eu/environment/chemicals/nanotech/faq/definition_en.htm).

DLS

Dynamic light scattering (also known as photon correlation spectroscopy or quasi-elastic light scattering) is a technique in physics that can be used to determine the size distribution profile of small particles in suspension or polymers in solution (Berne, B. J., & Pecora, R. (2000). Dynamic light scattering: with applications to chemistry, biology, and physics. Courier Corporation).

UF

A separation process whereby a solution containing a solute of molecular size significantly greater than that of the solvent molecule is removed from the solvent by the application of hydraulic pressure which forces only

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the solvent to flow through a suitable membrane, usually having a pore size in the range 0.001- 0.1 mum (http://purl.obolibrary.org/obo/CHMO_0001645).

ICP-AES

Inductively coupled plasma atomic emission spectroscopy (ICP-AES), also referred to as inductively coupled plasma optical emission spectrometry (ICP-OES), is an analytical technique used for the detection of trace metals. It is a type of emission spectroscopy that uses the inductively coupled plasma to produce excited atoms and ions that emit electromagnetic radiation at wavelengths characteristic of a particular element. It is a flame technique with a flame temperature in a range from 6000 to 10000 K. It is also a solution technique & standard silicate dissolution methods are employed. The intensity of this emission is indicative of the concentration of the element within the sample (Stefansson, A., Gunnarsson, I., & Giroud, N. (2007). New methods for the direct determination of dissolved inorganic, organic and total carbon in natural waters by Reagent-Free™ Ion Chromatography and inductively coupled plasma atomic emission spectrometry. Analytica chimica acta, 582(1).

Zeta potential

The potential difference between the bulk dispersion medium (liquid) and the stationary layer of liquid near the surface of the dispersed particulate (http://purl.bioontology.org/ontology/MNMo#MNMO_1302).

Refractive index (Ri)

A realizable entity which is defined as the ratio of the speed of light in vacuum to that in a substance. It is a measure of the speed of light in the substance (http://purl.bioontology.org/ontology/MNMo#MNMO_1850).

Absorbance index (Rabs)

A dimensionless logarithmic unit assigned to a measure of absorbance of light through a partially absorbing substance, defined as -log10(I/I_0) where I = transmitted light and I_0 = incident light (http://purl.obolibrary.org/obo/UO_0000269).

Abbreviations

CTRL: sample control

DLS: Dynamic Light Scattering

TEM: Transmission Electron Microscopy

UF-ICPAES: Ultrafiltration - Inductively Coupled Plasma Atomic Emission Spectroscopy

DMEM: Dulbecco's Modified Eagle's Medium

RPMI: Roswell Park Memorial Institute Medium

FBS: Fetal Bovine Serum

MNM: Manufactured nanomaterials

R.T.: Room Temperature

Ri: Refractive index

Rabs: Adsorption index

SOP: standard operating procedure

Procedure description (SOP for dissolution test by a multi-technique based method)

The procedure is validated testing NM-300K, NM-200 and NM-110, reported here as examples

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Behaviour of MNM in biological media.

The evaluation of ions release and size/charge profiles from tested MNM (NM-300K, NM-200 and NM-110) was performed at 37°C in neutral conditions, namely at pH 7 for both biological media DMEM and RPMI. Media were complemented with 10% FBS, 1% Glutamine and 1% Penicillin/Streptomycin. MNM were dispersed according to Nanogenotox. MNM were then incubated into cell culture media at the final concentration of 6.8 µg/mL, at 0, 24, and 48 hours. At each time point, MNM were processed via UF-ICPAES and DLS/zeta pot analyses (see above).

Dissolution test of MNM

MNM were dispersed according to Nanogenotox. The evaluation of ions release and size/charge profiles from tested MNMs (NM-300K, NM-200 and NM-110) was performed at 37°C over two days. On the first day, the artificial juices were synthetized as described by Walczak et al, 2013 and also presented by D5.2. In the Table 2, are reported the organic and inorganic components of the juices, at the concentrations at which they must be prepared, in the final volume of 1 L, as well as their relative pH.

The pH of the total digestion juice (1 mL Saliva, 2 mL Gastric, 2 mL Duodenal, 1 mL Bile and 28 mg (26.5-29.5 mg) NaHCO3) had to be 6.5 ± 0.5. The solutions were stored overnight at R.T.

On the second day, the digestion was performed, pre-warming at 37°C the juices for 2 hours as following:

1 mL of MNM suspension was injected into a 50 mL tube, at the starting concentration of 47.5 µg/mL and 950 µg/mL and mixed to 6 mL of saliva, by shaking for 5 min at 37°C. Then, 12 mL of gastric juice were added, at the pH of 2.5 ± 0.5, for 125 minutes at 37°C. Finally, 12 mL of duodenal juice, 6 mL of bile juice and 2 mL of NaHCO3 (84.7 g/L) were added, at the pH of 6.5 ± 0.5, for 145 minutes at 37°C.

At each time point, (0, 5, 125 and 145 minutes) MNM were processed via UF-ICPAES and DLS analyses (see above).

Table 24: Composition of the salivary juices for *in vitro* digestion model.

MOUTH		
Ions	**stock concentration (g/L)**	**mL per 1 L total**
KCl	89.6	10
KSCN	20	10
$NaH_2PO_4 \cdot H_2O$	102.1	10
Na_2SO_4	57	10
NaCl	175.3	1.7
$NaHCO_3$	84.7	20
Organic compounds	**stock concentration (g/L)**	**mL per 1 L total**
Urea	25	8
	mg per 1 L total	
Uric acid	15	
Proteins	**mg per 1 L total**	
Amylase	290	
Mucin	25	

Table 25: Composition of the gastric juices for *in vitro* digestion model.

STOMACH

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Ions	stock concentration (g/L)	mL per 1 L total
NaCl	175.3	15.7
$NaH_2PO4{\cdot}H_2O$	102	3
KCl	89.6	9.2
$CaCl_2$	30.2	10
NH_4Cl	30.6	10
Organic compounds	**stock concentration (g/L)**	**mL per 1 L total**
Glucose	65	10
Glucuronic acid	2	10
Glucosaminehydrochloride	33	10
Urea	25	3.4
Proteins	**mg per 1 L total**	
BSA	1000	
Pepsin	2500	
Mucin	3000	

Table 26: Composition of the small intestine juices for *in vitro* digestion model.

SMALL INTESTINE		
DUODENUM		
Ions	**stock concentration**	**mL per 1 L total**
NaCl	175.3	40
$NaHCO_3$	84.7	40
KH_2PO_4	8	10
KCl	89.6	6.3
$MgCl_2 \cdot 6\ H_2O$	5	10
$CaCl_2$	30.2	5
Organic compounds	**stock concentration**	**mL per 1 L total**
Urea	25	4
Proteins	**mg per 1 L total**	
BSA	1000	
Pancreatin	9000	
Lipase	1500	
BILE		
Ions	**stock concentration**	**mL per 1 L total**
NaCl	175.3	30
$NaHCO_3$	84.7	68.5
KCl	89.6	4.2
$CaCl_2$	30.2	5.5
Organic compounds	**stock concentration**	**mL per 1 L total**
Urea	25	10

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Proteins	mg per 1 L total
BSA	1800
Bile	30000

Apparatus and chemicals

Equipment

Dynamic light scattering and Zeta potential.

A Zetasizer Nano ZSP (Malvern) equipped with a 10 mW He–Ne laser operating at 633 nm and an avalanche photodiode detector was used. Measurements were made at 25°C in water. Each sample was measured 10 times and the results were analysed by Malvern Instruments Ltd software.

ICP-AES.

Elemental analysis was carried out by inductively coupled plasma optical emission spectroscopy (ICP-AES) with a Agilent Technologies 700 Series system. Samples were incubated overnight in 0.5 mL of concentrated HNO3, diluted with MilliQ water, and then analysed by ICP-AES.

MNM

MNM were from repository list of JRC from NANOREG

NM-300K.

Ag MNM, with nominal size of 106 nm (DLS measured) and nominal charge of -11 mV.

NM-200.

Si MNMs, with nominal size of 207.8 nm (DLS measured) and nominal charge of -47.5 mV.

NM-110.

ZnO MNM, with nominal size of 275 nm (DLS measured) and nominal charge of 24.3 mV.

All MNM were dispersed according to NANOGENOTOX protocol upon probe sonicator. This protocol produces a highly dispersed stock solution of any MNM, by ethanol pre-wetting followed by dispersion in sterile-filtered 0.05% w/v BSA-water at a fixed concentration of 2.56 mg/mL according to the SOP provided by NANOREG.

Chemicals

Protein components

Amylase (#A6380), BSA (#A2153), Pepsin (#P7000), Pancreatin (#P7545), Lipase (#L3126), Bile (#B3883) were purchased from Sigma-Aldrich.

Mucin (#8494.1) was purchased from Roth.

Organic solutions

Urea, Glucose, Glucuronic Acid and Glucosamine hydrochloride were donated by RIKILT. Uric Acid (#U0881) was purchased from Sigma-Aldrich.

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Inorganic solutions.

KCl, KSCN, NaH2PO4·H2O, Na2SO4, NaHCO3, NaCl,CaCl2, NH4Cl, KH2PO4 batch solutions were kindly donated from RIKILT

Ultrapure grade water with a resistivity of 18.2 MΩ ·cm at 25°C, was used in all experiments (MilliQ).

Final Considerations

This guideline are in a proof of concept state and need to be further improved due to the small quantity of data available up to now. We still need to better evaluate the protocols by generating a wider amount of data possibly under validating conditions such as inter-laboratory comparison analysis.

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