Standard Operating Procedure

Title	***NRCWE Protocol for Sensor Dish Reader (SDR) analyses of MNM hydrochemical reactivity and dissolution in in vitro medium HAM's F12+10%FBS***
Subtitle	NA
NANoREG Work package/task:	WP2 Synthesis, supplying and characterization
Owner and co-owner(s)	NRCWE
Date finalised	
Document name	NANoREG D2.08 SOP 09 For SDR analyses of MNM hydrochemical reactivity and dissolution in in vitro medium
Key words:	Dissolution, rate, Hydrochemical reactivity, pH, O_2, redox, in vitro, incubation

Version	***Date***	***Reason of change***

This project has received funding from the European Union Seventh Framework Programme (FP7/2007-2013) under grant agreement no 310584

NRCWE Protocol for Sensor Dish Reader (SDR) analyses of MNM hydrochemical reactivity and dissolution in *in vitro* medium HAM's F12+10%FBS

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Introduction

This method and specific procedure is developed to measure the particle-induced reactivity and dissolution of a material (nanomaterial) during the exact *in vitro* conditions under which *in vitro* toxicological studies are performed. The method is currently under CEN standardization and the SOP applied here is modified from:

Jensen KA, Kembouche Y., and Nielsen SH, 2013. Deliverable 4.7: Hydrochemical reactivity, solubility, and biodurability of NANOGENOTOX nanomaterials. Edited by Jensen K.A. and Thieret N., March 2013, 60 pp. http://www.nanogenotox.eu/files/PDF/Deliverables/d4.7_hydrochemical_reactivity_and_biodurability.doc#_Toc351405986

Chemicals and Equipment

- *In vitro* cell medium Hams´F12. (see section)
- Esco ESCCCL-170B-8 Cell Culture Incubator (5% CO_2; 37°C)
- Sensor Dish Reader 305 and 315 (PreSens Precision Sensing GmbH, Germany)
- 1 Oxydish® OD24 for O_2 measurements (PreSens Precision Sensing GmbH, Germany)
- 1 Hydrodish® HD24 for pH measurements (PreSens Precision Sensing GmbH, Germany)
- Software: SDR_v4.exe (methods and data-logging) (or newer version)
- Computer for the SDR_v. 4.0 software (or newer version)
- Amicon® Ultra-4 Centrifugal Filters, cut off 3 kDa (Z740186)
- Micropipettes and tips
- Centrifuge (SORVALL RC 6+ with SH-3000 Swinging Bucket Rotor head with insert)
- Weighing scale (±0.1 mg or better) for weighing mass of liquid filtered into Amicon® tubes)
- 2% ultra-pure HNO_3 in nanopure water to prevent reprecipitation growth in filtered sample

The specific equipments listed above are not mandatory, but similar equipment is required

For all preparations, only verified elementally clean Nanopure water is used as the source of water. Elemental water analysis is needed to ensure purity of water.

Specifications of Nanopure Diamond UV water:

Purification system is designed to 4 stages of de-ionization combined with UV light-treatment:

Resistivity: ≤ 18.2 MΩ-cm at 25ºC

Pyrogens: < 0.001 EU/ml

Total Organic Carbon: < 3.0 ppb

Other: nuclease-free (RNase and DNase).

Filter: 0.2 μm filter (γ-irradiated Barnstead D3750 Hollow fiber filter)

Solubility and hydrochemical reactivity screening using the SDR system

The SDR system can only be used for monitoring the pH reactivity and O_2 variation in MNM dispersions with a pH range between 5 and 9. Real-time monitoring is done using the SDR incubation dishes mounted with

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sensors for online pH and O_2 concentration measurements. Relevant test conditions are maintained by placing the whole system in a cell incubation oven with a well-controlled atmosphere (37°C and 5% CO_2 air supply).

Protocol

The measurements are made in order to measure the chemical reactivity of the particles in hydrous media (here: cell media HAM´s F12+10% FBS). The end-points are pH (dpH = $pH_{test\text{-}material} - pH_{reference}$) and oxygen concentration (O_2 ($dO_2 = O_{2,test\text{-}material} - O_{2,reference}$)). The tests are run as duplicate exposures in one dose (0.32 mg/ml) for each substance. The experiments run 24 hours in 24-well trays with internal sensors.

1) Dispense proportionate volumes of media (HAMS´F12+10%FBS) in SDR pH (white sensor) and O2 (orange-red sensor) plates

- Use well A1 to A5 and B1 to B5 for the dose 0.32 mg/ml
- Level A: 1,750 mL; series B: 1,750 ml; range C: 2ml and series D: 2 ml media (HAM`sF12+10%FBS)
- The last well A6 to D6 is always 2 ml of pure media control

2) Place the SDR plates on the respective readers (carefully ensure that the SDR plates are placed on the correct readers). Check that they are in the right position and make three point measurements using the program "single measurement" command. - This is done to confirm that the sensors are working and also establish a general 0-reference.

- Press 3 times the "single measurement" with Tab enabled reader 305
- Press 3 times on "single measurement" with Tab enabled reader 315).
- Set a measurement on pause by pressing "Start / Stop"

3) Dispense the appropriate doses into the wells of pH and O_2 plates

- Start with the A1 to A5 and B1-B5: 0,250 mL
- C1-C5 , D1-D5 and A6-D6.: 0,0 mL

4) Place the pH and O_2 SDR plates on SDS read and check that they are in the right position

5) Start measurements by pressing the "Start Measurement"

The pH and O_2 monitoring rate is 5 min

Liquid samples are collected after 0.25 hours, and again at 1, 2, 4 , and 24 hours duration into the experiment, 2 mL from A1 and 2mL from B1 of the dissolution medium for immediate 3 KDa centrifuge* and subsequent ICP-MS analysis

1. After 24 hours of incubation, Immediately withdraw the last 2 x 2 mL supernatant for ultracentrifugation* and subsequent ICP-MS analysis

7) After a total of 24 hours, end the measurement.

- stop data recording

Take the SDR plates out of the incubator at the end of the experiment (24-hour reaction)

Filtration and centrifugation

3 KDa centrifuge filtration is conducted immediately after each sampling of supernatant. The centrifugation is completed for 30 minutes at 4.000G (RCF) and fast acceleration (9) and deceleration (9). Immediately after filtration, the filter is removed from the vial and the vial is weighed with the screw-cap on. The weight is recorded (m_2) and $m_2 - m_1 = m_{sample}$. The m_{sample} is added 0,5 mL 2%

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ultrapure HNO_3 for stabilization of the supernatant and to prevent growth during storage. Samples are analyzed as soon as possible using either XRF, AAS or ICP-MS

6) The liquid samples are stored in darkness at room temperature until analyzed.

Test medium (HAM´s F12):

The test medium used for the 24-hour reactivity and dissolution studies is HAM´s F12+10%FBS. To prepare the dispersion, add FBS and pen/Strep to avoid growth, and filter the solution through a 0,8 µm sterile filter. The final solution is placed in darkness in the refrigerator for max 2 weeks.

Composition of the HAMs F12 cell medium

HAM's F12:

Gibco by life technologies 1x nutrient mix

F12-Nut Mix (Ham) (1x)

F12 nutrient Mixture (Ham)

(+) L-Glutamine

Pen/strep:

Penicillin/Streptomycin (10000U/ml/10mg/ml)

FBS:

Foetal Bovine Serum USA grade

150 mL of Hams F12 medie_

1.5 mL pen/strep

15 mL Inactive FBS to make10% Hams F12 medium at 150ml

Data treatment

The SDR pH, O_2 and temperature data are expported as #.txt files to enable data handling using e.g., EXCEL. The time-matched data obtained in the exposed wells and reference medium wells are used to calculate the effect on pH ($dpH = pH_{2,\text{test material}} - pH_{2,\text{medium control}}$) and O_2 ($dO_2 = O_{2,\text{test material}} - O_{2\text{medium control}}$) and to plot this data as function of time.

The dissolution data are analysed as suitable for the purpose of the investigation. Data may be plotted as function of time corrected or not corrected for medium and potential dissolution incurred during sample preparation and dosing.

An example of results are given below in Figure 1

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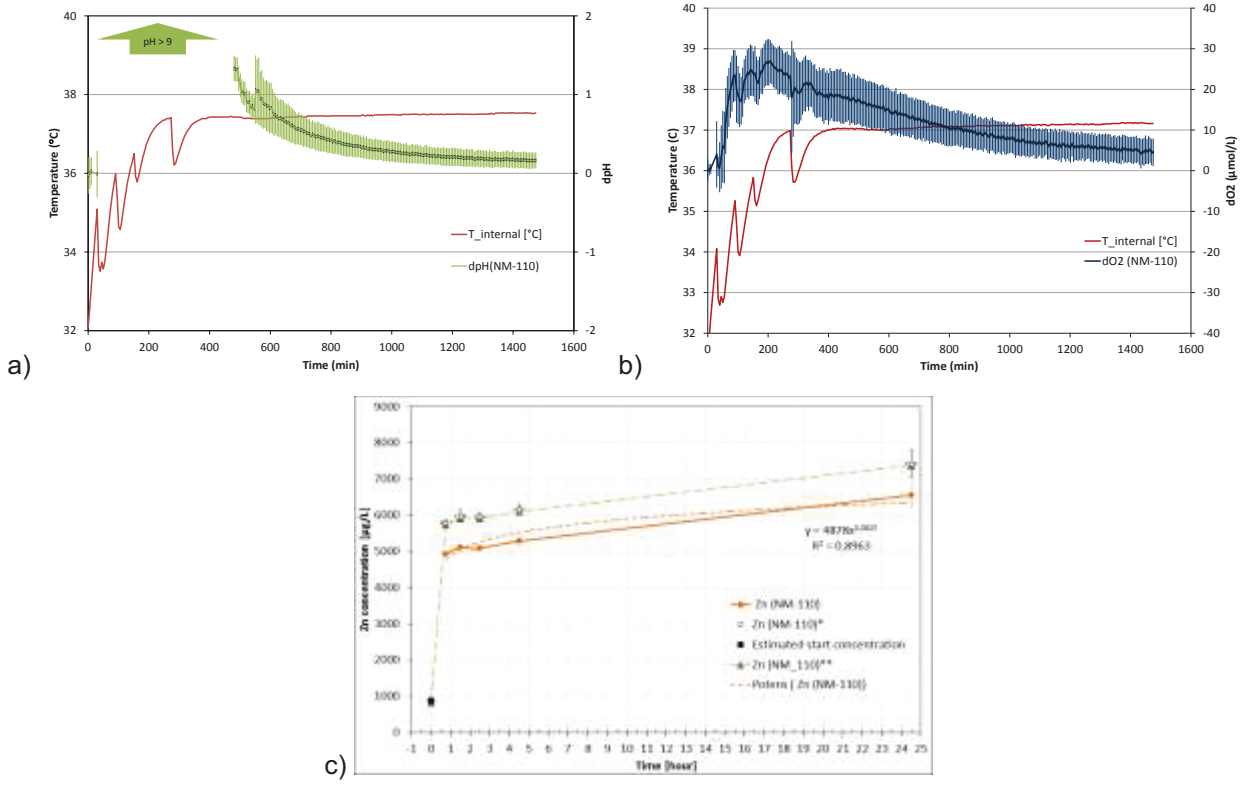

Figure 1. Example of results obtained by using the SDR system testing NM-110. A) Variation in pH (dpH) as function of time. B) Variation in dO2 as function of time. C) Variation in the concentration of Zn in cell medium during incubation, plotted as function of time.

Comments on use and applicability

The method is currently under establishment. There is no previous similar method. Therefore comparison and validation of measurement data are based on comparisons with conventional potentiometric measurement techniques for pH and redox potential.

Test conditions are currently mimicking *in vitro* testing, but in principle other conditions can be tested as well as well as different durations of testing. Longer test durations may be required to investigate potential alteration of low to very-low soluble MNM.

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