

Towards a method for detecting the potential genotoxicity of nanomaterials



Standard operating procedures for
characterization of the selected manufactured
nanomaterials types

Report

Standard operating procedures for
characterisation of the selected manufactured nanomaterials
and dispersions thereof
June, 2011



This document arises from the NANOGENOTOX Joint Action which has received funding from the European Union, in the framework of the Health Programme under Grant Agreement n°2009 21. This publication reflects only the author's views and the Community is not liable for any use that may be made of the information contained therein.



Grant Agreement n° 2009 21 01

WP 4 : Physicochemical Characterisation of Manufactured Nanomaterials (MNs) and Exposure Media (EMs)

Deliverable 2: Standard operating procedures for characterisation of the selected MN types

Deliverable leader : NRCWE

Keld Alstrup Jensen

The National Research Centre for the Working Environment (NRCWE),

Lersø Parkallé 105, DK-2100 Copenhagen, DENMARK



National Research Centre
for the Working Environment

Workflow	
<p>Author(s) Keld Alstrup Jensen, Per Axel Clausen, Renie Birkedal, Yahia Kembouche, Elzbieta Christiansen, Nicklas R Jacobsen, Marcus Levin, Ismo Koponen and Håkan Wallin (NRCWE) Pieter-Jan de Temmerman and Jan Mast (VAR) Camille Guiot and Olivier Spalla (CEA) Charles Motzkus (LNE) Boris Shivachev (CLMC) Davy Rousset and Olivier Witschger (INRS)</p>	<p>Reviewer(s) WP Leader: Keld Alstrup Jensen (NRCWE) Coordinator: Nathalie Thieret (ANSES)</p>

Document status: V.2 (Final)
 Creation date: 27/04/2011
 Completion date: 27/06/2011

Confidentiality level of the deliverable		
PU	Public	PU
CO	Confidential, only for members of the consortium (including the Commission Services)	

Contents

1. INTRODUCTION.....	6
2. NM DISPERSION.....	7
2.1 LIQUID DISPERSION IN ISOPROPANOL	8
2.1.1 General description	8
2.1.2. Preparation of dispersion.....	8
2.1.3. Particle dispersion.....	8
2.1.4 Comments on use and applicability.....	8
2.2 LIQUID DISPERSION USING ETHANOL PRE-WETTING AND BSA-WATER.....	8
2.2.1 General description	8
2.2.2 Preparation of stock dispersion.....	8
2.2.2 Production of sterile-filtered BSA water	10
2.2.3 Weighing nanomaterial powder (suggested procedure).....	11
2.2.4 Particle dispersion.....	13
2.2.5 Probe Sonication	13
2.2.6 Quality control.....	14
2.2.7 Comments on use and applicability.....	14
2.3 LIQUID DISPERSION BY pH-ADJUSTMENT AND STABILIZATION BY BSA-WATER.....	15
2.3.1 General description	15
2.3.2 Equipment needed.....	16
2.3.3 Procedure	16
2.3.4 Dispersion procedure for TiO ₂ nanomaterials.....	17
2.3.5. Dispersion procedure for SiO ₂ nanomaterials	18
2.3.6 Comments on use and applicability.....	19
2.4 DRY DISPERSION BY SMALL ROTATING DRUM DUSTINESS TESTING	19
2.4.1 General description	19
2.4.2. Materials and methods	19
2.4.3 Comments on use and applicability.....	23
3. PARTICLE SIZE-DISTRIBUTION ANALYSIS BY TRANSMISSION ELECTRON MICROSCOPY	24
3.1. PRIMARY PARTICLE SIZE DISTRIBUTION OF TiO ₂ AND SiO ₂ BY TEM	24
3.1.1 General description	24
3.1.2 Materials and Chemicals.....	24
3.1.3 Sample preparation	24
3.1.4 Data treatment.....	25
3.1.5 Comments on use and applicability.....	25
3.2. AGGREGATE/AGGLOMERATE SIZE DISTRIBUTION OF TiO ₂ AND SiO ₂ BY TEM.....	25
3.2.1 Description of method and data collection.....	25
3.2.2 Materials and Chemicals.....	25
3.2.3 Sample preparation	26
3.3.4 Data treatment.....	26
3.3.5 Comments on use and applicability.....	26
3.3 SEMI-AUTOMATIC DETECTION AND IMAGE ANALYSIS OF NANOPARTICLES.....	27
3.3.1 General description	27
3.3.2 Equipment.....	27
3.3.3 Instruction.....	27
3.3.4 Select classification parameters and export results.....	31
3.3.5 Comments on use and applicability.....	32
3.4 COATING OF DISPERSED NANOPARTICLES IN LIQUID ON GRIDS FOR TEM ANALYSIS	32
3.4.1 General description	32
3.4.1 Definition, abbreviations, references and norms.....	33
3.4.2 Materials and equipment.....	33
3.4.3 Instruction for coating and preparation of TEM samples.....	33

3.4.3 Storage of the grids.....	34
3.4.4 Specific safety measures	34
3.4.5 Comments on use and applicability.....	35
4. DETERMINATION OF SPECIFIC SURFACE AREA.....	36
4.1 NITROGEN ADSORPTION BY THE BRÜNAUER, EMMETT AND TELLER (BET) METHOD	36
4.1.1 General description	36
4.1.2 Materials and chemicals.....	37
4.2.3 Sample preparation	38
4.2.4 Measurement	38
4.2.5 Observations on use and applicability.....	39
4.2 SMALL-ANGLE X-RAY SCATTERING (SAXS) AND ULTRAFINE SAXS (USAXS)	39
4.2.1 General description	39
4.2.2 Materials and chemicals.....	40
4.2.3 Sample preparation	41
4.2.4 Measurement	42
4.2.5 Raw data treatment.....	43
4.2.6 Data analysis.....	44
4.2.6 Data fits	46
4.2.7 Observations on use and applicability.....	46
5. SURFACE CHARGE AND SIZE DISTRIBUTION ANALYSIS OF DISPERSIONS	47
5.1 SURFACE CHARGE AND ISOELECTRICAL POINT OF TiO ₂ AND SiO ₂ BY ZETOMETRY	47
5.1.1 General description	47
5.1.2 Chemicals and equipment.....	48
5.1.3 Procedures.....	48
5.1.5 Comments on use and applicability.....	50
5.2 DYNAMIC LIGHT-SCATTERING.....	51
5.2.1 General description	51
5.2.2 Chemicals and Equipment	51
5.2.3 Procedure	52
5.2.4 Observations on use and applicability.....	58
6. STRUCTURAL ANALYSIS	59
6.1 GENERAL SOP FOR PHASE IDENTIFICATION AND DETERMINATION OF CRYSTALLITE SIZE USING POWDER X-RAY DIFFRACTION ANALYSIS.....	59
6.1.1 General description	59
6.1.2 Materials and equipment	59
6.1.3 Sample preparation	59
6.1.4 General data treatment.....	60
6.1.4 Comments on use and applicability.....	60
6.2 IDENTIFICATION AND QUANTIFICATION OF CRYSTALLINE PHASES OF TiO ₂ POWDERS CRYSTALLITE SIZES USING THE X'PERT PRO MPD DIFFRACTOMETER	60
6.2.1 General description	60
6.2.2 Materials and methods	61
6.2.3 Preparation of the samples :.....	61
6.2.4 Measurement protocol.....	61
6.2.5 Observations on use and applicability.....	62
6.3 RAMAN SPECTROSCOPY	62
6.3.1 General Description	62
6.3.2 Materials and Chemicals.....	62
6.3.3 Sample preparation:	62
6.3.4 Analytical conditions and data collection	62
6.3.5 Data treatment.....	62
6.3.6 Comments on use and applicability.....	62
7. CHEMICAL ANALYSIS	63

7.1 THERMOGRAVIMETRIC ANALYSIS (TGA)	63
7.1.1 General description	63
7.1.2 Materials and Chemicals:	63
7.1.3 Procedure	63
7.1.4 Data treatment:	64
7.2 QUANTITATIVE ELEMENTAL ANALYSIS USING INDUCTIVE COUPLE PLASMA MASS SPECTROMETRY ANALYSIS OF IMPURITIES IN CNTs USING ICP/MS	64
7.2.1 General description	64
7.2.2 Materials and equipment	64
7.2.3 Samples pre-treatment	64
7.2.4 Impurity analysis	64
7.2.5 Comments on use and applicability	65
7.3 IDENTIFICATION AND ANALYSIS OF ORGANIC COATING	65
7.3.1 General description	65
7.3.2 Proposed procedure for identification of an organic fraction in unknown NM.	65
7.3.3 Comments on use and applicability	69
8. HYDROCHEMICAL REACTIVITY	70
8.1 OH-RADICAL FORMATION CAPACITY BY PARTICLES IN WATER USING BENZOIC ACID AS PROBE	70
8.1.1 General description	70
8.1.2 Materials	70
8.1.3 Sample preparation;	70
8.1.4 HPLC-analysis	71
8.1.5 Calculation of \cdot OH-formation capacity	71
8.1.6 Comments on use and applicability	71
8.2 pH- AND ATMOSPHERE-CONTROLLED STIRRED BATCH-REACTOR HYDROCHEMICAL AND BIODURABILITY TESTER	71
8.2.1 General description	71
8.2.2 Chemicals and Equipment	71
8.2.3 Procedure	72
8.2.4 Data treatment	75
8.2.5 Comments on use and applicability	75
9. REFERENCES	76

1. Introduction

This report fulfills deliverable 3 Standard operating procedures for characterization of the selected MN types. The report also includes methods for analysis of MN dispersions.

The report includes a presentation of the first standard operation procedures (SOPs) applied for the initial physicochemical characterization of the manufactured nanomaterials selected for the NANOGENOTOX project. The report focuses on applied and proposed sample preparation and characterization procedures. One of the major initial tasks was to identify or develop good methods for dispersing particles for specific physico-chemical analysis. Establishment of these common methods became the first important internal milestone. Pre-dispersion of particles is particularly important for size and charge analysis using photon correlation spectroscopy (PCS) alias dynamic light scattering (DLS) of suspensions and proper sample preparation for e.g., electron microscopy, atomic force microscopy.

Even though NM characterization is not a new scientific discipline, method development is often still required to obtain reliable and comparable characterization values. For example, official OECD SOPs are not suitable or need to be revised to enable characterization NM and their properties (OECD, 2009). ISO methods may also need revisions to achieve high quality results. This is exemplified by on-going developments in quantitative sizing by transmission electron microscopy.

For the initial characterization, the procedures applied in NANOGENOTOX were generally selected or modified from already used in-house SOPs in the different participating laboratories. The SOPs were subsequently evaluated internally for applicability and challenges were identified. Cross-comparison of data is the second stage of the evaluation and not part of this report.

2. NM Dispersion

Particulate matter may be dispersed in liquids or air for subsequent analysis. The efficiency and suitability of the methods depends on the type and size of the particulate matter as well as the analytical method to be employed. For liquid dispersions, stable liquid dispersions can be obtained into three different principle ways: 1) steric, 2) depletion (or polymeric) or 3) charge-stabilization. In method 1 and 2, the dispersion is stabilized using e.g. proteins or polymers, whereas surface charge is the causing stabilization in method 3 (Figure 2.1). Finally, there is a mixed type called, electrosteric stabilization.

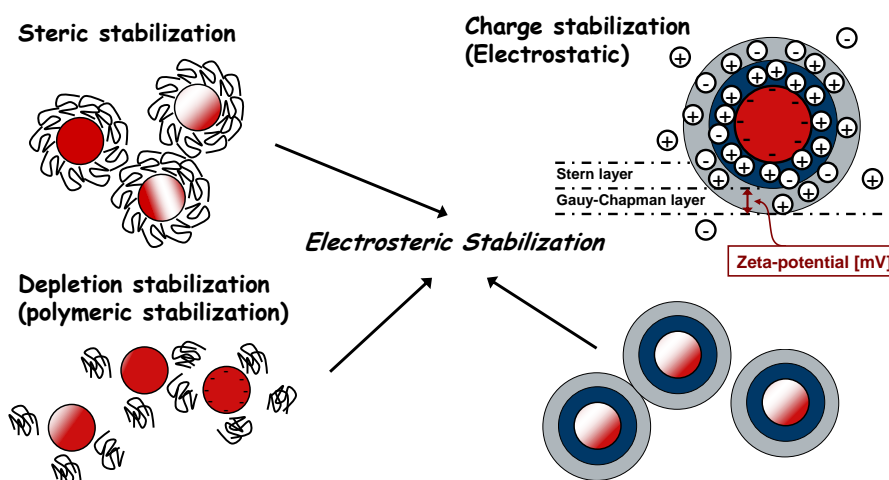


Figure 2.1. Principle types of stabilizing particles in liquid dispersions and the mixed electrosteric stabilization. For charge-stabilization, the different ionic layers are described with indication of characteristics resulting in a zeta-potential.

Particles may also be aerosolized to obtain a dust particle size-distribution. The size-distribution of dust may vary depending on e.g., the energy input as well as the particle concentration and residence time in the air.

In NANOGENOTOX, we have applied three different principle methods for achieving NM dispersion in liquids and two methods will be used for dust size-distributions by different types of dustiness testing of which one of the SOPs is presented in this report and the other is under final development.

2.1 Liquid dispersion in isopropanol

Davy Rousset (INRS),

2.1.1 General description

This is a simple method for particle dispersion, which may be applied for preparation of samples for characterization of general characterization and determination of primary particle size by scanning and transmission electron microscopy, atomic force microscopy as well as analysis by other wet-methods such as DLS.

2.1.2. Preparation of dispersion

Materials and methods

Ultrasonic bath (Annemasse)
2-propanol grade analysis
50 ml plastic vials (alternatively glass-vials can be used)
Spatula

2.1.3. Particle dispersion

A small amount (<0.1 mg) of powder (spatula tip) is added to about 15 ml isopropanol in a glass vial. The suspension is then treated 10 min in a ultrasonic bath to enhance NM's dispersion.

2.1.4 Comments on use and applicability

The procedure and additional detail of preparation is under evaluation for dispersing NM for electron microscopy.

2.2 Liquid dispersion using ethanol pre-wetting and BSA-water

Keld Alstrup Jensen, Yahia Kembouche, Elzbieta Christiansen, Nicklas Raun Jacobsen, & Håkan Wallin (NRCWE); Camille Guiot (CEA); Olivier Witschger (INRS)

2.2.1 General description

This SOP was initially developed for preparation of general batch dispersion protocol for in vitro and in vivo toxicity testing in NANOGENOTOX. The method aims to produce a highly dispersed state of any NM by ethanol prewetting to handle hydrophobic materials followed by dispersion in filtered 0.05% w/v BSA-water for stable to metastable steric to electrosteric stabilization at a fixed concentration of 2.56 mg/ml. The procedure was subsequently adopted for sample preparation for various size distribution analysis in the NANOGENOTOX project. The protocol is developed with the aim to use or characterize the dispersion immediately after its production.

2.2.2 Preparation of stock dispersion

A 2.56 mg/ml stock dispersion is prepared by prewetting powder in 0.5 vol% ethanol ($\geq 96\%$ purity) followed by dispersion in 0.05 wt% BSA-water during 16 minutes of probe sonication. For harmonization of dispersion energy and stabilities it is recommended to produce a 6-8 ml dispersion

in the 20 ml tall glass scintillation vials. It takes 15.36 mg to produce a 6 ml 2.56 mg/ml stock dispersion (see below for further details).

Materials

Pure and sterile-filtered water
Bovine Serum Albumin (sterile)
Ethanol (96 vol%)

1 flask for batch dissolution of BSA
1 flask for sterile-filtered 1% w/v stock BSA-water solution
1 flask for 0.05% w/v BSA-dispersion medium

Sterile filter (0.2 µm)

Vials: 20ml Scint-Burk glass pp-lock+Alu-foil (WHEA986581; Wheaton Industries Inc.) for weighing out NM and sonication [revision for precision of original draft protocol]

Steel and glass spatula

Pipette and pipette tips

Weighing boat/weighing paper

Electrostatic neutralizer

Weigh

Control or reference weights

Probe sonicator

Ice (Ice-water)

Water

It is recommended to use the purest available water. The protocol has been tested using both Nanopure Diamond UV and Millipore MilliQ-filtered water. No differences were observed in the quality of the batch dispersions. However, if chemical analyses of especially Fe and Zn are to be conducted, it may be recommended to use Nanopure or controlled water because water from some Millipore systems has been found to be contaminated with these elements at promille levels even after mounted after de-ionization units.

Quality control of the water may be done before use. Especially in case that analysis and experiments may be influenced by trace-elements at low concentrations.

For general sampling and validation, we suggest collection of water in acid cleaned chemically stable bottles suitable for element chemical analysis. Control the water quality (e.g., particles by DLS, elemental concentration, CFU and endotoxin) before use. If the water sample passes the quality test, the water may be evaluated pure and used for the experiment.

a. Nanopure Diamond UV water:

The water purification system is designed to 4 stages of de-ionization combined with UV light-treatment.

Resistivity: $\leq 18.2 \text{ M}\Omega\text{-cm}$ at 25°C

Pyrogens: $< 0.001 \text{ EU/ml}$

Total Organic Carbon: $< 3.0 \text{ ppb}$

Other: nuclease-free (RNase and DNase).

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

b. MilliQ-water:

The Millipore water purification system is designed to filter water that has already been de-ionized.

Resistivity: 18.2 M Ω -cm at 25°C

Pyrogens: <0.02 EU/ml

TOC: 5-10 ppb

1-5 ppb

2-5 ppb

Silicates: <0.1 ppb

Heavy metals \leq 0.1ppb

Microorganisms: \leq 1 cfu/ml

Filter: 0.22 µm MilliPark Gammagold (Millipore)

Bovine Serum Albumin (BSA)

Several types of albumin may be selected to fit specific toxicological test protocols. It is important that the selected albumin has passed tests for purity and sterility.

This protocol was developed using:

Bovine Serum Albumin (Fraction V), Sigma (catalogue number: A-9418)

Cell culture tested

Analysis: 16 wt% Nitrogen and 1.9 wt% H₂O.

Number of amino acid residues: 607

Molecular weight: 66,399 Da

Isoelectric point in water at 25 °C: pH = 4.7

Physical dimensions: 140 x 40 x 40 Å³ (prolate ellipsoid where a = b < c)

2.2.2 Production of sterile-filtered BSA water

The production of the 0.05% w/v BSA-water (the dispersion medium) is done in two steps: 1) Preparing a sterile-filtered 1% w/v BSA stock solution and 2) Dilution to reach a 0.05% w/v BSA dispersion medium.

a. Procedure for making a 1% w/v BSA water stock solution:

- 1) Add from pipette 50 ml water Nanopure (or MilliQ) to a 100 ml mixing flask (e.g, re-useable acid-washed blue-cap flasks).
- 2) Weigh out 1 g BSA (powder) in a weighing boat and pour it into the flask with 50 ml water, rinse the weighing boat into the mixing bottle with Nanopure (or MilliQ) water to retrieve as much BSA as possible into the mixing flask.
- 3) Fill Nanopure water (or MilliQ) into the mixing flask up until 100 ml to reach a 1 % w/v BSA-water solution.
- 4) Gently stir or swirl the BSA-solution for a few minutes (be careful to avoid foam by not using agitated stirring) and leave the mixing flask in the refrigerator over-night for complete dissolution of the BSA.
- 5) Sterile filter the solution into a new flask through a 0.2 µm sterile disposable filterware with collection flasks after complete dissolution of BSA in the mixing flask. A subsequent step of

sterile filtration of the volume to be used for each toxicity test is recommended to ensure no bacterial contamination in the tests. Sterile filtration causes about 28% loss of BSA resulting in a true BSA concentration of 0.036% w/v in the stock dispersion as determined by a Pierce BCA protein Assay Kit for microplate reading.

After sterile filtration the BSA stock solution can be kept in a refrigerator for 2 weeks.

b. Procedure for making a 0.05% w/v BSA-water solution for nanomaterial dispersion:

The 0.05% w/v BSA solution to be used for test item preparation is achieved by simple dilution of the 1% w/v batch solution.

Example: 2 ml 1 % w/v BSA is diluted with 38 ml Nanopure water (or MilliQ) (Dilution factor = 20x) to reach a batch solution of 0.05 w/v%.

2.2.3 Weighing nanomaterial powder (suggested procedure)

Weighing should be done in a ventilated weighing box, a glove box or a fume hood designated for sensitive weighing with an accuracy of at least 0.1 mg or better.

Materials

Microweigh with accuracy of 0.1 mg or better

Reference or control weights with masses within the scale the weighing project

Wet and dry wipes for cleaning

Weighing boat

Steel and glass spatula's

Vials: 20ml Scint-Burk glass pp-lock+Alu-foil (WHEA986581; Wheaton Industries Inc.)^a

Vials with nanomaterials

Tray for storage of vials

Argon source.^b

^a Can be re-used after acid washing or regular washing followed rinsing in ethanol. Add new pp-lock+Alu-foil lids.

^b Maybe needed for handling NM-series materials distributed from JRC. See below.

Preparation of weighing area.

- 1) Turn on the weighing box, glove box, fume-hood 15-30 minutes before use.
- 2) Ensure wearing appropriate work dress (two- or three layer gloves*, lab-coat, laboratory shoes) and that personal respiratory protection equipment is easily accessible in case of accident.
- 3) Ensure all material to be used for weighing and storage is present (nanomaterials, bottles/vials for weighing material in, cleaning tissue (both wet and dry wipes) before commencing the work.
- 4) Calibrate the weigh with traceable reference weight and log the data. Check that accuracy is within acceptance.

** It is recommended to use two- or three layers of gloves for dermal protection. 1) Inner glove in textile 2) and or inner glove using long powder free nitril or latex rubber glove, 3) powder free nitril glove.*

Weighing out the nanomaterial

- 5) a. Open a clean empty vial for preparation of the stock dispersion and place it on the weigh.
- b. Tara the weigh.
- 6) Carefully open the vial without shaking it (NM-materials are packed in argon atmosphere and a special strategy may required for weighing and potential re-use for NM-samples – see below).
- 7) Remove the electrostatic charge on the vial using a neutralizer (e.g., ionization blower) and carefully weigh out the required mass with a spatula in steel or glass.
- 8) Close the lid on both vials
- 9) After completion of weighing materials, clean the weigh and work area for potential spills using wet and dry wipes.
- 10) Waste is packed in a suitable waste bag and discarded according to local or institutional directions.
- 11) Ventilate the work-area (ventilated weighing station, fume hood, glove box etc.) for 15 minutes after weighing and cleaning has been completed.

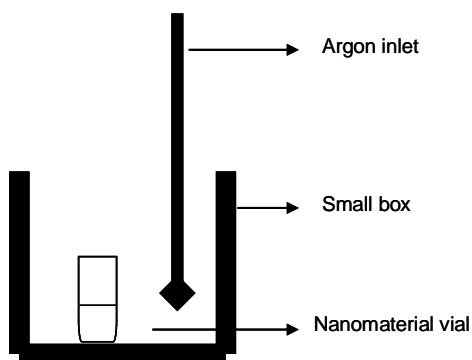


Figure 2.1. Illustration of a simple setup for sample retrieval and re-sealing NM material vials under argon.

Calculations for preparing the 2.56 mg/ml stock dispersion

For preparing a 2.56 mg/ml stock dispersion in a 6 ml EtOH and BSA-water, each vial must contain at least 15.36 mg nanomaterial. For harmonization of the dispersion energy it is recommended to stay as close as possible to 6 ml EtOH and BSA-water.

Calculation of the correct volume is done simply according to equation 1:

$$1: V = m / c$$

m = mass of nanomaterial (mg)

c = concentration (normally 2.56 mg/ml)

V = volume of dispersion medium (ml)

6 ml EtOH + BSA-water is required to disperse 15.36 mg powder.

0.5 vol% EtOH (96% or higher) is used for pre-wetting: $6 \text{ ml} \times 0.5/100 = 0.03 \text{ ml}$ (30 μl) EtOH

99.5 vol% sterile-filtered BSA-water (0.05% w/v) is used as dispersion medium: $6 - 0.03 = 5.97 \text{ ml}$ BSA-water

2.2.4 Particle dispersion

Particles are pre-wetted using ethanol (EtOH). This procedure was previously introduced to enable dispersion of hydrophobic materials in water-based systems^[1]. In this protocol, we have introduced EtOH pre-wetting for all materials to harmonize the treatment for all powder materials.

Pre-wetting procedure (volumes for 15.36 mg powder)

- 1) Carefully open the glass scintillation vial with pre-weighed powder (ideally 15.36 mg).
- 2) Tilt the scintillation vial ca. 45° and add 30 µl EtOH drop-by-drop onto the particles in the vial by pipette.
- 3) Screw on the lid and gently mix the EtOH and powder by simultaneous gently tapping the vial on the table-top while rotating the tilted 45° vial from side to side between your fingers for approximately one minute.
- 4) Add 5,970 µl 0.05 % BSA water by pipette while slowly rotating and swirling the 45° tilted scintillation glass. Be careful to avoid foaming of BSA. The last ml BSA-water or so is added along the top of the inner wall of the vial to collect the powder in the fluid at the vial bottom.

2.2.5 Probe Sonication

The protocol was developed using a 400 Watt Branson Sonifier S-450D (Branson Ultrasonics Corp., Danbury, CT, USA) equipped with a standard 13 mm disruptor horn (Model number: 101-147-037). The horn can be seen at www.sonifier.com/pdf/DISRUPTO.PDF and the sonifier can be seen at http://www.sonifier.com/s450_digital.asp.

The particle dispersions are continuously cooled in an ice-water bath to minimize heat development during sonication (Fig. 2.2).

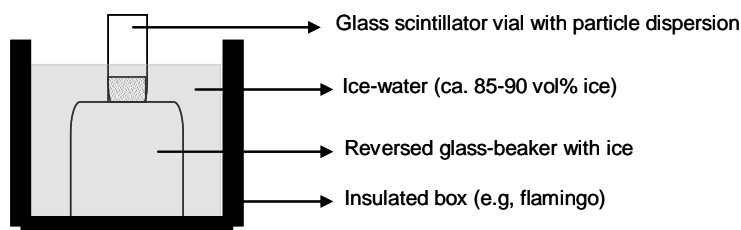


Figure 2.2. Illustration showing a principle set-up for placement of sonication vial in ice-water bath. Different configurations are possible.

Sonication procedure

- 1) Fill a 250 ml glass beaker with ice and place it upside-down in a insulation box (flamingo)
- 2) Add ca. 85-90 vol% ice into the insulation box
- 3) Add ca. 10-15 vol% cold water into the insulation box
- 4) Carefully place the glass scintillation vial with powder on top of the upside-down glass-beaker and pack the ice-water around the vial to keep the dispersion cooled.
- 5) Insert the sonication probe (between the upper quarter and upper half of the BSA-water volume in the scintillation beaker (e.g., find the correct height using a vial with BSA-water alone).
- 6) Start sonication and run it for 16 min at 400 W and 10% amplitude while controlling that the sonication probe does not touch the walls of the scintillation vial.
- 7) Remove the scintillation vial and add the lid.

- 8) Clean the sonication probe by sonication for 5 minutes (similar sonication settings) with the probe fully immersed in a 50:50 water-EtOH (>96%) mixture followed by rinsing in EtOH using a dispenser and a collection bottle underneath. The probe is allowed to air-dry in the fume-hood.

Sonication energy

To enable comparison of sonication energy between different instruments, the temperature increase during 16 min sonication of 6 ml un-cooled Nanopure water was measured in two triplet runs (Table 3.1). The result showed that the average temperature increase of Nanopure water in the scintillation vial was $72.7 \pm 0.8 - 22.9 \pm 1.6 = 49.8 \pm 1.6^\circ\text{C}$ (Table 3.1) corresponding to $1.25 \cdot 10^3 \text{ J}$ according to the equation $Q = c \cdot m \cdot \Delta T$, where c is the specific heat capacity for the material (4.187 J/kgK for water), m is the mass of water, and ΔT is the temperature difference. In this calculation we neglected the heat loss from surfaces during the sonication procedure.

Table 2.1. Start and end temperature of Nanopure water after sonication of 6 ml at standard protocol settings

Sonication series 1	A1	B1	C1
Ti initial i °C	20.9	24.1	25.1
Tf final i °C	71.2	72.7	73.4
$\Delta T = T_f - T_i$ i °C	50.3	48.6	48.3
Sonication series 2	A2	B2	C2
Ti initial i °C	21.9	21.8	23.5
Tf final i °C	72.8	72.8	73.1
$\Delta T = T_f - T_i$ i °C	50.9	51.0	49.6

2.2.6 Quality control

After sonication, the dispersions should be checked. The **dispersion is quickly characterized using dynamic light-scattering techniques, DLS** (see SOP for dynamic light scattering).

Fast **indication of dispersion quality** can also be obtained by optical microscopy. For preparation of slides for optical, place e.g. 10 μl on an objective glass and add a cover glass. Quickly check the dispersion by optical microscopy to assess the presence of large agglomerates and aggregates. The presence of such large particles should be relatively low and well-dispersed particles are partly visible at high magnification by vibrating small dots.

2.2.7 Comments on use and applicability

For sample storage and handling, the original protocol erroneously wrote use nitrogen under the materials and chemicals section for keeping samples in inert air. Nitrogen gas is slightly lighter than air and is therefore not recommended as the inert gas for the sample vials. It should have said Argon, which is also written under the procedures and recommended by JRC in order to avoid any surface reaction.

Regarding vials for sonication, it has been found that the sonication efficiency and dispersion stability may vary with the vial type. It is now not only suggested, but enforced for harmonization that batch dispersions are made in 20ml Scint-Burk glass pp-lock+Alu-foil (WHEA986581; Wheaton Industries Inc.). Increased stability may sometimes be achieved by using polymer vials instead (see section 3.3)

Regarding sonicators and sonication energies, experience has shown that some probe sonicators has 20% amplitude as the minimum setting and different powers. In this case, one should keep the probe dimensions and reduce sonication time to 12 minutes. Matching exact energy used for the sonication

can be done using a watt-meter, which will be described in an upcoming report: The NANOGENOTOX batch dispersion protocol with analysis of size distributions and stabilities of NM batch dispersions.

The SOP contains a note on how NANOGENOTOX nanomaterials may behave. It is important to note that the generic protocol may not always be the most optimal dispersion technology, but ensures that the dispersions are reasonable and made in the same way with the same amount of dispersants etc. It should also be noted that NM's are often aggregated and agglomerated and dispersion of these are not always possible or relevant.

Regarding quality control of the dispersions it will be clarified that the magnification in optical microscopy can normally not exceed 1000 times. This means that it is just possible to detect a 100 nm particle if the refractive index is different from the medium and the microscope has good contrast properties. The use of conventional optical microscopy is only suggested for fast evaluation of large particles and agglomerates.

2.3 Liquid dispersion by pH-adjustment and stabilization by BSA-water

Camille Guiot and Olivier Spalla (CEA)

2.3.1 General description

This method may be used for determination of aggregate size in individually designed particle water/BSA suspensions (steric stabilization). Initial dispersion of NM is achieved by sonication under electrostatic stabilization conditions, followed by addition of BSA according to the specific surface area and final adjustment of pH to physiological conditions (e.g., pH 7.4).

The considerations in this protocol are based on the physico-chemical properties of both BSA and the NM to be tested. Bovine serum albumin is a protein of about 66 kDa. Its charge varies depending on pH, being positive for acidic pH and negative for basic pH with an inversion of charge or isoelectric point (IEP) at pH about 4.5-5 (depending on authors). In its native conformation (from pH 4 to 7) the protein forms an equilateral triangle of 8 nm side, with a thickness of 3 nm.

BSA adsorbs on the surface of oxide nanoparticles, therefore its addition to suspensions modifies their stability and surface charge properties over the pH range and the available surface area. For example, stabilization of TiO₂ suspensions can be achieved at pH 7 (near IEP of TiO₂) by addition of BSA, with a negative zeta potential. However, the adsorption of BSA is partly irreversible, so the conditions of addition (pH, order of addition) and the amount may be of great importance to obtain well dispersed and stable suspensions. The amount of BSA added is chosen according to the specific surface area of NP, in order to ensure a full coverage of NP surfaces and achieve complete steric stabilization. Thus, a material specific dispersion protocol could be developed using 3 preparation steps. However, the method is material dependent and was developed to disperse SiO₂ and TiO₂. It was developed to enable dispersion of CNT, which is usually poorly dispersible in water.

In short two different general procedures can be used for TiO₂ and SiO₂, respectively as shown below.

a. For TiO₂ nanomaterials:

- 1) Dispersion by sonication directly in acidic media (HNO₃ 10⁻² mol/L, pH 2): These conditions should ensure dispersed and more stable suspensions of positively charged TiO₂ particles.
- 2) Addition of BSA: BSA adsorbs onto TiO₂ particles, which stays positively charged (as well as BSA) at this pH, well dispersed and stable.
- 3) Adjustment of pH to the target value of pH 7.4 by addition of NaOH solution: a charge reversal occurs but the particles are protected from important flocculation due to adsorbed BSA. It is important not to stop around pH 5 which is a critical pH where BSA is uncharged and leads to fast aggregation of the particles.

b. For SiO₂ nanomaterials:

- 1) Dispersion by sonication in pure water (or pH around 7, possibly some adjustment by addition of NaOH is needed for some samples). These conditions should produce better dispersed and more stable suspensions and consist of negatively charged particles.
- 2) Addition of BSA: BSA adsorbs onto SiO₂ particles, which stays negatively charged (as well as BSA) at this pH, well dispersed and stable.
- 3) Adjustment of pH to the target value of pH 7 by addition of NaOH solution: no charge reversal occurred in the whole process. It is important that the suspensions keep a pH higher than 5, otherwise the charge reversal of BSA leads to instability and aggregation of the particles.

2.3.2 Equipment needed

Balance (0.1 mg precision)

Ultrasonicator, with 13 mm probe, placed in a sound abating enclosure. (Sonicator Sonics & Materials, VCX500-220V, 500 W, 20 kHz, standard 13 mm disruptor horn)

pH-meter with conventional glass electrode (better if the electrode is dedicated to NP suspension measurements)

standard 20 mL glass vial of internal diameter 24 mm

volumetric glassware (10 mL jauged vial) and pipettes

2.3.3 Procedure

In short two different general procedures are used for TiO₂ and SiO₂, respectively as shown below. First, the preparation of the medium is described and then the individual methods.

Calculation of the amount of BSA to be added

If we consider a full coverage of nanoparticles surface by BSA molecules in their native form, using the projected surface area of the equilateral triangle (27.7 nm²), and a molecular weight of 66500 g/mol, the maximum surface area occupied by BSA at the surface is 250 m² per gram of BSA.

In order to determine the right amount of BSA to be added to the suspensions, this value has to be compared with the specific surface area of nanomaterials (NM). For NM of high specific surface area of 250 m²/g, the amount of BSA added must be at least equal to the amount of NM to ensure a full surface coverage.

In the NANOGENOTOX project, the SiO₂ nanomaterials studied display a high specific surface area of up to 230 m²/g (as reported by the producer for NM202). Therefore it seems reasonable to have a mass ratio BSA/ SiO₂ of 1:1, i.e. 5.12 g/L of BSA for 5.12 g/L SiO₂ suspensions.

TiO₂ nanomaterials studied have a smaller specific surface area of about 60 m²/g, therefore a BSA/TiO₂ mass ratio of 1:4 should be sufficient to fully cover the nanoparticles surface. However, we suggest using the same ratio as for SiO₂ nanomaterials (1:1), not to change compositions too much and enable applicability for materials with even higher specific surface area. For a 2.56 g/L TiO₂ suspensions, addition of 2.56 g/L BSA is suggested, keeping in mind that there should be an excess of about 2 g/L of “free” BSA. It is assumed that this should not be a problem considering the much higher concentration of BSA in serum used for toxicological studies.

2.3.4 Dispersion procedure for TiO₂ nanomaterials

Main steps:

- 1) Sonication of 3.41 mg/mL NM10X suspension in HNO₃ 10⁻² mol/L acidic medium
- 2) Addition of BSA concentrated solution (10.24 g/L) (volume ratio ¼ : ¾) leading to a suspension of 2.56 mg/mL of NM10X and 2.56 mg/mL of BSA (mass ratio BSA/NM 1:1).
- 3) Adjustment of pH to a suitable value (pH 7) for subsequent dilution into biological media

Solutions to be prepared preliminary:

HNO₃ 10⁻² mol/L in ultrapure water (Milli-Q)

NaOH 1 mol/L in ultrapure water (Milli-Q) (or 0.1 mol/L)

BSA stock solution of 10.24 g/L, kept in refrigerator when not used and no longer than 2 weeks.

Detailed protocol:

- 1) Wear lab coat, protective gloves, mask and glasses, and damp paper towel around the weighing scale.
- 2) Weigh 34.1 mg of NM10X on weighing-paper or dedicated weighing-funnel,
- 3) Transfer NM10X into a 10 mL gauged vial; make sure that all the weighed powder is introduced by rinsing the funnel with a few drops of HNO₃ 10⁻² mol/L,
- 4) Complete the gauged vial with HNO₃ 10⁻² mol/L until the line is reached (gently shake the suspension before addition of the total volume to allow for volume change),

(Remark : if the weighed NM exceeds the target value on step a; it is possible to adjust the total volume by adding a suitable volume of dispersion medium, calculated by proportionality law. For example, for 36 mg of NM, the total volume needed is (36 mg)/(3.41 mg/mL) = 10.557 mL, so 557 µL of HNO₃ 10⁻² mol/L should be added.

In this case, after shaking, remove 557 µL of suspension to have exactly 10 mL).

- 5) Transfer the suspension into a 20 mL standard glass of internal diameter 24 mm (height of 10 mL liquid, 22 mm), making sure that all the settling material is recovered,
- 6) Prepare the sonication set up with the vial and the sonicator fixed on some support in a way that the sonication probe is dipped in the suspension with its bottom at the middle of the suspension volume (height of liquid 11 mm). For easiness and better reproducibility, marks can be drawn on the vial and on a scale.
- 7) Place a water/ice bath under the vial and adjust its level to best immerged the vial,
- 8) Ultrasonicate for 20 min at 40% of power,

- 9) Note down the time of the end of sonication (called t_0) and the energy released during the sonication process if available (press energy button on sonicators Sonics & Materials),
- 10) Remove 2.5 mL of suspension (which can be used for some characterization such as DLS),
- 11) Add 2.5 mL of 10.24 g/L BSA solution to the 7.5 mL of suspension remaining,
- 12) Place the vial on a magnetic stirrer with bar and insert pH-meter electrode
- 13) While stirring, dropwise add NaOH 1 mol/L (or 0.1 mol/L) solution until the pH reaches 7 (typically add 60 μ L of NaOH 1 mol/L)
- 14) The suspension is ready for characterization or dilution into biological media.

2.3.5. Dispersion procedure for SiO₂ nanomaterials

Main steps:

- 1) Sonication of 6.82 mg/mL NM20X suspension in ultrapure water
- 2) Addition of BSA concentrated solution (20.48 g/L) (volume ratio $\frac{1}{4}$: $\frac{3}{4}$) leading to a suspension of 5.12 mg/mL of NM20X and 5.12 mg/mL of BSA (mass ratio BSA/NM 1:1).
- 3) Adjustment of pH to a suitable value (pH 7) for subsequent dilution into biological media

Solutions to be prepared preliminary:

NaOH 1 mol/L in ultrapure water (Milli-Q)

HNO₃ 1 mol/L in ultrapure water (Milli-Q)

BSA stock solution of 20.48 g/L, kept in refrigerator when not used and no longer than 2 weeks.

Detailed protocol:

- 1) Wear lab coat, protective gloves, mask and glasses, and damp paper towel around the weigh-scale.
- 2) Weigh 68.2 mg of NM20X on weighing-paper or dedicated weighing-funnel,
- 3) Transfer NM20X into a 10 mL gauged vial; make sure that all the weighed powder is introduced by rinsing the funnel with a few drops of ultrapure water (Milli-Q),
- 4) Complete the gauged vial with ultrapure water (MilliQ) until the line is reached (gently shake the suspension before addition of the total volume to allow for volume change)

(Remark : if the weighed NM exceeds the target value on step a; it is possible to adjust the total volume by adding a suitable volume of water, calculated by proportionality law.

For example, for 70 mg of NM, the total volume needed is $(70 \text{ mg}) / (6.82 \text{ mg/mL}) = 10.264 \text{ mL}$, so 264 μ L of ultrapure water should be added

In this case, after shaking, remove 264 μ L of suspension to have exactly 10 mL).

- 5) Transfer the suspension into a 20 mL standard glass of internal diameter 24 mm (height of 10 mL liquid, 22 mm), making sure that all the settling material is recovered,
- 6) Prepare the sonication set up with the vial and the sonicator fixed on some support in a way that the sonication probe is dipped in the suspension with its bottom at the middle of the suspension volume (height of liquid 11 mm). For easiness and better reproducibility, marks can be drawn on the vial and on a scale.
- 7) Place a water/ice bath under the vial and adjust its level to best immersed the vial,
- 8) Ultrasonicate for 20 min at 40% of power,

- 9) Note down the time of the end of sonication (called t_0) and the energy released during the sonication process if available (press energy button on sonicators Sonics & Materials),
- 10) Remove 2.5 mL of suspension (which can be used for some characterization),
- 11) Add 2.5 mL of 20.48 g/L BSA solution to the 7.5 mL suspension remaining,
- 12) Place the vial on a magnetic stirrer with bar and insert pHmeter electrode
- 13) While stirring, dropwise add NaOH 1 mol/L solution until the pH reaches 7 (typically add 10 μ L = 1 drop), if necessary adjust with HNO₃ 1mol/L.
- 14) The suspension is ready for characterization or dilution into biological media.

2.3.6 Comments on use and applicability

For a lighter procedure, NM can be weighed directly in 20 mL vials and 10 mL solvent added with volumetric pipette.

This method improves the stability of some TiO₂ suspensions as compared to the generic NANOGENOTOX protocol. For SiO₂, the suspensions had the same quality as compared to the dispersion using NANOGENOTOX protocol. SiO₂ also disperses well in pure water.

It should be noted that added BSA amount added is considered here as a ratio of NP/BSA, so the absolute concentration of BSA is 5 times higher than using NANOGENOTOX protocol for TiO₂ suspensions at 2.56 mg/mL and 10 times higher for SiO₂ suspensions at 5.12 mg/mL. This could result in a higher degree of inhibition of surface reactivity. It should also be noted that pre-dispersion in acidic solutions required for TiO₂ may alter organic functionalizations and surface coatings.

2.4 Dry dispersion by small rotating drum dustiness testing

Keld Alstrup Jensen, Marcus Levin, Ismo K Koponen (NRCWE)

Modified EN15051 Standard for rotating drum dustiness testing according to Schneider and Jensen (2008)

2.4.1 General description

This procedure is used to analyze the dustiness levels of powders using a standardized procedure for agitating powders. The SOP was developed to determine the propensity of powders to generate dust by simulated handling. The dustiness is determined gravimetrically by filter collection and ranked based on the amount (mg) of size-specific dust generated per kg powder. Dust particle size-distributions may be measured using various different fast-reading online and offline techniques. The EN15051 rotating drum was downscaled by 1/3 to to 5.9 L enabling safer testing the dustiness of nanoparticles powders as well as nanostructured materials (Schneider and Jensen, 2008). The particle size distributions of the dusts are thought to be the agglomerate sizes of powder dusts and dustiness data may be used for risk assessment.

2.4.2. Materials and methods

The dustiness system is illustrated in Figure 2.3 and 2.4 and required equipment is listed below.

Equipment

- Balance used with local exhaust ventilation or better.
- Fume hood
- Humidifier with water-trap (enabling 50% RH at 11 l/min)
- Temperature sensors
- Humidity sensors
- PC's for online monitors and controlling humidity
- Pressurized air-supply for drum ventilation
- Pump for filter sampling at 4.2 l/min (only respirable dust)
- HEPA filters
- 5.93 L down-scaled EN15051 drum
- Diluters (Palas VKL-10)
- Particle sizing monitors
 - Fast Mobility Particle Sizer (TSI FMPS Model 3091)
 - Aerodynamic Particle Sizer (TSI APS Model 3321)
 - Condensation Particle Counter (TSI CPC Model 3022A or GRIMM CPC Model 5.403) (only used in the set-up for collection of respirable dust)
- Sampler for respirable or inhalable dust
- Sampler for TEM analysis (optional)
- Fume-hood or glove-box
- Powder (optimally at 4 x 6 g for each dust fraction; 6 g for saturation test and 3 x 6 for dustiness testing)
- Cleaning agents (wet-wipes, waste bin etc.)
- HEPA filtered vacuum cleaner for designed for dangerous dust (asbestos)
-

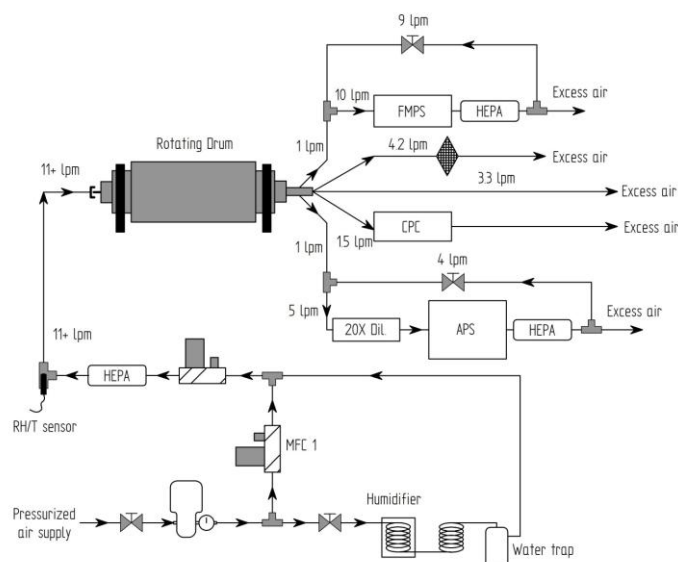


Figure 2.3. Principal sketch of the down-scaled EN15051 rotating dustiness drum test system applied for dust particle size-distribution analysis and collection of respirable dust.

Procedure for dustiness testing

The test procedure was as follows.

- 1) Assemble the set-up according to collection of respirable (Fig. 2.3) or inhalable dust (Fig. 2.4).
- 2) Start on-line measurement equipments. The FMPS should be started at least 1 hour (preferently over-night) prior to start of testing to stabilize electrometers.
- 3) Establish and control the relative humidity to 50% RH.
- 4) Weigh out 6 g of test material (bulk density may be determined)
- 5) Remove the exit cone part of the drum positioned with one of the three lifter vanes in the lowest position.
- 6) Carefully place a weighed amount of test material in a pile at the centre and at the upward moving side of the lowest lifter vane.
- 7) Assemble the drum
- 8) Mount filters
- 9) Check flows of pressurized air and pump for filter collection
- 10) Assemble sampling lines and turn on sampling pumps (pressurized air and filter(s))
- 11) Wait 180 s for humidity equilibration of powders and check that the particle concentration has reaches “zero” background levels
- 12) Start the dustiness test and rotate the drum at 11 rpm for 60 sec. and stop.
- 13) Pause with pumps still running for 120 sec. after ended rotation.
- 14) Remove sampling filter and store them in dedicated clean boxes.
- 15) Disassemble the drum and pour the remaining material out and remove the remaining loose material by tapping on the drum as specified in EN15051.
- 16) Repeat the dustiness test using one run for saturation of drums and tubing and three runs for statistical repetition of the test.
- 17) After completion of test clean the drum and tubing thoroughly using vacuum-cleaner, wet-wiping with ethanol wetted lint-free paper and discard material according to national regulation and institutional decisions.
- 18) Air-dry the drum in the fume-hood.

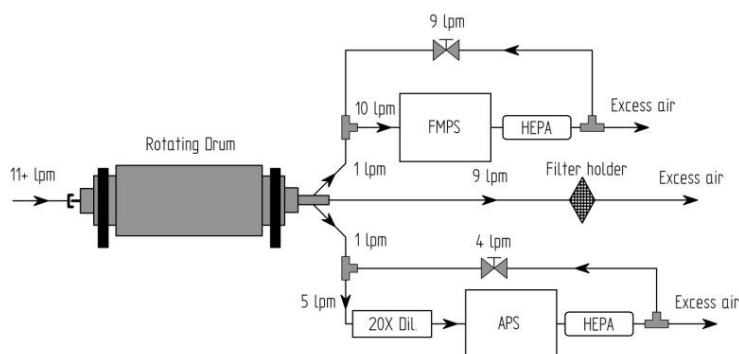


Figure 2.4. Principal sketch of the down-scaled EN15051 rotating dustiness drum test system applied for dust particle size-distribution analysis and collection of inhalable dust.

Determination of dustiness index

The gravimetric dustiness index is used to categorize the potential exposure potential during handling a powder.

- 1) Filters are stored in a clima-controlled ($22^{\circ}\text{C}\pm 10\%$ and $50\% \text{RH}\pm 5\%$) weighing room for at least 24 hours
 - a. Millipore Nitrocellulose membrane filters (OD = 90 mm; pore size $0.8 \mu\text{m}$) are used for inhalable dust
 - b. Millipore Teflon filters (OD = 37 mm; pore size $1.0 \mu\text{m}$) are used for respirable dust
- 2) Filters are pre-weighed using a micro-balance together with 1 – 3 control filters and results are stored in a dedicated weighing program.
- 3) Mount filters
 - a. Filters for respirable dust are mounted in Millipore cassettes and pressure tested
 - b. Filters for inhalable dust is mounted in in-line steel holder in the dustiness laboratory
- 4) After completion of dustiness testing filters are brought back to the weighing room for 24-hour humidity equilibration.
- 5) Exposed and control filters are weighed using the micro-balance and results are stored in a dedicated weighing program
- 6) Mass-concentrations of dust is determined by difference corrected by variation observed on control filters.
- 7) The dustiness index is calculated as the mass of dust (mg/kg powder used in the test) considering the sampling flow for the specific size-fraction ($4.2/11$ and $9/11$ l/min for respirable and inhalable dust, respectively)
- 8) The dustiness levels are categorized very low, low, moderate and high according to EN15051

Determination of particle size distributions and release concentrations

The particle number concentrations are measured using a CPC Model 3022A, which measures the total number of airborne particle aggregates/agglomerates from 1 nm to more than $1 \mu\text{m}$ at 1 sec resolution after growth in a butanol-saturated chamber.

Particle size distributions of fine and coarse particles were measured using the APS 3321, which measures the aerodynamic diameter, D_a . The particle sizing was based on measurements in 51 diameter classes with midpoints ranging from 0.52 - $18.43 \mu\text{m}$. A separate class of particles smaller than $0.52 \mu\text{m}$ was also recorded. Experience have shown that results below ca. $0.7 \mu\text{m}$ should be used with great care due to insufficient sampling efficiency of the APS and are omitted in standard analysis.

Nanoparticles and small submicrometer-size particles between 5.6 and 560 nm were measured by the FMPS. The FMPS measures particle sizes according to their electrical mobility diameter, D_m , in diameter classes with midpoints ranging from 6.04 to 523 nm.

Both the CPC, APS and FMPS instruments were set to collect data at one-second time resolution.

CPC particle release concentrations are calculated accumulating the back-ground corrected particle concentration measured from start of rotation to end of sampling taking the sampling volume into consideration ($1.5/11$ l/min)

Total FMPS size distributions are calculated accumulating the back-ground corrected particle concentration in each bin measured from start of rotation to end of sampling

Total APS size distributions are calculated accumulating the back-ground corrected particle concentration in each bin measured from start of rotation to end of sampling

The data may be presented in different ways (e.g., total, total per kg test material, release rate per time unit) as shown in e.g, Schneider and Jensen (2008) and Jensen et al. (2009).

2.4.3 Comments on use and applicability

The method is used as “routine” analysis in this project. Reduction of the amount of some test material has, however, been enforced due to their high dustiness levels. A procedure evaluating the dust concentration from the saturation test and subsequently deciding the mass of test material required has successfully been employed. It may be beneficial to add an impactor before the CPC to avoid accumulation of coarse particles in the CPC sampling train. This will be implemented in the future.

3. Particle size-distribution analysis by transmission electron microscopy

Three different methods were applied for analyzing the particle size distribution by transmission electron microscopy.

3.1. Primary particle size distribution of TiO_2 and SiO_2 by TEM

Davy Rousset (INRS)

3.1.1 General description

The grids are directly observed by TEM (Zeiss EM 910 120 kV). For the determination of primary particles size distribution, particles are observed at a magnification of x 100 000. Images were digitized with a digital camera (ProgRes® JENOPTIK CF Scan Digital Microscope Camera Jenoptik – resolution 2k x 2k approx.) and associated software. The size distribution is based on 100 measurements (approx. 5 measurements x 20 images). Using a free processing image software (ImageJ, NIH), particles' outlines are manually drawn, so surface areas are reachable, and the diameter is calculated assuming primary particles spherical.

3.1.2 Materials and Chemicals

TEM Zeiss EM 910 120 kV operating at 100 kV

Ultrasonic bath (Annemasse)

ImageJ (free download at <http://rsb.info.nih.gov/ij/>)

Sputtering system: Balzers model SCD 040

TEM grid

Sterile pipette

Oven

2-propanol grade analysis

50 ml plastic vials

Spatula

For BSA see dispersion protocol

For aerosol see SOP Nanodustiness by the vortex shaker method

3.1.3 Sample preparation

Dispersion in isopropanol

A small amount of powder (spatula tip) is added to about 15ml isopropanol in a glass vial. The suspension is then sonicated during 10 min in ultrasonic bath to enhance NM's dispersion. Just at the end of sonification, about 3 to 10 droplets of suspension are put with disposable sterile pipettes onto pre-carboned TEM grids under vacuum (vacuum enhances droplet drying). Grids are then dried during few minutes in an oven at 50°C before TEM observation.

Dispersion in BSA

WP4 dispersion protocol is used. Just after sonification, drops of suspension are put with disposable sterile pipettes onto microscope glass slides. A pre-carboned TEM grid is then put onto the drop surface. After about 2 min, the grid is dried during few minutes in an oven at 50°C before TEM observation.

Aerosol

The powder is weighted in a stainless steel tube and mixed with bronze balls. The tube is closed then shaken using the vortex shaker (see SOP for nanodustiness using vortex shaker) to produce airborne nanoparticles. The aerosol is collected using a closed-faced cassette fitted with a 0.4 µm in porosity Nuclepore (polycarbonate) membrane. In the center of the membrane is stuck a precarboned TEM grid.

3.1.4 Data treatment

Regular spread-sheet or statistical program capable of calculating and plotting data for size distribution analysis. Size distribution data are presented in histogram.

3.1.5 Comments on use and applicability

This SOP is operator-dependant as it was not possible to computerize primary particles identification using our image processing software. This has to be hand-made.

3.2. Aggregate/agglomerate size distribution of TiO₂ and SiO₂ by TEM

Davy Rousset (INRS)

3.2.1 Description of method and data collection

The grids are directly observed by TEM (Zeiss EM 910 120 kV). For the determination of the aggregate/agglomerate size distribution, particles are observed at a magnification of x 25000 or x 50000 according to the size of the objects. Images were digitized with a digital camera (ProgRes® JENOPTIK CF Scan Digital Microscope Camera Jenoptik – resolution 2k x 2k approx.) and associated software. The size distribution is based on 100 measurements (approx. 20 images). Using a processing image software (Visilog, NOESIS), images are digitized and particles are identified automatically by the software which yields several morphological parameters for each aggregate/agglomerate, such as area, perimeter, Feret diameter... The unit of software is in pixel but could be easily converted in nm from internal calibration.

3.2.2 Materials and Chemicals

TEM Zeiss EM 910 120 kV operating at 100 kV
Ultrasonic bath (Annemasse)
Visilog (NOESIS)
Sputtering system: Balzers model SCD 040
TEM grid
Sterile pipette
Oven

2-propanol grade analysis
50 ml plastic vials
Spatula
For BSA see dispersion protocol
For aerosol see SOP Nanodustiness by the vortex shaker method

3.2.3 Sample preparation

Dispersion in isopropanol

A small amount of powder (spatula tip) is added to about 15ml isopropanol in a glass vial. The suspension is then sonicated during 10 min in ultrasonic bath to enhance NM's dispersion. Just at the end of sonification, about 3 to 10 droplets of suspension are put with disposable sterile pipettes onto pre-carboned TEM grids under vacuum (vacuum enhances droplet drying). Grids are then dried during few minutes in an oven at 50°C before TEM observation.

Dispersion in BSA

WP4 dispersion protocol is used. Just after sonification, drops of suspension are put with disposable sterile pipettes onto microscope glass slides. A pre-carboned TEM grid is then put onto the drop surface. After about 2 min, the grid is dried during few minutes in an oven at 50°C before TEM observation.

Aerosol

The powder is weighted in a stainless steel tube and mixed with bronze balls. The tube is closed then shaken using the vortex shaker to produce airborne dust particles. The Vortex dustiness method is under development and is anticipated described in the final SOP report. The aerosol is collected using a closed-faced cassette fitted with a 0.4 µm in porosity Nuclepore (polycarbonate) membrane. In the center of the membrane is stuck a precarboned TEM grid.

3.3.4 Data treatment

Size distribution (histogram)

3.3.5 Comments on use and applicability

Aggregate/agglomerate size distribution for aerosol to be done.

Need to check the influence of:

- Dispersion media (isopropanol, BSA-water, aerosol)
- Magnification

3.3 Semi-automatic detection and image analysis of nanoparticles

Pieter-Jan de Temmerman & Jan Mast (VAR)

3.3.1 General description

This protocol provides a step-by-step guide for semi-automatic detection and image analysis of nanoparticles that can be distinguished from the background based on their grey values corresponding with electron density. The protocol is conform with the ISO 13322-1:2004(E) "Particle size analysis – Part I: Image analysis methods. In principle the current method allows to detect and analyze any kind of nanoparticles and has successfully been applied for: Ag, Au, SiO₂, TiO₂, Fe₂O₃ and Fe₃O₄. However, on-going work suggests that even more information can be extracted from quantitative measurements than anticipated from the ISO norm.

During an automatic particle analysis, the image analysis program automatically detects particles on an image. The gray value, corresponding with electron density, is the criterion for the recognition of a particle. Therefore, in order to have successful particle detection, the particles must clearly stand out from the background. All detected particles can be semi-automatically measured. A wide array of measured parameters can be chosen.

A typical particle analysis consists of following steps:

- Image preparation
- Setting and adjusting the threshold value
- Defining the detection area
- Definition of the detection parameters and detection of the nanoparticles
- Selection of the particle parameters
- Defining the classification schemes
- Classification of the particles according the selected parameters
- Selection of the parameters for a class measurement
- Exporting of results in excel spreadsheets and storage of the images in the nanoparticles database

3.3.2 Equipment

In order to perform analyses, the 'Analysis solution' of the iTEM software (Version 5.0, Olympus, Münster, Germany) installed on a powerful computer is indispensable. The iTEM software can directly be integrated in a database in which relevant fields can be accessed. Alternatively separate files in TIF-format can be analysed if a correct calibration of pixel size can be assumed.

3.3.3 Instruction

Image preparation

- (Optional) Verify whether the image background (e.g. due to heterogeneous image illumination during image acquisition) is homogenous. The mean intensity profile should have no specific tendency.
- [Measure – Intensity Profile – Horizontal Mean]
- (Optional) Modify the gray values based on the graph displaying the frequencies of different gray values such that only relevant gray values are maintained.
- (Optional) Apply the Separator filter to separate neighbouring particles.
- Set the zoom level of the image in the viewport to 100%.

- Select the “Oper > Define Filter Separator...” command.
- Select the “Step option” in the “Boundary shape” group.
- Set both “Smooth” and “Fine/Coarse” slide controls to 1.
- Switch on the preview to be able to observe how adjusting settings alters the image.
- Move the Fine/Coarse slide control incrementally higher until you have found the optimal setting.
- If an image has a lot of noise, increase the value Smooth.
- Select the “Burn white option” in the Result group to be able to sketch white dividing lines on the original gray value image.
- Click the “Execute” button to separate particles.
- The resulting image in the target image buffer will display the image with the particles outlined in white (a gray value of 1).
- The separating line generated has a width of one pixel.
- Please keep in mind that the gray value range which defines the particles must not contain a gray value of "1".
- Introduce the obtained intermediate image into the nanoparticle database, attached to the original micrograph for later reference.

Setting thresholds

- Set the gray value range interactively and manually to define the particles so that they are distinguishable from the image background. The thresholds are comprised of the lowest and greatest gray values. Comment 1
- Load the image you wish to analyze into the active image buffer.
- Select the “Image > Set Thresholds...” command.
- Select the “Manual” tab. Define only one gray value phase.
- Select the “Histogram” entry from the “Diagram” group. The diagram now shows the gray-value distribution within the image frame you have set. The current threshold values are shown in the diagram as two perpendicular lines. The lower threshold is blue and the upper is red.
- Mark the “Current” option in the “Preview” group. The active phase is shown in color within the image so that you can view what affect your settings have.
- Define the gray value range directly in the diagram such that all particles belonging to that phase are shown in color. Move the mouse pointer over one of the two threshold lines. The mouse pointer will change into a double arrow. Hold down the left mouse button and pull the threshold to the desired value. The set gray value range will be colored within the image.
- Confirm the new threshold setting by clicking on “OK”.

Defining detection area (ROI)

- Define the detection area. This is the region of interest (ROI) to which the particle analysis is restricted. Comment 2
- Select the “Analysis > Define ROI” command.
- To delete previously-set ROIs, click the Delete All button.
- Define the ROI selecting one of the shapes in Tools group.
- Make the ROI smaller than the picture, otherwise the Exclude border particles command will distort the result
- Right click to return to the dialog box. The new ROI will be numbered and added to the Active ROI list.
- Exit the Define ROIs dialog box via the “Close” button.

- Use the “Analysis > Draw ROIs > Into Overlay” command to have the defined ROIs displayed in the overlay.

Defining detection

- Set the parameters for detection in the “Define Detection” dialog box. Settings generally do not change between analysis for the same type of particles. Exclude border particles, limit the search area to the ROI, set the particle filter (minimal pixel size) to reduce background interferences. Indicate whether the analysis should be restricted to a range of any selected measurements. Indicate whether holes in particles should be taken in account. Select the type of pixel connectivity (adjacent borders versus include diagonals) that results in the best overall particle detection.
- Select the “Analysis > Define” Detection... command.
- The Define Detection dialog box has three tabs for defining detection and classification parameters.
- Select the “Detection” tab to set particle-detection parameters.
- Enter the minimum number of pixels which may be detected as comprising a particle into the Minimum field of the Particle filter group.
- This is an excellent way to exclude noise particles.
- Clear the Use ranges check box to detect all particles occurring within the thresholds set of measurements. The Total count field shows the number of particles which were detected during the previously-conducted detection.
- If particles have inclusions which are to be taken into account when calculating particle parameters, clear the “Fill holes” check box. This is necessary for particle holes to be recognized as such.
- Indicate that the ROI should be used for detection in the Search area group.
- Define that particles, which are not located entirely within the search area are excluded from analysis in the “Border particles” group.
- Define inter-particle connectivity in the “Connectivity” group. If the separation line between two particles is only one pixel in width (e.g. when using a separator filter), then select the Adjacent borders (4) option. Otherwise the separated particles will be considered one particle when detected.
- Click the “Execute” button to conduct particle detection.
- All particles detected will be shown in the image overlay in color. Determine how particles are shown in the Classification tab. Label the particles on the micrograph with their particle ID such that they also can be identified in the data sheet.
- The most recently-defined classification will be applied.
- Click on “OK” to close the dialog box.

Selection of the particle parameters

- Set the particle parameters in the “Define Measurements” dialog box. This dialog box contains three tabs for defining measurements of particles, classes and ROIs. First choose the “Particles” tab. Here all the parameters listed that can be measured. These are divided in categories: Area, Density, Dimension, Distance, Features, ID, Perimeter, Position and Shape some with their according subcategories.
- Select the “Analysis > Define Measurements...” command
- Select the “Particles” tab and select the parameters you want to include in your particle analysis. The easiest way to do this is to click on “All”, hereby showing all parameters

alphabetically. From the list that is now exposed on the right in the dialog box, you can include your desired parameters by clicking on the check boxes.

- In the middle part of the dialog box, the selected parameters are listed.
- By clicking on a parameter an upper and lower limit can be set in the Filter range area that becomes activated in the lower part of the dialog box).
- Click “OK” or “Detect” to perform analysis.
- (Optional) You can do the same for Classes and ROI.

Definition of the classification schemes

A classification scheme consists of a name, a unit and a class division. Particles are sorted via the classification scheme according to certain parameters (e.g. into 10 size classes). You can determine the number of particles per size class, for example. You define a new classification scheme in the Define Classification dialog box. You have numerous possibilities in opening the dialog box:

- Use the “Measure > Define Classification...” command.
- Click the “Define Classification” button located in the “Analysis” button bar.
- Use the “Analysis > Classify...” command. Click the “Classification...” button.

Automatic Classification

The last used classification scheme will automatically be loaded when a new image is acquired in iTEM? Change the classification if it is not applicable to the new image. The last used classification scheme will be automatically saved.

Defining a classification scheme

- Select the “Measure > Define Classification...” command to define a suitable classification scheme.
- Select the “Show sample objects” check box to be able to evaluate classification.
 - The current overlay will disappear from view and the analysis program will show a number of sample objects for visual reference purposes.
- Enter the name of the classification into the “New Classification” field. The “new” button will become available.
- Click the “New” button to create the new classification scheme.
- Click the “Unit...” button to select the unit desired for the classification. The unit you select will depend on the classification criterion. Classifying according to area is possible by select a unit of μm^2 , for classification according to particle length, chose a length unit (e.g. m). Classify by particle characteristics is possible by selecting ‘no unit’.
- Click the “Compute...” button to define the number and value range of each class. The value range is determined by the minimum and maximum particle parameters.
 - It is possible to automatically calculate a suitable classification. In the “Compute Classification” dialog box, enter the criteria and the amount of classes and click the “Auto” button.
- Click the “OK” button to return to the “Define Classification” dialog box.
- Go to the “Define Classification” dialog box to edit the class divisions interactively:
- To alter the color of a class select the line number of that class and select one of the 16 colors available in the “Set color” color palette.
 - To give names to classes, left click in the “Name” field of the class. Enter the class name into this field.

- To interactively alter class divisions of a class, double click on one of the numeric values and enter a new one.
- Click on “OK” to exit the dialog box.

Classifying particles

Applying classification

- Select the “Analysis > Classify...” command to divide detected particles up into classes.
- Select the desired particle parameter for classification within the “Criterion” list in the “Classify” group (e. g., “Area”).
- Select “No classification” in the list of classification criteria if no classification is desired. The class parameters will then refer to all detected particles. If the classification is not available in “Classification” check in “Define Classification” whether the appropriate unit is selected (e.g. nm for a length criterion, nm² for an area criterion, no unit for a characteristics criterion).
- Select the desired classification scheme in the “Classification” list. This list will show all classifications that can be used with the unit selected.
 - Should a suitable classification scheme not be available, click the “Classification...” button to create a new classification scheme or to edit an already existing one.
- Click the Execute button to have the classification applied to the image.
 - The particles in the overlay will be divided up into classes and shown in their class color. Any particles that do not belong to any of the classes will remain crosshatched.
- If “classification” does not produce the desired results then try out other classification schemes before generating a sheet for results.
- Click on OK to close the dialog box.

3.3.4 Select classification parameters and export results

- Select the “Analysis > Define Measurements...” command.
- Activate the Classes tab to determine the parameters to be include in the measurement sheet.
- Select the parameters desired: e. g., “ID Class” and “Particle Count”. To do this, select the check box next to the parameter desired. It will appear in the “Selected Measurements” list.
 - The parameters selected will be moved to the “Selected Measurements” list and will appear in the results sheet for each class.
 - It is advisable to include the “ID Class” parameter in the results sheet to be able to see what results go with which classes.
 - The lower part of the dialog box shows a text and graphic definition of any parameter you select.
- Click on “OK” to close the dialog box.
- Select the “Analysis > Class” Results command.
 - A sheet containing the results of the class measurement will be generated.
- All particles which cannot be assigned to a class will be put in the “0” class and included in the sheet as well.

Exporting of results

After analysis is finished, iTEM shows the results in (a) spreadsheet(s), depending of the type of analysis which is performed. These include a sheet with particle results, a sheet with class results and

a sheet with frame/ROI results. Furthermore, the pictures obtained (called annotated micrographs) can be stored in the database integrated in the software. Extra information can be indicated in the appropriate fields. One extra field with additional information about a particular picture is also included.

3.3.5 Comments on use and applicability

Regarding setting thresholds. A Particle is a quantity of connected pixels within a defined gray value range. This is why you must define a suitable gray or color value range before particle detection. Successful particle detection requires a clear correlation between gray or color values and particle structure.

Regarding defining the detection area. If no detection area is defined, the entire image is analyzed by default. The image analysis program provides various possibilities for restricting image analysis to a specific area within an image. Mostly regions of interest (ROI) are chosen. A ROI is an area of arbitrary shape within the image allowing to restrict the area for particle analysis.

Regarding analysis of NM, it was found that length dimensions of CNT could not be adequately measured using automatic or semi-automatic analysis.

3.4 Coating of dispersed nanoparticles in liquid on grids for TEM analysis

Pieter-Jan de Temmerman and Jan Mast (VAR)

3.4.1 General description

This procedure aims to coat nanoparticles suspended in a liquid on EM-grids. To be suitable for further TEM analyses, the NP should be evenly distributed over the grids, while the fraction of the attached NP represents the dispersed NP optimally. The method allows coating of dispersed nanoparticles in liquid on EM grids for further TEM analyses

- The nanoparticles can be metallic (Ag, Au), metal oxides (SiO_2 , TiO_2 , Fe_2O_3 , Fe_3O_4 or other)
- The medium can be polar (water, phosphate buffered saline,..) or apolar (hexane, acetone,...).
- TEM analyses can be qualitative or quantitative.

Dispersed particulate NP are brought in contact with an EM-grid and are allowed to interact with its surface. When excess fluid is drained and grids are air-dried, a fraction of the NP remains attached to the grid by different types of interactions (electrostatic, apolar, van der Waals, ...). The concentrations of NP, and the type and charge of the grid are chosen such that the fraction of nanoparticles attached to the grids optimally represents the dispersed NP, and that (aggregates of) nanoparticles can be detected individually.

3.4.1 Definition, abbreviations, references and norms

- Solution: Solutions are mixtures of dispersed particles, sizes lower than 2nm, in a liquid medium.
- Colloid: Colloids are mixtures of dispersed particles, sizes between 2 nm and 1000 nm, in a liquid medium.
- Suspension: Suspensions are mixtures of dispersed particles, sizes greater than 1 µm, in a liquid medium.
- Primary- or single particle: An individual particle recognized by suitable physical means
- Aggregate: Primary particles assembled face-to-face; their surface area is smaller than the sum of the surface area of the primary particles (DIN 53206).
- Agglomerate: Primary particles and/or aggregates not permanently joined together but attached e.g. at the edges and corners. Their surface area does not differ markedly from the sum of that of the individual units (DIN 53206).
- Liquid: The state in which a substance exhibits a characteristic readiness to flow with little or no tendency to disperse and relatively high incompressibility.
- Medium: The liquid vehicle in which nanoparticles can be dispersed.
- PBS: Phosphate buffered saline
- TEM: Transmission electron microscopy
- NP: Nanoparticles
- NM: Nanomaterial

3.4.2 Materials and equipment

- Eppendorf microcentrifuge tubes
- Pioloform-coated and carbon-shaded copper grids 400 mesh (Agar Scientific Ltd., G2400C). Both home-made and commercially available grids can be used.
- Large (120 mm diameter) polyethylene petridish
- pipette tips of 200µl (Gilson Diamond) and 200-1000µl (VWR)
- Tweezers
- Filter papers Ø 70 mm (Whatman, 54 hardened) and tape
- 4" Parafilm M (American National Can, Freewich, CT06830)
- Permanent, waterproof marker or a ball point to indicate references on filter paper
- Water (double distilled), Hexane or PBS
- Alcian blue stock solution: 2 % in water
- Alcian blue working solution: Dilute the Alcian blue stock solution with an equal amount of water to obtain a 1 % working solution. The latter is stable for 1 month at 4 °C.
- Micropipettes of 20 µl, 200 µl and 1000 µl (Gilson)

3.4.3 Instruction for coating and preparation of TEM samples

Alcian blue pre-treatment of the grids

- Increase the hydrophilicity of EM-grids by pre-treating them with Alcian blue (Comment 1).
- Fix a strip of parafilm to a flat and clean surface by wetting the surface with some drops of water, press the parafilm with the film side to the surface and remove the protective cover carefully.
- Place an EM grid with the carbon-coated side on a drop of about 20 µl Alcian blue working solution and incubate for 10 minutes. Avoid air drying of the grids.

- Grip the grid carefully with a pair of tweezers and wash most of the blue stain away by transferring it to 5 drops of water placed on the parafilm. Remove excess fluid by blotting its edge on a strip of filter paper, leaving a rest of humidity.
- Use the grids immediately in the next step.

Coating the nanoparticles on the grid

- Homogenize the dispersed nanoparticles by shaking, vortexing stirring or pipetting.
- Dilute the dispersed NP by adding a suitable dilution medium (Comment 2).
 - Make a two to ten-fold dilution series by pipetting dispersed NP into an eppendorf-type microcentrifuge tube and dilute by adding medium.
 - Repeat this pipetting step with the last dilution another three times.
- Allow the NP to interact with the grid surface by either the drop-on-grid method, or the grid-on-drop method (Comment 3)

Grid-on-drop method: Place the EM-grid on a droplet of dispersed NP.

- Fix a strip of parafilm to a flat and clean surface by wetting the surface with some drops of water, press the parafilm with the film side to the surface and remove the protective cover carefully.
- Place a droplet of 2 to 50 µl of dispersed NP on the parafilm
- Transfer the grid to the droplet and indicate the appropriate references with a waterproof marker.
- Float the grids, with coated surfaces down, on the droplets for 1 to 10 minutes.
- Remove excess fluid by blotting its edge on a strip of filter paper, leaving a rest of humidity.

Drop-on-grid method: Place a droplet of dispersed NP on an EM-grid.

- Put the grid on the parafilm and then pipette a droplet of 2 to 50 µl of dispersed NP on the grid. Leave this for 1 to 10 minutes.
- Remove excess fluid by blotting its edge on a strip of filter paper, leaving a rest of humidity.

Extra rinse step (Comment 4)

- Rinse the grids, with coated surfaces down, by placing them on a droplet of water for 30 seconds to remove excess material
- Remove excess fluid by blotting its edge on a strip of filter paper, leaving a rest of humidity.

3.4.3 Storage of the grids

- Place the grids in a grid box or on a filter paper in a petri-dish.
- Make sure to always note the appropriate references in order to be able to recover the grids later on

3.4.4 Specific safety measures

- Because of the possible toxicity of the reagents and NP and the possible presence of unknown contaminants, it is highly recommended to wear gloves during this procedure.
- All waste materials generated during this procedure should be disposed in the suitable container for chemical waste.
- Hexane is explosive (see MSDS file)

3.4.5 Comments on use and applicability

Comment 1: The interaction of NP with an EM-grid is strongly determined by the charge of the grid. In general, carbon-coated coated grids are negatively charged, although dependent on the preparation of the grid these charges tend to disappear leaving a hydrophobic surface. The hydrophilicity of EM-grids can be increased by pre-treating them with Alcian blue. The resultant positive charge strongly increases attachment of negatively charged nanoparticles. In case the charge of the NP is unknown, it is wise to evaluate both Alcian blue and non-treated grids.

Comment 2: PBS can be diluted by water and vice versa. Dilution with water is preferred, to lower salt concentration. If the dispersability of the NP is not negatively altered.

Comment 3: A concentration of NP should be chosen such that the number of particles per picture is optimal. It should be taken into account that the NP do not touch or overlap each other. Optimal concentrations vary from sample to sample. A typical concentration for colloidal spherical unaggregated Silica NP is 0,05 mg/ml. For colloidal aggregated Silica NP a concentration of 0,5 mg/ml can be used. For non colloidal aggregated Silica NP a concentration of 2,5 to 25 mg/ml can be used. Grid on drop provides good results for colloidal NP. For NP in suspension, apolar NP and NP in low concentration the drop-on-grid method is preferred since it improves contact between grids and NP.

Comment 4: The additional washing step reduces the background signal of the grid by removing excess salt and material of large aggregates. It also prevents the dry-out of the grid and the crystallization of the salts.

4. Determination of specific surface area

4.1 Nitrogen adsorption by the Brünauer, Emmett and Teller (BET) method

Boris Shivachev (CLMC)

4.1.1 General description

Surface area and porosity are important characteristics, in understanding the structure, formation and potential applications of different natural materials. For this reason it is important to determine and control them accurately. The most widely used technique for estimating surface area is the so-called BET method (Brünauer, Emmett and Teller, 1938). The concept of the theory is an extension of the Langmuir theory, which is a theory for monolayer molecular adsorption, to multilayer adsorption with the following hypotheses: (a) gas molecules physically adsorb on a solid in layers infinitely; (b) there is no interaction between each adsorption layer; and (c) the Langmuir theory can be applied to each layer.

$$\frac{V}{V_m} = \frac{C \phi}{1 - \phi} \quad (1)$$

$$\frac{V}{V_m} = \frac{C \phi}{1 - \phi} \quad (2)$$

Equation (1) is an adsorption isotherm and can be plotted as a straight line with $1/v[(P_0/P)-1]$ on the y-axis and $\phi = P_0/P$ on the x-axis according to experimental results (BET plot). The value of the slope, A and the y-intercept, I of the line are used to calculate the monolayer adsorbed gas quantity V_m and the BET constant C . The following equations can be used:

$$\frac{1}{V_m} = \frac{C}{V_m} \quad (3)$$

$$I = \frac{1}{V_m} \quad (4)$$

A total surface area S_{total} and a specific surface area S are evaluated by the following equations:

$$S_{total} = \frac{V_m N_A s}{V} \quad (5)$$

$$S = \frac{V_m N_A s}{V a} \quad (6)$$

where v_m is in units of volume which are also the units of the molar volume of the adsorbate gas, N is Avogadro's number, s is the adsorption cross section of the adsorbing species, V is the molar volume of adsorbate gas, a is the mass of adsorbent (in g).

4.1.2 Materials and chemicals

Apparatus

BET surface area and pore size analyzer (in our case Coulter SA 3100 Surface Area and Pore Size Analyzer, which uses the static dosing method). SA 3100 has three vacuum out-gassing stations and one analysis port. It provides single and multipoint surface area analysis, multigas capability for surface area and full adsorption capability.

BET analyser operates by measuring the quantity of gas adsorbed onto or desorbed from a solid surface at some equilibrium vapor pressure by the static volumetric method. The data are obtained by admitting or removing a known quantity of adsorbate gas (in our case Nitrogen) into or out of a sample cell containing the solid adsorbent maintained at a constant temperature below the critical temperature of the adsorbate. As adsorption or desorption occurs the pressure in the sample cell changes until equilibrium is established. The quantity of gas adsorbed or desorbed at the equilibrium pressure is the difference between the amount of gas admitted or removed and the amount required to fill the space around the adsorbent (void space).

Table 4.1.2 Coulter SA 3100 specifications

Gas Purity Requirement - Adsorbates:	99.9% or better
Gas Purity Requirement - Helium:	99.995%
Manifold Temperature:	45 °C (± 0.1°C)
Mesopore Size Distribution Range:	3 nm to 200 nm
Micropore Analysis:	Micropore Volume and Area by t-Plot Analysis
Minimum Measureable Relative Pressure:	0.00006
Minimum Sample Pressure Resolution:	0.046 mmHg
Outgas Furnace Settability:	1 °C
Outgas Furnace Stability:	5 °C
Outgas Furnace Temperature Accuracy:	± 5 °C
Outgas Furnace Temperature Range:	30°C to 350 °C
Outgas Ports:	3
Overall Pressure Range:	Vacuum to 1000 mmHg
Specific Surface Area Range:	From > 0.01 m ² /g, dependent upon sample density
Storage Temperature Range:	0°C to 50 °C
Transducer Linearity:	< 0.1% BFSL
Transducer Repeatability:	0.0125% BFSL
Typical Reproducibility:	< 2% CV
Ultimate Vacuum:	10 ⁻³ mmHg
Vacuum Pump Volume Displacement:	1.5 m ³ /hr

Equipment needed

BET analyzer (Coulter SA 3100 Surface Area and Pore Size Analyzer),
Sample cell,
Microweigh with accuracy of 0.1 mg or better*,
Weighing box,
Fume-hood.

Materials and chemicals

Liquid Nitrogen,
Helium** ,
weighing box,
glove box,
Weighing boat,
Wet and dry wipes for cleaning,
Steel and glass spatula's,
Vials with nanomaterials.

* *It is recommended to follow the procedure described in 2.2.3.; use two- or three layers of gloves for dermal protection, glasses and mask.*

** Only if reference method requires it; SA3100 reference method uses helium to measure the sample tube free-space for highly accurate data.

4.2.3 Sample preparation

For NANOGENOTOX materials (TiO₂, SiO₂, ZnO, CNT) no special preparation is required. Unless the samples are sensitive to the adsorbate gas or pressure variation no special preparation is needed. Weigh approximately 0.1 g of the material and place it in the appropriate cell size (the volume of the sample may vary from sample to sample due to difference in density etc.).

4.2.4 Measurement

The time of acquisition necessary for BET experiment depends on the sample properties. NANOGENOTOX samples, two measurements were performed. All experiment parameters are monitored and recorded on PC providing data reprinting, archiving, graphic overlays and other features. Typical setup parameters and results output are listed below. In order to obtain accurate results the correlation coefficient should be greater than 0.999.

Elapsed Time	3 hrs 16 min
Sample Wt	0.1089 g
Outgas Time	15 min
Profile	FULLUN
Outgas Temperature	120 C
Surface Area Report	
BET Surface area	56.261 sq.m/g
Correlation Coefficient	0.99996

4.2.5 Observations on use and applicability

The SOP is under evaluation for its applicability.

4.2 Small-Angle X-ray Scattering (SAXS) and ultrafine SAXS (USAXS)

Camille Guiot and Olivier Spalla (CEA)

4.2.1 General description

This section describes the general procedure applied at CEA LIONS to perform Small Angle X-ray Scattering measurements and to treat the data. This procedure was applied in the framework of NANOGENOTOX to characterize TiO_2 and SiO_2 manufactured nanomaterials as raw powders and in aqueous suspensions.

Small-Angle X-ray Scattering is a technique based on the interaction between X-rays and electrons to probe the structure of materials. The processed data is the number of X-ray scattered by a sample as a function of angular position of a detector (

Figure 4.1).

The experimental set up (X-ray source, optical elements, detectors, etc) and the procedure for absolute scaling of data has been thoroughly described by Zemb (2003) by Né (2000).

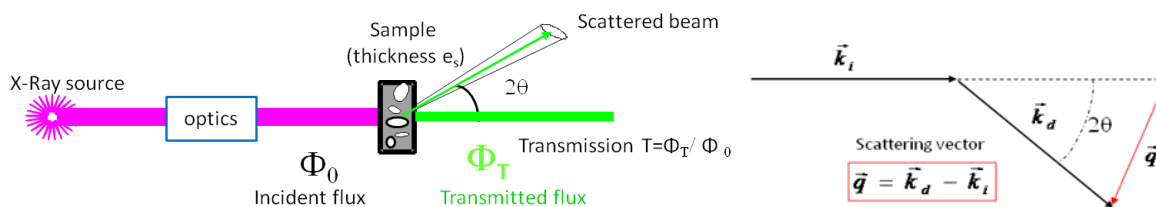


Figure 4.1: Schematic set up for SAXS and physical quantities

2D raw data images are converted into diffractograms displaying the **scattered intensity I as a function of scattering vector q** defined by:

λ : X-ray wavelength

The experimental scattering intensity is defined as the differential scattering cross-section per unit volume of sample and can be expressed as follows:

σ : scattering cross-section

V : volume of sample

C_{ij} : number of counts detected on a pixel ij during dt

η_1 : detector quantum efficiency when measuring the direct beam

η_2 : detector quantum efficiency for the count C_{ij}

(ϕ_0ST) : flux (in detector unit counts/s) integrated over the whole beam transmitted by the sample

T: transmission of the sample

$\Delta\Omega$: solid angle covered by one pixel seen from the center of the sample ($\Delta\Omega = p^2/D^2$ with p the pixel size and D the sample to detector distance)

The intensity is then expressed in **absolute scale** (in cm^{-1}) to be independent of the experimental set up parameters (X-ray wavelength, experimental background, time of acquisition, sample thickness, etc).

General theorems of experimental physics have been developed to extract different properties of nanostructured material from the diffractograms, such as, shape of nanoparticles, surface area, interactions occurring, etc. $I(q)$ curves can also be theoretically calculated from assumed nanostructures to fit the experimental curves.

In the simple case of binary samples, the scattering intensity is proportional to:

- the electronic contrast, more precisely the square of scattering length density difference between the two materials $(\Delta\rho)^2$,
- the concentration of the scattering object (in volume fraction), in case of suspensions for example.

Ultra Small Angle X-ray Scattering (USAXS) measurements give access to X-ray scattering data for a range of smaller q and then complement the SAXS diffractograms. It requires a specific and very precise set-up, different from the one used for SAXS.

4.2.2 Materials and chemicals

Apparatus

The main set up components used for SAXS and USAXS experiments at CEA/LIONS are listed hereunder:

- X-ray generator : Rigaku generator RUH3000 with copper rotating anode ($\lambda = 1.54 \text{ \AA}$), 3kW
- Home made optic pathways and sample holders (with two channel-cut Ge (111) crystals in Bense/Hart geometry for USAXS set up, cf Lambard (1992).
- Flux measurement for SAXS set up : pico amperemeter Keithley 615
- Flux measurement for USAXS set up : DonPhysik ionization chamber
- Detector for SAXS set up : 2D image plate detector MAR300
- Detector for USAXS set up: 1D high count rate CyberStar X200 associated to a scintillator/ photomultiplier detector.

All experiment parameters are monitored by computer thanks to a centralized control-command system based on TANGO, and interfaced by Python programming. 2D images are treated using the software *ImageJ* supplemented with some specific plugging developed at CEA/LIONS. This control-command system have been achieved by Olivier Taché and is detailed in reference Taché (2006).

Calibration

A sample of 3 mm of Lupolen® (semi crystalline polymer) is used for the calibration of the intensity in absolute scale, the maximum intensity being adjusted to 6 cm^{-1} .

A sample of 1 mm of octadecanol is used for the calibration of the q range (calculation of sample-to-detector distance), the position of the first peak standing at 0.1525 \AA^{-1} .

Calibrations in intensity and in q range are performed before each series of measurements.

4.2.3 Sample preparation

Almost any kind of material can be analyzed by SAXS, whether it comes as a powder, a colloidal suspension, a gel, or even self-supported hybrid materials, and as long as the sample prepared meets some requirements of transmission and scattering properties.

Indeed, depending on the X-ray absorption coefficient of the material and its scattering properties, the sample thickness have to be adjusted to get a transmission as close as possible to the target transmission of 0.3 (optimal absorption/transmission ratio).

The sample thickness e is directly linked to the transmission T by the following equation:

—

μ : X-ray absorption coefficient of the material,

T : transmission, $T = \text{transmitted flux} / \text{incident flux of the direct beam}$

If not self-supported (liquids, powders or gel), the material to be analyzed is inserted in a cell, which can be made of glass (capillary), or X-ray transparent material such as Kapton® (polyimide). In any case, a measurement of the empty cell is performed and subtracted as a background for the sample measurement. See Figure 4.2 for examples of cells used at CEA/LIONS.

Powders

Nanomaterials in the form of powder are stored in glass vials of about 2 g sealed under argon. Once a vial has been opened to air for experiments, it is filled by N_2 after each use and the period of experiments should not exceed a few months. All experiments involving handling of powders are conducted under a fume hood with protective gloves, mask and glasses.

The coefficient of absorption depends on the material and on the energy. For the Cu $K\alpha$ emission (8 keV) that is used on our setup, the coefficient for TiO_2 is $\mu_{\text{TiO}_2} = 470 \text{ cm}^{-1}$ and $\mu_{\text{SiO}_2} = 77 \text{ cm}^{-1}$ for SiO_2 . The optimal sample thickness (equivalent thickness of dense material) to get a transmission of 0.3 is 25 \mu m for TiO_2 and 150 \mu m for SiO_2 .

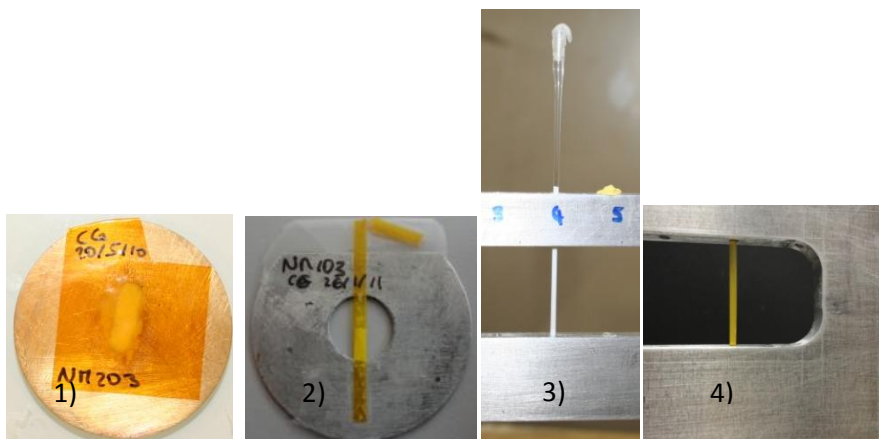


Figure 4.2 : Examples of different type of cells used for SAXS measurements, 1) double sticky kapton® cell for powders, 2) 1.5 mm flattened polyimide capillary for powders, 3) 1.5 mm glass capillary for powder or liquid samples, 4) 1.5 mm polyimide capillary for powder or liquid samples

In a first place, the TiO₂ powder samples were prepared between two sticky kapton® films pressed on a 0.4 mm brass cell (typical thickness of dense material around 30 µm). However, it was inferred that the presence of glue may affect the calculation of specific surface area of powders. Therefore, in a subsequent step, all the TiO₂ powder samples were measured in a flattened polyimide capillary, mounted on a circular sample holder (Figure 4.2, 2). The typical equivalent thickness of dense material obtained is 30 µm.

Most of the SiO₂ powder samples were prepared in 1.5 mm glass capillaries leading to typical equivalent thickness of dense material from 100 to 200 µm. However, NM203 powder is very sticky and was very difficult to insert into capillaries, so it was measured in a double sticky kapton® cell.

Aqueous suspensions

All aqueous suspensions prepared for NANOGENOTOX were measured in 1.5 mm polyimide capillaries, leading to transmissions of about 0.25.

4.2.4 Measurement

In order to calculate the sample transmission, the flux of incident and transmitted beam are measured and averaged over 200 s before running the SAXS measurement.

The time of acquisition necessary for SAXS experiment depends on the sample properties. For SiO₂ and TiO₂ powders, two measurements were performed: one with a short time of 200 s or 150 s to get unsaturated data for small angles (low q), and one for a long time of 1800 s to get data in the high q region with low signal/noise ratio.

All aqueous suspensions were measured for 3600 s (typical concentration in oxide 3 g/L).

4.2.5 Raw data treatment

SAXS data

Radial averaging of 2D image (ImageJ)

2D images from the detector are converted into Intensity = $f(\text{scattering vector } q)$ graphs thanks to the software ImageJ together with SAXS plugging. The process follows mainly these steps:

- Determination of the center coordinates (direct beam position)
- Application of a mask to remove pixels corresponding to the beam stop and around the photodiode
- Radial averaging of the intensity, knowing pixel size, sample-detector distance and wavelength (example of parameters in Figure 4.3), conversion of pixel position into scattering vector q , and creation of a .rgr file containing with $I(q)$ data.

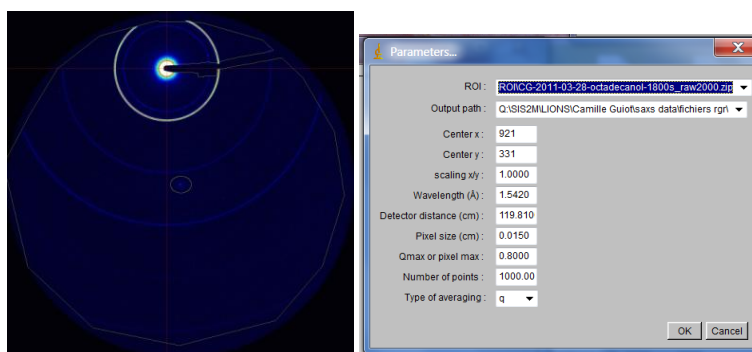


Figure 4.3 : Example of raw 2D image (octadecanol) and parameters used for radial averaging with ImageJ

Absolute scaling of $I(q)$ (pySAXS)

In order to scale the data to the absolute scale in cm^{-1} , $I(q)$ data generated by ImageJ as .rgr files are treated thanks to a homemade program called pySAXS and based on python programming.

The scaling involves a subtraction of the detector background and normalization by exposition time, sample transmission, sample thickness and K constant. The K constant is calibrated with Lupolen® sample and allows conversion of intensity in photon/s into absolute intensity in cm^{-1} . Example of parameters used for the scaling is shown on Figure 4.4.

The subtraction of the empty cell signal and the normalization by the sample thickness are then done in a subsequent.

USAXS data

Raw USAXS data are generated as intensity vs angle data in .txt files. Data treatment is achieved using PySAXS and involves the following steps:

- Subtraction of the “rocking curve” (signal with empty cell) normalized by the intensities at 0° (transmission).

- Desmearing, taking into account the effective size of the “punctual” detector (cf Lambard 1992)
- Conversion of angle into q range
- Normalization by the sample thickness.



Figure 4.4: Example of SAXS scaling parameter file from PySAXS software

4.2.6 Data analysis

General theorems of X-ray scattering have been developed to analyze SAXS data. Here are presented some simple laws for **binary systems** (two phase samples), that may be of use in NANOGENOTOX framework.

Porod’s Law

In the high q range, sample diffractograms display an intensity decreases in a q^{-4} trend, called the “Porod region”. This region corresponds in the “real space” to the scale of the interfaces (for smooth interfaces).

Therefore, for a binary sample, the asymptotic limit of the so-called “Porod’s plateau”, when data are represented in Iq^4 , is related to the total quantity of interface Σ (in m^2/m^3) between the two phases, as follows:

Where $\Delta\rho$ is the difference in scattering length density between the two phases. For a binary sample of **known thickness**, the volume fraction of a material ϕ_A , its specific surface area S_A/V_A (surface developed/ volume of A in the binary sample) and Σ are linked by the following relation:

For example, for a suspension of oxide in water, the determination of Porod plateau gives access to the concentration of the sample if the specific surface area of particles suspended is known (and vice versa).

Specific surface area determination from SAXS on powders

To treat raw SAXS data and get absolute intensities, one needs to normalize the intensity by the thickness of the scattering material. However, in powder samples, the sample thickness is not clearly defined and cannot be precisely controlled since it depends on the powder compaction and the different scales of porosity. To elude this problem, a model system is used, in which we consider the effective thickness of material crossed by X-rays, called e_B , corresponding to an equivalent thickness if all the material was arranged in a fully dense (no inner or outer porosity) and uniform layer .

The sample transmission is related to this equivalent thickness by the following equation:

—

Where μ is the material absorption coefficient for X-Ray - $\mu_{TiO_2} = 470 \text{ cm}^{-1}$ - $\mu_{SiO_2} = 77 \text{ cm}^{-1}$ and T_{exp} is the **experimental transmission** (transmitted flux Φ_T / incident flux Φ_0), i.e. transmission of the sample with regard to the transmission of the empty cell (kapton® alone, empty capillary, etc). The intensity scaled by this thickness e_B is called I_1 . The Porod's law can then be applied for I_1 to access the specific surface area of the powder. Cf Spalla (2003) for more details on the data analysis.

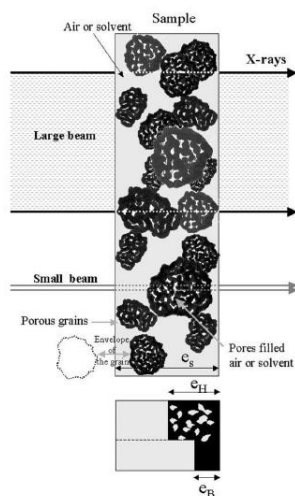


Figure 4.5 : Schematic representation of a powder sample for SAXS measurement, and definitions of equivalent thicknesses e_H and e_B .

Invariant theorem

When $I(q)$ can be extrapolated to zero values of q (no interaction at a large scale, i.e. a flat signal for low q) and infinite q (usually with the Porod law), the following invariant theorem can be applied:

This implies that the invariant Q is a constant for a defined composition, which gives access to the volume fraction φ , or to the evolution of interactions for a fixed composition.

Guinier regime

For **diluted samples of monodispersed objects** (negligible position correlation between scattering objects, i.e. structure factor 1), the intensity in the low q region ($qR_G \ll 1$) can be approximated to:

Which gives access to the **radius of gyration of the particles R_G** with the slope of $\ln(I)=f(q^2)$.

4.2.6 Data fits

Assuming values of parameters such as volume fraction, size, shape and polydispersity of scattering objects for a model sample, it is possible to calculate theoretical curves of $I(q)$. Therefore, the adjustment of such parameters to fit theoretical curves with some experimental data allows for the modelisation of the sample properties.

4.2.7 Observations on use and applicability

The SOP is under evaluation for refinements

5. Surface charge and size distribution analysis of dispersions

5.1 Surface charge and isoelectrical point of TiO_2 and SiO_2 by zetametry

Camille Guiot and Olivier Spalla (CEA); Keld Alstrup Jensen (NRCWE)

5.1.1 General description

Particles dispersed in a liquid are subject to an equilibrium between attractive (mainly van der Waals) and repulsive (electrostatic or steric) interactions. As shown in Chapter 2, stable suspension can be obtained in different ways, either electrostatic, steric, depletion stabilization or as a result of mixed conditions, called electrosteric stabilization. A stable electrostatic stabilization of small sub- μm -size particles is obtained if the repulsive interactions overcome the attractive ones, responsible for agglomeration. Zeta potential is a good index of the magnitude of the repulsive interactions between charged particles. The charge at the very surface of the particles is not accessible. Zeta potential corresponds actually to the potential at the shear plane; i.e. the boundary between the bulk dispersant and the double layer of solvent and ions moving together with the particles (outer limit of Gouy-Chapman layer (Fig. 2.1). κ^{-1} , called the reciprocal Debye length, represents the thickness of this double layer.. This zeta-potential varies with pH due to protonation-deprotonation of the material surface. From colloid science, a suspension of small particles is considered stable if the zeta-potential exceed $|30|$ mV.

In acidic medium, the surface of metal oxide materials is protonated (MOH_2^+) leading to positively charged particles, whereas for high pH the deprotonation results in negatively charged particles (MO^-). The pH-value at the point of zero-charge determines the so-called isoelectric point (IEP) at which the dispersion is highly unstable. The IEP is usually determined by titration, but can also be measured by manual preparation of different dispersions. The zeta potential can be greatly influenced by the properties of the medium, such as ionic strength (by compression of the double layer), or adsorbing molecules or ions (especially multivalent ions)

The zeta potential (ζ) is calculated from the measurement of electrophoretic mobility U_E using Henry's equation:

$$U_E = \frac{2 \varepsilon \zeta f(\kappa a)}{3 \eta}$$

ε : dielectric constant of medium

η : viscosity

κ : inverse of the Debye length, a : radius of a particle

$f(\kappa a) = 1.5$ for aqueous suspensions in the Smoluchowski approximation

In practice, the sample is submitted to an electric field which induces the movement of charged particles towards the opposite electrode.

5.1.2 Chemicals and equipment

- HNO₃ (analytical grade)
- NaOH (analytical grade)
- NaNO₃ (analytical grade)
- Purified water (MilliQ or Nanopure water)
- Ultrasonic probe (e.g. Sonics & Materials, VCX500-220V, 500 W, 20 kHz, 40% amplitude for 20 min or Branson Bransonic 400W, 10% amplitude for 16 min, both equipped with a standard 13 mm disruptor horn.
- Zetasizer Nano ZS (e.g. Malvern Instruments), equipped with laser 633 nm
- Autotitrator (Malvern MPT-2) –*optional for automatic determination of IEP*
- Zetasizer software (DTS 5.03 or higher)
- Clear disposable zeta cells (DTS1061)
- Computer

5.1.3 Procedures

Preparation of dispersions

Samples for zeta potential measurements are prepared as aqueous suspensions of 0.5 g/L for TiO₂ nanomaterials, and 1 g/L for SiO₂ nanomaterials with constant ionic strength of 0.036 mol/L (monovalent salt) and controlled pH. They are prepared by dilution of concentrated sonicated stock suspensions of 10 g/L into pH and ionic strength controlled “buffers”.

Stock suspensions

20 mL of stock suspensions of 10 g/L NM (SiO₂ or TiO₂) in pure water are prepared using the following steps:

- 200 mg of NM are weighed and introduced in a 20 mL gauged vial (with protective gloves, mask and glasses, and damp paper towel around the weigh-scale).
- The 20 mL gauged vial is completed with ultrapure water (MilliQ)
- The suspension is transferred into a 40 mL large-neck glass flask of internal diameter 38 mm (height of 20 mL liquid, 20 mm), making sure that all the settling material is recovered.
- The suspension is dispersed by ultrasonication following selected protocols (Sonics & Materials sonifier, VCX500-220V, 500 W, 20 kHz, 40% amplitude for 20 min or Branson Bransonic 400W, 10% amplitude for 16 min, both equipped with a standard 13 mm disruptor horn; both with contained in a ice-water bath. Probe, sample and bath are placed in a sound abating enclosure, and inside a fume hood.

Preparation of buffer solution

Denominated *buffer* solutions are aqueous ionic solutions of Na⁺, H⁺, NO₃⁻ and OH⁻, designed to display the same ionic strength with a modulated pH.

- A first set of concentrated buffer solutions (0.1 mol/L of salt, various pH) are prepared by addition of HNO₃, NaOH and NaNO₃ in various proportions in ultrapure water.

- Then, 20 mL of these concentrated buffers are poured into 50 mL gauged vials completed with ultrapure water, to lead to a new set of buffers with a salt concentration of 0.04 mol/L and a pH ranging from 1.5 to 12.5. The combination of two consecutive buffers gives access to the necessary intermediate pH.
- By this procedure, acidic buffers contain 0.04 mol/L of NO_3^- and various ratios of Na^+ / H^+ as counter ions; likewise, basic buffers contain 0.04 mol/L of Na^+ and various ratios of $\text{NO}_3^- / \text{OH}^-$.

Preparations of suspensions for zeta potential measurement and determination of isoelectric point

In this SOP Zeta potential measurements are performed on:

- 0.5 g/L suspensions for TiO_2 samples
- 1 g/L suspensions for SiO_2 sample (the lower refraction index of SiO_2 requires higher concentration).

For TiO_2 samples, freshly sonicated stock suspensions are first two-fold diluted in ultrapure water to get 5 g/L concentrated suspensions. For SiO_2 samples, 10 g/L suspensions are used right after sonication.

Series of samples are prepared by addition of 400 μL of concentrated NM suspension (10 g/L for SiO_2 and 5 g/L for TiO_2) and 3.6 mL of 0.04 mol/L buffer solutions in a 5 mL glass flask. This lead to samples of 0.5 g/L TiO_2 or 1 g/L SiO_2 and a constant ionic concentration of 0.036 mol/L in monovalent salt.

For each NM, an additional sample is prepared in MilliQ or Nanopure water with the same NM concentrations, i.e. by addition of 400 μL of concentrated NM suspension and 3.6 mL of water.

Optional: If results looks suspicious or samples are known to contain larger particles, it may be filtered through a 0.8 or 3 μm syringe before addition to the cuvette to reduce interference caused by stokes sedimentation during the electrophoretic transport in the cuvette.

Measurement

Zeta measurements are performed on the “general purpose” mode at 25°C with automatic optimization of laser power, voltage settings, the number of runs (10 - 100) and run duration. The analysis is repeated 3 times with no equilibration time (CEA) or 6 times with 1-2 min equilibration time (NRCWE) on the same vial, depending on temperature setting in the zetasizer. The Smoluchowski model ($F(\kappa a)=1.5$) was used and hence assuming a thin double layer around the particles.

For the dispersant one may use the refractive index R_i , absorption R_{abs} , viscosity and di-electric properties for water or calculate the properties of the specific test solution using the in-built calculation tool in the DTS software. In this SOP version, we consider the use of the reference water parameters and material properties as listed in Tables 4.1 and 4.2.

Table 4.1 Conditions used at CEA

	Water (STP)	TiO_2	$\text{SiO}_2(\text{amorphous})$
R_i	1.33	2.49	1.5
R_{abs}		0.01	0.01
Viscosity [cP]	0.8872	-	-

Table 4.2 Conditions used at NRCWE

	Water (STP)	Rutile	Anatase	SiO ₂ (amorphous)	CNT
R _i	1.33	2.903	2.49	1.544	2.02
R _{abs}		0.10	0.10	0.20	2.00
Viscosity [cP]	0.8872	-	-	-	-

Data treatment

Electrophoretic mobility is measured by a technique called Laser Doppler Velocimetry, and based on the phase shift of the laser beam induced by the movement of particles under an electric field. This technique is combined to a “mixed mode measurement” (M3). During the fast field reversal mode, the electro-osmose effect is negligible, allowing to determine an accurate mean zeta potential, whereas the slow field reversal mode helps modeling the distribution of potentials. An example of the main data plots returned by DTS software from zeta potential measurements is shown (phase plot and corresponding electric field applied, mean zeta potential and zeta potential distribution).

More details on the results produced in zeta potential measurements with M3-PALS technique are available in the documentation from Malvern Instruments. The reported value is the average of zeta potential values from the 3 (CEA) or 6 (NRCWE) measurements (determined during the fast field reversal step), with possible exclusion of diverging data. At CEA, degradation of the signal was observed on the phase plot during the measurement for several TiO₂ samples. In this case, only the first measurement was used. This phenomenon might be avoided by filtration for large aggregates/agglomerates.

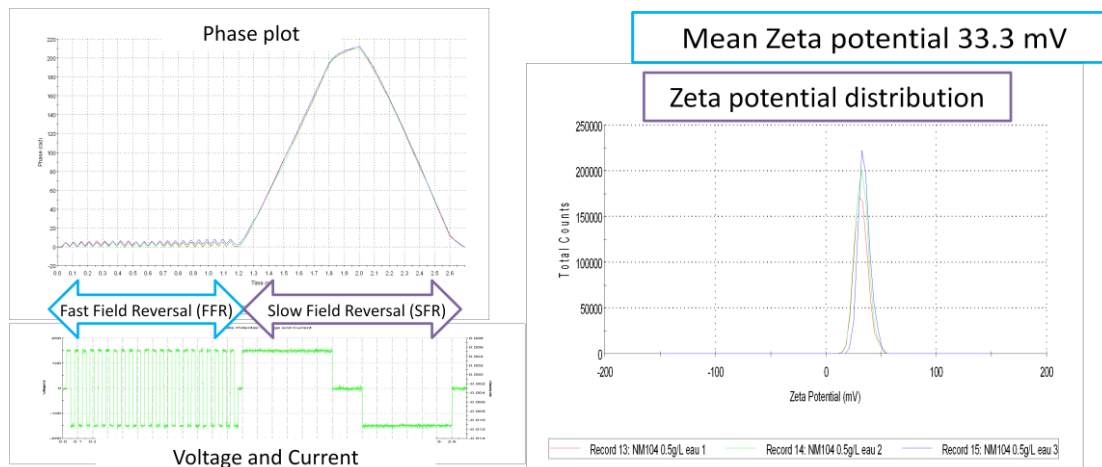


Figure 5.1: Data plots retrieved from zeta potential measurements on Nanosizer ZS, example of 3 consecutive measurements on a suspension of NM104 at 0.5 g/L in pure water.

5.1.5 Comments on use and applicability

The current methods description is a first harmonization of the procedures applied at CEA and NRCWE. Initial test analyses have been conducted at CEA and comparative analyses will be made at NRCWE using the Malvern autotitrator an in-house procedures. Hence, the SOP for determination of IEP and surface charge will be further developed and harmonized in the final report.

5.2 Dynamic light-scattering

Camille Guiot and Olivier Spalla (CEA), Keld Alstrup Jensen (NRCWE)

5.2.1 General description

Dynamic Light Scattering (DLS), also called Photon Correlation Spectroscopy (PCS) or Quasi-Elastic Light Scattering (QELS), is a technique of characterization of colloidal systems based on the scattering of visible light resulting from the difference in refractive index between the dispersed colloids and the dispersion medium. The method may be applied for sizing particle suspended in a liquid in the range from about 0.6 nm to about 6 μm depending on the optical properties of material and medium.

The principle in DLS is measurement of fluctuations in laser light scattered by vibrating particles suspended in a liquid as function of time. The vibration is due to Brownian motion caused by collision with solvent molecules of the liquid. The Brownian motion varies as a function of particle size and causes variation in the intensity of scattered light as function of time. A correlator compares the signal measured at a time t_0 with different very short time delays dt (autocorrelation). As the particles move, the correlation between t_0 and subsequent dt signals decreases with time, from a perfect correlation (1) at t_0 , to a complete decorrelation (0) at infinite time (order of milliseconds). In case of big particles, the signal changes slowly and the correlation persists for a long time, whereas small particles have high Brownian movement causing rapid decorrelation.

In fact a DLS instrument measures the velocity of Brownian motion, defined by the translational diffusion coefficient D of the particles. The particle size, or more precisely its hydrodynamic diameter d_h , is then estimated using the Stokes-Einstein equation assuming spherical shape:

$$d_h = \frac{kT}{3\pi\eta D}$$

k : Boltzmann's constant

D : translational diffusion coefficient

T : absolute temperature

η : viscosity

It should be noted that even if a particle is really spherical, the spherical DLS size is fundamentally different from the physical spherical size. The hydrodynamic size includes the double-layer with highly polarized water molecules around the physical particle. When the particle morphology is highly nonspherical, the hydrodynamic size should be understood as the equivalent hydrodynamic spherical size. Similar concept is used in aerodynamic measurement of airborne particles. Establishment of mean hydrodynamic size and size distributions (intensity, number, volume) is reached by DTS software algorithms, by fitting the correlation function (cf data treatment section).

5.2.2 Chemicals and Equipment

Samples for DLS are measured in 1 cm transparent cuvettes of glass or polymer. DLS measurements rely on non-invasive back scatter (NIBS[®]) technology developed by Malvern Instruments, in which the signal is detected at 173° (Figure 5.2). The signal is treated by a digital correlator, and transmitted to

the computer. DTS software enables the fitting of correlation data either by a single exponential, called the cumulant analysis (as defined by ISO 13321 Part 8) to obtain mean size (Z-average diameter) and polydispersity index (PDI), or by a multiple exponential known as the CONTIN method to obtain a distribution of particle sizes.

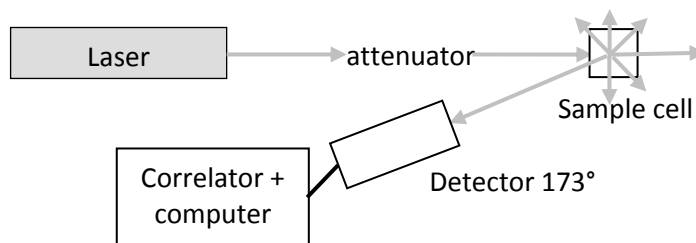


Figure 5.2: Simplified sketch of the optical configuration for DLS measurement on Zetasizer Nano ZS

Specific materials and equipment

- Test material or chemical
- Dispersion medium
- Ultrasound bath or Ultrasonic probe (e.g, Sonics & Materials, VCX500-220V, 500 W, 20 kHz, 40% amplitude for 20 min or Branson Branson 400W, 10% amplitude for 16 min, both equipped with a standard 13 mm disruptor horn.
- Zetasizer Nano ZS (Malvern Instruments), equipped with laser 633 nm
- Zetasizer software (DTS 5.03 or higher)
- Viscosimeter (e.g, Malvern Inc., SV-10 Vibro Viscometer) *Optional for measurement of true viscosities*
- DLS cuvette of clear disposable polymer or glass cells or folded capillary zeta cells
- Pipette and pipette tips
- Syringes and syringe filters or filter paper
- Computer

5.2.3 Procedure

Sample preparation

Dispersions for analysis are prepared by mixing particulate material into a dispersion medium. A sub-sample of a suitable concentration is added to suitable measurement cuvettes. Dispersions are typically produced by sonication in a dispersion medium. The dispersion medium must be filtrated before use to avoid any dust contamination. This can be done by using syringe filters or filter paper with high efficiency. Usually filters with a 0.2 to 0.45 μm pore-size are sufficient for filtration of dispersion media.

The concentration required for analysis depends in part on the relative refractive index between particles and dispersion medium, the particle size and polydispersity and the sample absorption. Malvern apparatus is designed to measure samples over a large range of concentration and size of particles. Specifications of sample properties (concentration range, size of nanoparticles, medium)

can be found in the documentation from Malvern Instrument accessible on their website. Overview of measurement conditions used at CEA and NRCWE is listed in Table 5.3 and 5.4.

For this analysis, the dispersions protocols presented in chapter 2, section 5.1 or different protocols may be used depending on purpose of the measurement. It is mandatory that the dispersion is stable within the time-frame of the measurement.

Table 5.3 Conditions used at CEA

	Water (STP)	TiO ₂	SiO _{2(amorphous)}
R _i	1.33	2.49	1.50
R _{abs}		0.01	0.01
Viscosity [cP]	0.8872	-	-

Table 5.4 Conditions used at NRCWE

	Water (STP)	Rutile	Anatase	SiO _{2(amorphous)}	CNT
R _i	1.33	2.903	2.49	1.544	2.02
R _{abs}		0.10	0.10	0.20	2.00
Viscosity [cP]	0.8872	water	water	water	variable

Measurement

DLS measurements are performed in disposable polystyrene cuvettes or alternatively glass cuvettes. The measurements are repeated 3 (CEA) or 6 (NRCWE) times to establish an average diameter and intensity size distribution, which can be converted to number or volume size-distribution. The repeated analysis are conducted to enable omission of measurements with bad correlation data or abnormal solutions to the correlation function (must be carefully considered).

The following procedure is recommended as the general approach for DLS measurement of NM dispersions.

1. Turn on the computer and DLS instrument
2. Allow the instrument to warm up according to the manufacturer's recommendation
3. *Optional: Complete viscosity measurement using the SV-10 Vibro Viscometer mounted with the 10 ml flow-reactor placed in a thermostatted water jacket. The measured dynamic viscosity is used as input data for the specific dispersion measured in the DTS software.*
4. Upload the DTS software and the "Measurement" window for entering material specific data on dispersion mediums and test materials as well as specific analytical settings.
 - Refractive index and absorption values for dispersant and NM are found and entered
 - Temperature conditions and equilibration time for measurement is entered
 - The General purpose model is selected for initial evaluation of data and is the most generic model for calculation of size.
5. Select a sample cuvette and ensure that it does not contain dust, nor defects or scratches in the measurement area of the cuvette. Some producers have been found to deliver cuvettes with scratches or folding structure in the measurement area at one side of the cuvette. Dust may be cleaned out by rinsing the cuvette in dispersion medium.
6. Fill in a suitable volume of the dispersion into a suitable measurement cuvette using a pipette
7. Place the sample cuvette in the sample holder in the DLS instrument
8. Run analysis

9. If the analysis suggests that the sample has a bi- or multimodal size-distribution, the result should be recalculated using the high-resolution algorithm selected under “Data processing” in the “Edit result...” menu.
10. The size analysis may be immediately accepted if the DTS Expert advice denotes the result quality as “Good”. If the result is not of good quality, the sample should be further analyzed for presence of dust, cuvette errors, large particles, sedimentation, wall-deposition etc.
11. If the sample contains particles with large spread in size-distribution, one may consider filtering the sample through different syringe filters to investigate presence of small nm-size particles. Small nm-size particles may not be fully resolved when coarser particles are present due to the large drop (10^6 per decade) in scattered light intensity with size.
12. If parameters such as refractive indexes, absorption coefficient or viscosity were wrong or unknown at the measurement time, the correction can be made afterwards using the command Edit (right click on the measurement) in DTS software.

Data treatment

The actual raw data obtained from a dynamic light scattering experiment is the autocorrelation function, which is an exponential decaying with a characteristic time related to the size of the diffusing object. An example of correlation data is shown on Figure 5.3 for two NM104 samples (0.5 g/L TiO_2 , 0.036 mol/L of monovalent salt), one stable suspension at pH 2.8 (red curves) and the supernatant of an aggregated sample at pH 10.1 (green curves). The data used are the averaged data for 3 consecutive measurements.

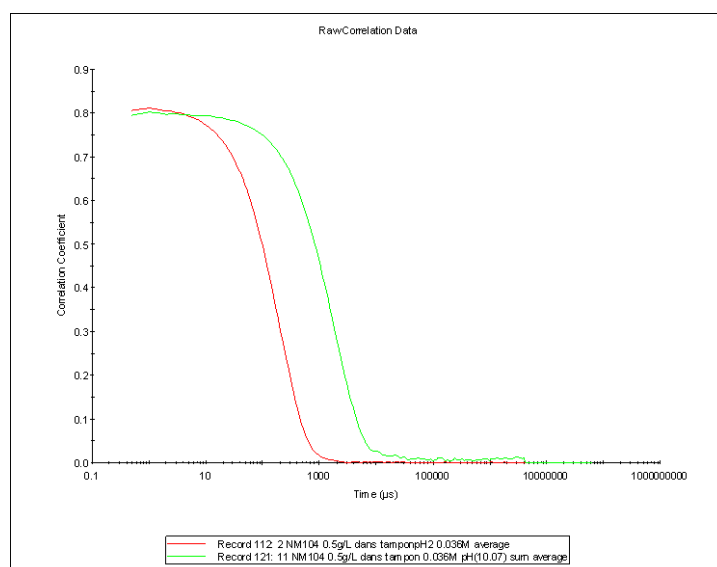


Figure 5.3 : Example of raw correlation data for two NM104 samples (0.5 g/L TiO_2 in 0.036 mol/L ionic buffer), one stable suspension of relatively dispersed particles at pH2.8 (red curve), and one unstable sample of big aggregates at pH10.1 (green curve, measure on supernatant).

The raw correlation data are analyzed to extract information on size and distribution. Various algorithms can be used. The simplest one is called the *Cumulants analysis*. It fits the data by a single exponential and provides a **Z average mean** value, which corresponds to the particle size diffusing

with the highest intensity, and a **polydispersity** for this monomodal distribution. In DTS software, the corresponding graph is entitled “Cumulants fit” (Figure 5.4). This analysis method applies for monomodal distributions with polydispersity lower than 0.25, and is in agreement with ISO 13321 standard.

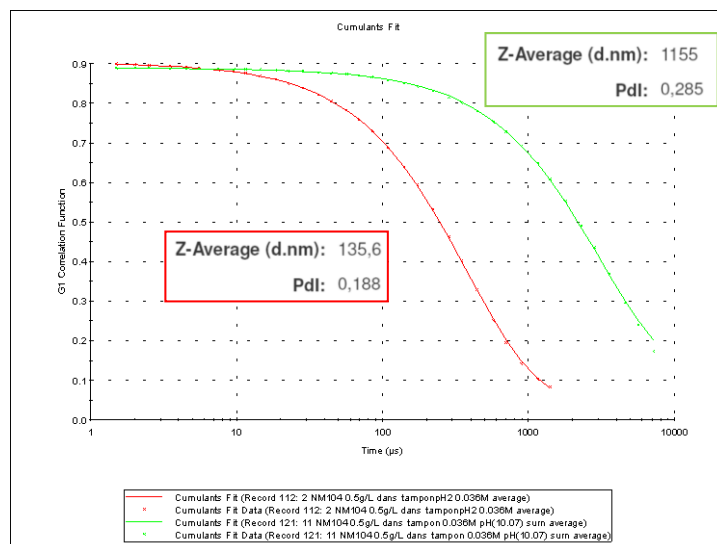


Figure 5.4: Data and fits by the Cumulant method, together with calculated values of Z average and polydispersity, for two NM104 samples (0.5 g/L TiO_2 in 0.036 mol/L ionic buffer, stable suspension at pH2.8 in red and unstable sample of big aggregates at pH10.1 in green).

The correlation data can be better analyzed by the *CONTIN method*. It consists in fitting the correlation data by the best combination of a set of 24 exponential functions. In DTS software, this fit is denominated as “distribution fit”, “data fit” or “size fit” (Figure 5.5).

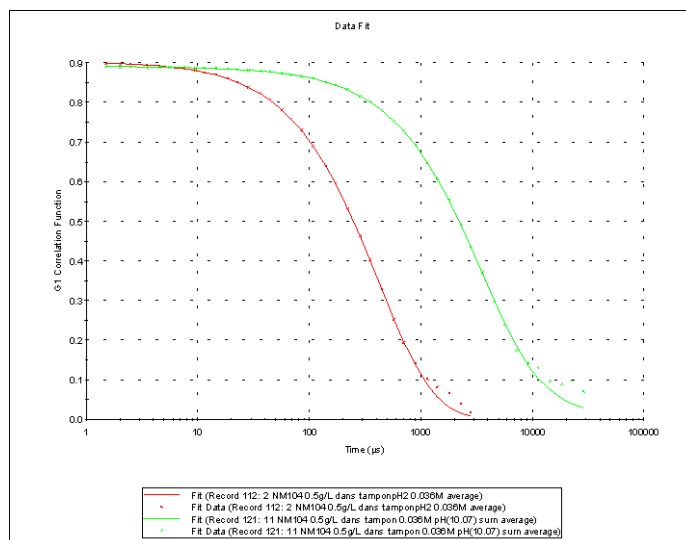


Figure 5.5: Data and fits by the CONTIN method, for two NM104 samples (0.5 g/L TiO_2 in 0.036 mol/L ionic buffer, stable suspension at pH2.8 in red and unstable sample of big aggregates at pH10.1 in green).

Taking into account the refractive indexes of material and dispersant, Mie Theory can be applied to represent size distribution in **volume**. The **number** size distribution can then be calculated from simple geometrical considerations (Figure 5.6). Distribution data can be retrieved from DTS software in the form of tables of diameter, percentage and width for the three main peaks.

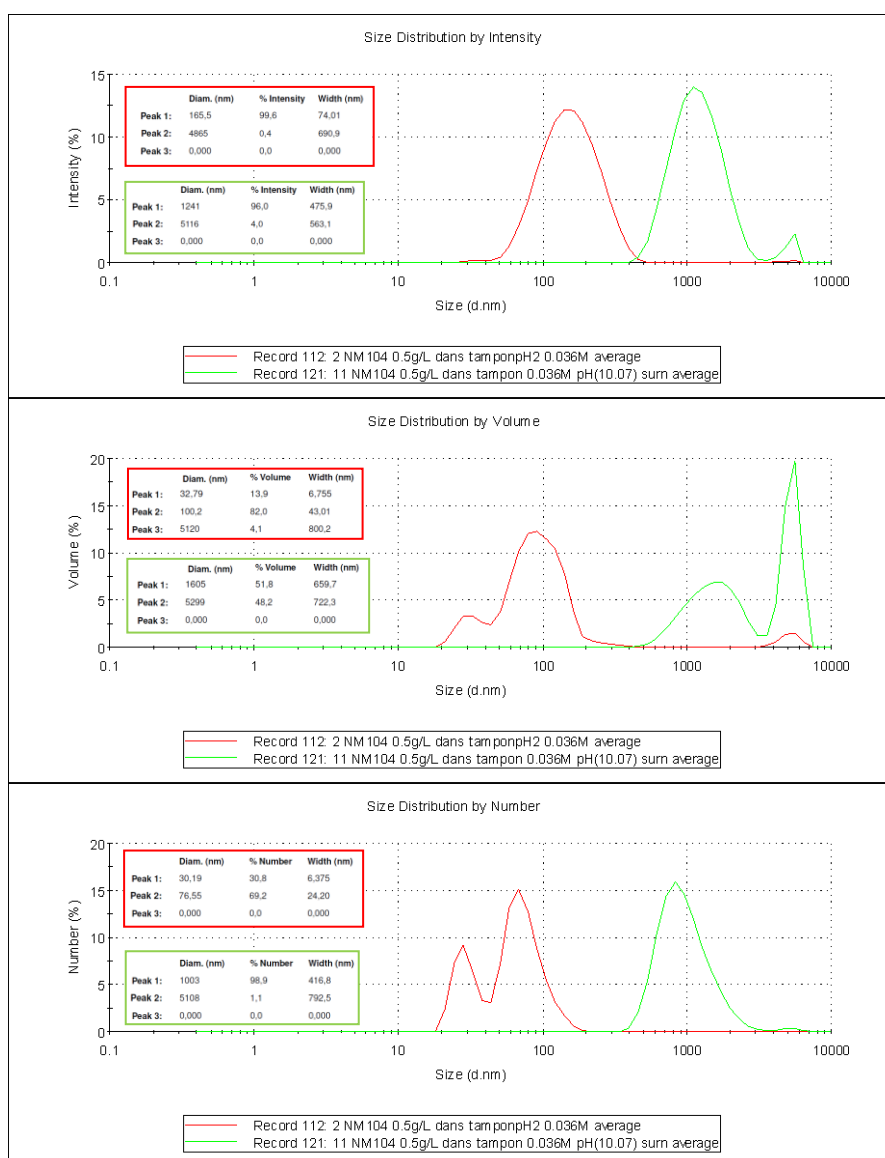


Figure 5.6: Size distributions by intensity, by volume and by number, together with tables of numerical values for the three main peaks of each distribution, for two NM104 samples (0.5 g/L TiO_2 in 0.036 mol/L ionic buffer, stable suspension at pH2.8 in red and unstable sample of big aggregates at pH10.1 in green).

It has to be noticed that for 2 particles with a size ratio of 10, the contribution of the bigger particle to the volume distribution is 10^3 times more than the smaller one, and it reaches 10^6 for the distribution by intensity. Since the measurement done by DLS is based on the intensity, this means that the light scattered by a few large particles may totally cover the signal from the smaller ones.

After controlling correlation data and fits, an average measurement is calculated with the software. As an example, the main graphs observed for the 3 initial measurements of a sample of NM104 at pH 2.8 (0.5 g/L TiO₂, 0.036 mol/L of monovalent salt) are displayed on Figure 5.7. Since the correlation data are good, all 3 measurements are taken into consideration for the averaged data.

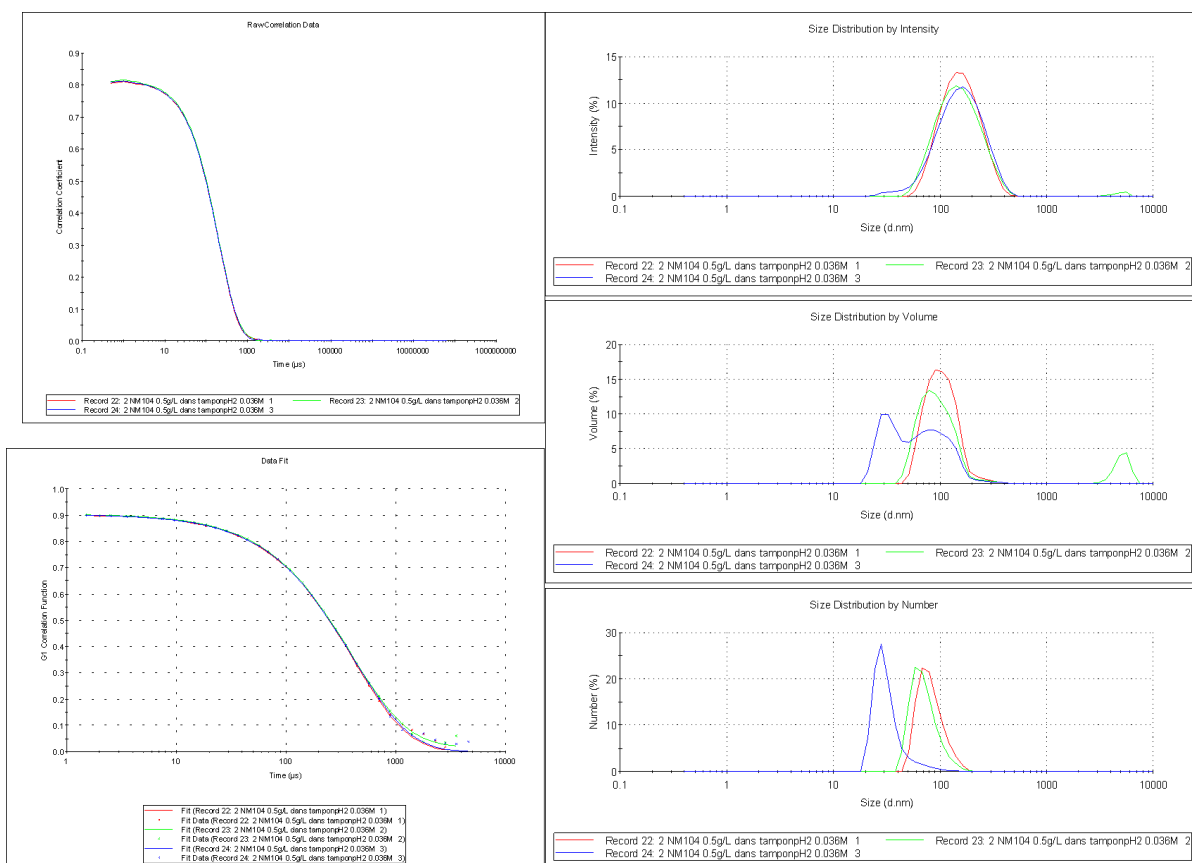


Figure 5.7: Main graphs reported by DTS software for 3 consecutive measurements of a NM104 sample (pH 2.8, 0.5 g/L TiO₂ in 0.036 mol/L aqueous ionic medium)

The main parameter reported in the results section is “Z average”, which represents the mean size contributing to the major part of the signal **in intensity**. Therefore, this value mostly gives a hint on the aggregation state of the particles but does not reflect the hydrodynamic size of most of the dispersed particles (in number), which of course is much lower. When Z average is higher than approximately 500 nm, it can only be deduced that there are big aggregates in suspension but the numerical value is totally meaningless.

5.2.4 Observations on use and applicability

DLS is very suitable for size and stability analysis of particles in liquid dispersions. However, great care should be taken in interpretation of data; especially when the sample contains both μm - and small nm-size particles.

For better accuracy of size-determination, it is important to obtain true values of the optical properties and viscosity of the dispersion liquid.

Currently, there is high uncertainty in understanding the signal obtained during analysis of dispersions of CNT.

Further analysis of applicability and improvements of analytical procedures will be conducted as part of the NANOGENOTOX project.

6. Structural analysis

The structure of crystalline phases can be analyzed using a number of different methods. In this work most of the analysis has been X-ray diffraction (XRD). However, electron diffraction done during transmission electron microscopy and RAMAN spectroscopy was also employed. Here we present SOPs for different XRD-analysis and RAMAN spectroscopy.

6.1 General SOP for phase identification and determination of crystallite size using powder X-ray diffraction analysis

Renie Birkedal (NRCWE)

6.1.1 General description

This method is a general procedure for identification of crystalline materials in powders and calculation of average crystallite size using powder X-ray diffractometry.

The principle is that crystalline materials diffract X-rays in a characteristic pattern, which is unique for each material. XRD can therefore be used to identify different polymorphs, e.g. rutile and anatase. The width of the reflections can also give information about the size of the diffracting crystals (not necessarily the same as the particle size).

XRD can be measured in different setups and depending on the information wanted, the setup must be chosen. Different wavelengths are also possible, but for standard measurements this is less important, as long as it is remembered and taken into account. Most databases are based on irradiation using Cu X-rays. The step length (if using Cu) is recommended to be 0.15. (Hill, 1986)

Reflection data is usually chosen for a fast identification of phases. It has the advantages that very small samples can be used (though larger is recommended) and the scatter is usually to high values of 2theta, so unit cells can be determined with high accuracy.

Transmission data are better for further calculations, such as analysis and determination of atomic structure and strain, because in this case, the X-rays have passed through the whole sample.

6.1.2 Materials and equipment

- Powder material
- X-ray diffractometer
- Sample holder (e.g. Al-holder, [001] quartz-holder or plastic holder)
- Glass-plate to flatten powder surface
- Data-collection software
- Data treatment software (e.g. TOPAS and Fullprof)

6.1.3 Sample preparation

The powder sample is placed in the sample holder. For reflection data the sample holder is usually flat, app 1.5 cm in diameter and 2 mm thick. The sample is pressed in the sample holder to ensure a flat sample with the correct height. For transmission the sample is placed in the sample holder – usually a capillary tube, between two thin sheets of foil or on tape.

If the powder is too coarse the sample should be grinded prior to analysis to minimize preferred orientation effects. If the crystal size is to be calculated this is not an option.

Sample time depends on the detector, the X-ray tube and the sample.

6.1.4 General data treatment

Identification of materials is done using databases, whereas the phase composition and crystallite size are calculated using dedicated software. Most diffraction programs can be used for calculating the ratio between phases (if the phases have been identified).

Some programs (e.g. TOPAS and Fullprof) can be used for calculating the average crystallite size. Almost all programs can also be used for refining and listing FWHM (full width at half maximum), alternatively this can be measured. FWHM can be used together with Scherrers formula to calculate an approximate average crystal size. The most accurate result is obtained, if the K-factor (the shape factor) in Scherrers formula is found by calibration with the same material as the measured.

6.1.4 Comments on use and applicability

Estimation of amorphous content based on addition of material is not recommended. It is difficult to ensure an effective mixing and by adding a crystalline material one may shadow the presence of other materials or the dopant.

Results from quantitative determination of bulk phase composition (proportions) and average crystallite sizes may be affected by the settings chosen to mathematically fit the X-ray diffractograms as well as by the type of reference or standard used to obtain the diffractogram. Observations indicating these phenomena have been made in NANOGENOTOX and are currently under investigation.

6.2 Identification and quantification of crystalline phases of TiO₂ powders crystallite sizes using the X'pert Pro MPD diffractometer

Charles Motzkus (LNE)

6.2.1 General description

This procedure describes a method for identification of crystalline phases and crystallite sizes of TiO₂ powders using the X'pert Pro MPD diffractometer. This diffractometer has a $\theta - \theta$ goniometer configuration enabling the characterizing of powders at high diffraction angles. The secondary optical focusing of X'pert Pro MPD can deliver analysis mode "skimming" for very low diffraction angles. The powders are prepared and placed in a rotating sample holder; the Spinner, which increase the bulk analysis by obtaining diffractions from a very high fraction of the material in the irradiated sample volume. Thus the value of the ratio intensity/resolution is high.

The linearity of the incident beam diffraction is regularly checked. The verification of the position of the goniometer which is performed using a single crystal of silicium allows to determine the identification of crystalline phases.

6.2.2 Materials and methods

- Test material (powder)
- X-ray diffractometer Type X'pert Pro MPD
- Anode X-Ray tube Cu 50kV 35mA
- Multiple sample holders and automatic changer 15
- Primary and secondary optical focusing
- Software X'pert High Score + Stress & Texture
- Database ICDD 2009

Verifying the alignment / single crystal Si:

- Results 4 / 1000
- Value of acceptance linearity 4 / 100
- Adjustment of optimum settings of the beam by fluorescence : intensity / resolution

6.2.3 Preparation of the samples :

The number of samples for analysis for each reference is equal to 2 in order to estimate the repeatability of the measurement.

These samples were stored at 20°C. For analysis, nano-TiO₂ powders were prepared and placed in sample holders for Spinner. During the diffraction analysis, the samples were rotated in order to increase the diffraction phenomena on all the crystals in the sample. Thus, the value of the ratio resolution/intensity was high and allowed treatment of the diffractograms. The analyses were performed for two samples (two bottles) of each reference (NM).

6.2.4 Measurement protocol

The diffractograms were obtained with a scan on range of 2theta from 3 to 140°. The stepping of the goniometer was fixed for these tests to 0.003° for an acquisition time of 300 s. The chamber temperature was 25°C. Analyses were performed at 50kV and 35mA.

For different spectral analysis, peak areas were preferred against intensity: the background was subtracted and only the most intense peaks without recoveries were identified.

Quantification is recommended using the "Reference Intensity Ratio (RIR)" method. The principle of this method is based on the determination of the ratio between the intensities of main peaks of each phase in relation to corundum for a mixing 50/50. The values constitute the RIR recorded for some of the phases in the ICDD database (the International Centre for Diffraction Data).

The phase identification and quantification are made with the ICDD database on the crystalline phases. This method can be considered quantitative if there are only two main phases in the TiO₂ powder: e.g, anatase and rutile. The number of samples for analysis for each concentration must be at least 2 to estimate the repeatability of the measurement.

We determined the association of Nickel filter, masks, slot and anti-scatter since these conditions leads to better results resolution / intensity spectrum exclusively for analysis on specifics powders. The average size of crystallites is obtained from the Scherrer equation by treatments of spectra.

6.2.5 Observations on use and applicability

Preliminary internal analysis report of 6/11/2009 indicated a shift of the beam equal to 4/1000. This shift is not significant because the accepted value provided by the manufacturer is equal to 4/100.

Variability analysis of NM samples may require more than analysis of 2 different sub-samples or vials as indicated in this procedure.

6.3 RAMAN spectroscopy

Renie Birkedal (NRCWE)

6.3.1 General Description

Monochromatic (laser) light interacts with molecular bonds or the electron clouds of the test material and the energy of the photon is changed (there is a shift up or down). The shift is unique for each material and Raman can therefore be used as a « finger print identification » method.

6.3.2 Materials and Chemicals

Test material (powder or slurry to be dried out)

Glass plate as sample substrate (should have unique or non-interfering RAMAN signal)

6.3.3 Sample preparation:

The material is placed on a glass plate and flattened onto the plate with a spatula.

6.3.4 Analytical conditions and data collection

It is very important to ensure shielding towards light of any kind. The raman spectrometer must be equipped with shielding otherwise light will interfere with the measurements – especially for white samples. As each RAMAN measurement is very fast several measurements each measurement should consist of 2 or 3 scans. This removes spikes from “cosmic radiation”. Measurements should be performed on different places on the sample.

6.3.5 Data treatment

By comparing several dataset from the same sample, the sample is analyzed for inhomogeneity. Various databases are used for the identification by Raman spectroscopy, e.g. are many inorganic materials and minerals are found in: <http://rruff.info/>

6.3.6 Comments on use and applicability

RAMAN spectroscopy is highly suitable for diameter sizing and structural characterization (D and G-bands) of single-walled CNT. However, in-depth categorization of structural type and defect levels requires analysis using several different laser wavelengths. RAMAN spectroscopy was found only partially suitable for structural analysis of multi-walled CNT. Whereas the D and G bands are clearly resolved and can be used to analyze sample homogeneity, there is currently no clear procedure for structural categorization of multi-walled CNT.

7. Chemical analysis

7.1 Thermogravimetric Analysis (TGA)

Renie Birkedal (NRCWE)

Based on NIST Recommended Practice Guide, Special Publication 960-19

7.1.1 General description

TGA is short for thermogravimetric analysis. The principle is measuring sample weight as a function of temperature in a given atmosphere at a given heating rate.

TGA is measured according to information wanted and material investigated.

If information about evaporation is wanted heating in N₂ is recommended. If information about organic content is wanted heating in O₂ or air is recommended, as this will insure combustion of all organic material. In order to make sure e.g. all organic material is decomposed, it is recommended to run to 1000 C.

7.1.2 Materials and Chemicals:

Powder (may be conditioned in a specific atmosphere and humidity conditions)

Laboratory weigh (scale)

Apparatus for thermogravimetric analysis. Sensitivity must be better than

7.1.3 Procedure

Sample preparation:

- Weigh container.
- Fill container with material. Do not stamp it, as this may affect the evaporation/decomposition temperature.
- Weigh container and material.

For inorganic powder materials a minimum of 10 mg should be used – if possible more. These samples are usually quite homogeneous and this is usually a representative fraction of the sample.

CNT samples are somewhat different. They are in many cases bundles, and these bundles may be different. At the same time these compounds often have a low density, and it is therefore difficult to measure a representative fraction in one or two measurements. The solution is many measurements and comparison of the data.

Selection of heating rate.

For inorganic materials only a minor fraction is expected to decompose, and a heating rate of 10 C/min is recommended. It is not assumed that there will be large weight losses for these materials, so this heating rate ensures a fast measurement and most likely still well defined weight losses. If the weight losses are not well defined a slower heating rate can be chosen.

The NIST Recommended Practice Guide, Special Publication 960-19, Measurement Issues in Single Wall Carbon Nanotubes, recommends a heating rate of 5 C/min. This is chosen as a compromise between time and avoiding too much spontaneous combustion. For some carbon nanotubes 5 C/min is not slow enough to avoid spontaneous combustion. There is no spontaneous combustion with a heating rate of 2.5 C/min. The measurement time is very long, app 7 hours per measurement, but this is still recommended. In order to minimize measuring time it may be an option only to heat to 900 C or even lower.

7.1.4 Data treatment:

Compare TGA curve and curve for first derivative to find steps of weight loss. It is recommended to obtain several measurements to calculate the mean and standard deviation of the weight loss and the evaporation/decomposition temperatures. (the last is most easily found from the curve of the first derivative). The test of multiple samples also enables evaluation of sample homogeneity.

7.2 Quantitative elemental analysis using inductive couple plasma mass spectrometry Analysis of impurities in CNTs using ICP/MS

Charles Motzkus (LNE)

7.2.1 General description

This method has been tested to investigate its feasibility for analysis of inorganic catalysts associated with CNT.

7.2.2 Materials and equipment

No specification at this point.

7.2.3 Samples pre-treatment

Under normal conditions Inductively Coupled Plasma – Mass Spectrometer (ICP/MS) works on liquid samples (Jarvis et al., 1992). Nano Carbon Tubes, NCT, have to be dissolved. Due to the very small sizes of the particles this kind of solid materials appears to be very difficult to put in solution. Some works have been done on this subject. So the LNE procedure to digest the NCTs samples will be according to the work performed by the NCNT (Chen et al., 2008).

7.2.4 Impurity analysis

External calibration of ICP/MS

For routine analysis, ICP/MS is calibrated with a linear regression curve using synthetic standard solutions of the elements to be analysed (ISO, 2007a,b). In this case accuracy of the results depends of the comparability between the sample matrix and the standard solutions. Sample matrix effect is generated in the ion source, inductively coupled plasma, by the major components present in the sample. In case of CNTs, carbon will produce this effect inducing a bias in the accuracy of typically - 20 % to - 30 %. To overcome this problem closed matrix matching similar to the sample can be added to the standard solutions but in case of carbon it is complicated (high purity needed). Another way is the use of a Certified Reference Material (CRM) but in the case of CNTs it doesn't exist currently.

Standard addition method

Analysis of metals using ICP/MS can be performed with the standard addition method, internal calibration of ICP/MS. This method consists in adding in the sample several small amounts of the element of interest, typically 0,5 – 1 – 2 times of the preliminary estimated residual amount in the unknown sample. A linear regression curve can be also established and the intercept point allows determining the amount of the metals in the sample. This method overcomes the matrix effect and gives in some cases good accuracy but its use is sample and time consuming.

Isotope dilution, primary method

Isotope dilution is recognized as a primary method by the Comité Consultatif pour la Quantité de Matière (CCQM) from the Bureau International des Poids et Mesures (BIPM). This method is based on the addition of one unique amount of the element to be analysed directly in the sample. This amount has the particularity to present an artificial modified isotopic composition from the natural isotopic abundance of this element (de Bievre, 1994). This adding is called "Spike".

By the measurement of the ratio of two selected isotopes in a blend sample-spike, the concentration of the element of interest in the sample can be directly determined. This method is very accurate for trace metals analysis, with expanded uncertainties of few percents, and free from matrix effect. It can be applied if the element of interest has more than one natural isotope, poly-isotopic, and if enriched spike for this metal is available. Isotope dilution needs a metrological approach and it is time consuming.

7.2.5 Comments on use and applicability

Test analysis have shown that challenges in extraction of metal catalyst material from CNT may be substantial and cause. Alternatively, other matrix effects are in play. Therefore, standard addition or isotope dilution methods will also be considered. Another issue is sample homogeneity of CNT. The appropriate sample size may be larger than anticipated.

7.3 Identification and analysis of organic coating

Vivi Kofoed-Sørensen and Per Axel Clausen (NRCWE)

7.3.1 General description

Today several NM are coated or functionalized to improve their general applicability, functionality or miscibility in matrices. Currently, there are no standard methods available proposing the pathway and analytical equipment required to identify and characterize the multitude of coatings, which is already in use. Unfortunately, there is no single analytical method that can cover analysis of all possible organic compounds used for functionalization and coating of NM. To close this gap, a procedure identification of an organic fraction and subsequent analysis is under development in NANOGENOTOX and a draft version is presented hereafter.

7.3.2 Proposed procedure for identification of an organic fraction in unknown NM.

Thermogravimetry (TGA) is used to verify whether the nanomaterial (NM) is coated or not (see SOP for TGA). If the TGA analyze shows a positive response a more specific characterization will be carried out. There are number of analytical techniques which can be used, and a standardized protocol for

determination of the coating is not easy to make and will be in constant development. Experience and technics in house will be very important for the choice of method. ASE (Accelerated Solvent Extraction) extraction or TD (Thermal Desorption) would be a good choice to extract the coating from the particle. A solvent extraction can be used for several analytical technics and will also give the possibility to make quantitative determination, but it is a more time consuming method. To get a quick overview of the coatings on the particles TD-GC-MS will be a good choice. For each NM the thermal stability and the solubility of the coating must be considered before extraction or desorption. The identification of the organic coating requires as many information of the NM as possible which will also determine the choice of method. If for example the coating material consists of high molecular component MALDI-TOF-MS (Matrix Assisted Laser Desorption Ionization – Time Of Flight) or ESI-MS (Electrospray Ionization) would be good choices and not GC-MS.

Figure 7.1 shows a decision tree to help determination of the organic coatings and functionalization of particulate nanomaterial.

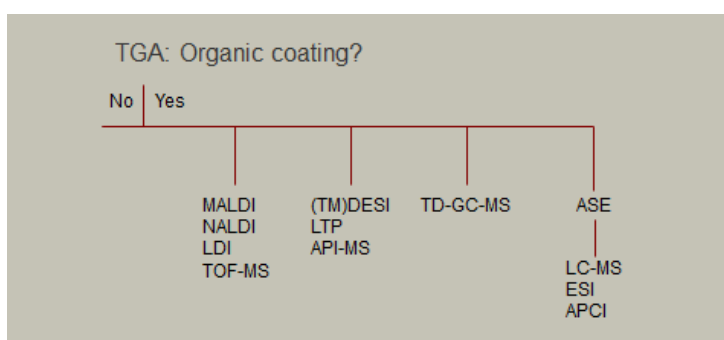


Figure 7.1. Proposed decision tree for analysis of organic coatings and functionalization of NM.

ASE (Accelerated Solvent Extraction) or PLE (Pressurized liquid extraction) is a fast automatic system for extracting of organic compounds from solid and powder samples. The ASE saves solvent while it accelerates the traditional extraction using high pressure and high temperature (above the boiling point of the solvent). The samples are extracted in cells (1. 5 and 11 ml) under pressure to keep the solvent liquid during the extraction. After heating the extracts are flushed into collecting vials. Optionally, temperature, pressure, extraction volume (mixed solvents) can be varied.

Sample preparation

Cell size has no effect on the extraction time, but strongly influences the amount of the solvent. The cell is filled with solvent during the extraction, therefore, large cells and cells with big dead volume will use more solvent and dilute the sample. Therefore, consider the following: Choose the smallest cell size, which may contain enough sample material to provide a good extraction results.

Remember to clean the cells, sand and filters before use. Place a clean filter on the bottom of the cell and weigh the samples into the cells (about 100 to 500 mg) and fill with sand (Ottawa sand) to reduce the amount of solvent during the extraction. To know the exact volume of the extract it is necessary to weigh the collection vials before and after the extraction.

Standard parameters

2000 psi and 200 °C heated for 10 minute with methanol as solvent in 5 ml cells.

Materials

Ottawa sand (Fisher), Methanol 99.9% (Fluka Chromasolv)

Different organic solvents can be used for extraction and therefore it is very important to read the MSDS and Workplace Instruction before starting the extraction.

TD-GC-MS (analytical thermal desorption (TD) used with gas chromatography (GC) and mass spectrometry (MS) in one systems)

TD is used to detect trace levels of volatile and semi-volatile organic compounds in a wide variety of samples, as for examples environmental pollutants, emissions from materials, food analysis, flavors and dust. The GC in this system is used to separate the component before they entered the MS. If the MS is running in the full scan mode there will typical be monitored a broad range of fragments from 50 to 400 m/z. The most common ionization form is electron ionization (EI). The molecules enter the source where the molecules are bombarded with free electrons emitted from a filament. The most common source is a quadrupole or ion trap and the fragments are monitored by a multiplier.

With a TD-GC-MS system there will be a lot of possibilities to vary the parameters as cold-trap, desorption temperature and time, column type, oven temperature, types of ionization, scan modes etc.

The samples can be added into a desorption tube containing a small plug of glass wool and direct desorbed in the TD or this tube can be desorbed in a heated block and transferred the volatile components to an absorbent tube containing e.g. Tenax TA which can then be analyzed with TD-GC-MS. Also a solvent extract from the ASE can be injected into a tube containing Tenax TA (from 5 to 50µl) and subsequently be desorbed in the TD and analyzed with GC-MS.

For quantification a 6 point calibration curve containing the component must be prepared. The components are diluted in methanol and injected on a Tenax TA tube and purged with 60 ml/min nitrogen for 3 min. to evaporate the methanol. These standards are analyzed like the samples.

Standard parameters

TD: The absorbent tube was desorbed at 275 °C in 20 min (oven) and the second desorption (cold trap) flash heated from -20 to 300 °C and hold for 1.5 minute. Split 1:10 and the temperature on the valve and transfer line 250°C. Pressure about 16 psi corresponding to 1.3 ml/min trough the column with Helium as the mobile phase (carrier gas).

GC: The GC is equipped with a 60 m capillary column with a diameter of 0.32 mm and stationary phase containing 5 % phenyl poly dimethylsiloxane. The initial GC oven temperature of 40°C was held for 4.0 min, followed by a ramp of 4°C/min to 120°C and a ramp of 8°C to a final temperature of 250 °C hold for 4 min.

MS: EI (electron ionization) in positive mode. Scan from 50 to 500 m/z. CI (chemical ionization) with methane or ammonia could be a good choice, because the softer ionization can give some information about the molecular ion. CI can be run in both negative and positive mode.

Materials

Sampling tubes is made of stainless steel (Perkin Elmer) containing 220 mg 60-80 mesh Tenax TA. All compounds used for standards must be of analytical grade.

On-column GC-MS

A variation of the above method is direct injection on the column with an auto sampler using liquid samples like the extracts from the PLE. The samples are injected directly into the column by a syringe and swept onto the column.

Sample preparation

Liquid samples in a solvent from for example from PLE extraction. The choice of solvent should be considered.

Standard parameters

Temperature program for the oven is as in the above GC-method. The injection volume from the autosampler varies from 0.5 to 5 μ l samples. The flow is 1 ml/min. helium through the column and the injector temperature at 50°C is hold for 2 min. and then flash heated to 250°C by a ramp of 50°C/min.

MS: EI (electron ionization) positive mode. Scan from 50 to 500 m/z.

Materials

All compounds used for standards and solvents must be of analytical grade.

MALDI (Matrix Assisted Laser Desorption/Ionization)

MALDI is a soft ionization technique used for analyzing large molecules. A nitrogen laser beam (337 nm) is ionizing the sample in the matrix.. The matrix absorbs the laser energy and it is thought that primarily the matrix is ionized by this event. The matrix is a chemical that easily absorbs the laser energy and a wide range of different chemicals are used for this purpose. The ionized matrix transfer the charges to the sample and the ions will be accelerated in an electro-static field. The mass will be measured in the flight tube in high vacuum by a very precise measurement of the time they are flying (mass analysis of ions with the time-of-flight mass spectrometer).

Sample preparation

There are different kinds of sample preparations for MALDI. The easiest preparation is the dried droplet method, where the sample is mixed with a pure solvent e.g. MeOH with or without matrix and applied (0.5 μ l) onto a target and dried at room temperature. The target can also consist of different material such as stainless steel or MALDI (nanostructured laser desorption ionization targets) where the surface is covered with a deposited layer of inorganic nanostructures.

Standard parameters

Dried drop on stainless steel target suspended in MeOH. Laser beam at 60% and mass range from 200 to 4000 m/z in positive mode. For a better ionization NaCl can be added to the sample.

Materials

Solvents must be of MS grade and standards of analytical grade.

Q-TOF (Quadruple Time of Flight mass spectrometer)

The Bruker microTOF-Q mass spectrometer is an ESI Hybrid Quadruple Time of Flight mass spectrometer equipped with a LC-system (liquid chromatography) from Agilent 1200 series. There

are different kinds of sources, but the most common is the ESI (electrospray ionization) which generates ion under atmospheric pressure. The formed ions are transferred by an electrical field into the capillary tube and the first stage of the vacuum system. The Q-TOF uses several types of ion guides from the capillary tube to the analyzer passing several vacuum stages. In the TOF part the ions will be pushed into the flight tube by the accelerator unit. The ions fly through the flight tube with different speed depending on their mass until they hit the detector and the time of the flight will be measured exactly. The squared flight time is directly proportional to the mass of the ions. The LC-system is used for separating the component in the samples before they enter the Q-TOF. If the ESI does not work APCI (Atmospheric Pressure Chemical Ionization) could be tried.

Sample preparation

Sample preparation depends on the matrix and the sample, but often some cleanup had to be done. Extracts from PLE in a solvent like MeOH can be centrifuges (20000G in 30 min.) to separate out residual particles and the supernatant injected into the LC-system or directly into the ESI on the Q-TOF. If the samples contain very small particles it will be necessary to filter the sample before analysis. SPE (solid phase extraction) can be necessary if the sample for example contains salts.

Sample analysis

The chromatographic separation and analysis of the clean extracts were done by using reversed-phase chromatography with a C18 Agilent Zorbax C18 column (4.6 x 12.5 mm particle size 3.5µm) at room temperature with a flow rate of 0.3 ml/min. There are two types of separations on the LC-system, the isocratic or the gradient procedure. It can be a good idea to start with the isocratic run in which the mobile phase does not change during the run and then slowly changing to a gradient run. The mobile phase normally consist of two solvents; solvent **A** is generally HPLC grade water with 0.1% acid and solvent **B** is generally an HPLC grade organic solvent such as acetonitrile or methanol with 0.1% acid. The acid is used to improve the chromatographic peak shape and to provide a source of protons in reverse phase LC/MS. Analytes that are basic in character are generally analyzed in positive ion mode with ESI source. The ionization parameters of the ESI source could be the following: nebulizer gas 1 bar, dry gas 4L/min temperature 190°C. The mass range of the detector is usually set to 50 to 3000 m/z. However, detection of masses up to 20,000 m/z may be achievable.

Materials

Solvents must be MS-grade and standards analytical grade

7.3.2 Additional methods

Additional methods are under development for the SOP. These include DESI, LTP, LDI, and API-MS

7.3.3 Comments on use and applicability

SOP under establishment

8. Hydrochemical reactivity

8.1 OH-radical formation capacity by particles in water using benzoic acid as probe

Vivi Kofoed-Sørensen, Keld Alstrup Jensen, Per Axel Clausen (NRCWE)

Revision of SOP published by Jung et al. (2006)

8.1.1 General description

This method is used for specific measurement of OH radicals formed by hydrochemical reactions when particles are dispersed in phosphate buffered water. OH-radicals ($\cdot\text{OH}$) reacts with the probe (benzoic acid) forming 4-HBA (4-hydroxy benzoic acid). The test is conducted at pH 7.4 and 37 °C and is a simple simulation of the potential radical formation occurring immediately after particle deposition in the lung-lining fluid of human lungs. The present method is a simplified modification of the method of Jung et al. (2006).

8.1.2 Materials

Balance

Benzoic acid (>99.9%) obtained from Aldrich

4-hydroxy benzoic acid (>99.9%) obtained from Aldrich

acetonitrile (>99.9%) obtained from Aldrich

Eppendorf centrifuge tubes

Vortex shaker (IKA Vibrax)

Incubator set to 37°C

Centrifuge (20,000G)

High Performance Liquid Chromatograph (HPLC)

HCl (analytical grade)

Internal positive control [NIST SRM1649a urban dust]

8.1.3 Sample preparation;

Each particle is weighed into 2-mL Eppendorf centrifuge tubes containing four particle loadings: 2, 5, 10 and 15 mg and wetted with 10 μl Ethanol. The particles are suspended in 1 ml of a solution of 10 mM benzoic acid and 10 mM phosphate buffer pH 7.4 to obtain four different concentrations (2,5,10 and 15mg/ml). The tubes were vortexed for 10 seconds and placed on a vigorous orbital shaker (IKA Vibrax, set to 1,000 rpm) in an incubator at 37 °C for 24 hours and 96 hours. After the incubation suspensions were centrifuged (20,000G at 30 minutes at 23° C) and 250 μl of the aqueous supernatant transferred to a glass vial with addition of 10 μl 5 M HCl to optimize HPLC conditions (High performance liquid chromatography).

Blank samples are the buffered benzoic acid solutions without particles incubated for 24 and 96 hours and NIST 1649a urban dust is a positive control included in each analysis sequence.

8.1.4 HPLC-analysis

The HPLC system consisted of a Waters Pump 1525, Waters autosampler 2707 and Waters UV/visible detector 2488 and two Xterra MS 5 μm -C18 (3.9 x 150 mm) columns in series. The system is controlled by the software Breeze.

25 μl of an acidified sample was injected into a HPLC system with UV detection. For each sequence a 6 point standard curve, containing 4-HBA is prepared. The mobile phases consisted of water adjusted to pH 2 with HCl (A) and acetonitrile (B) with the following eluent program: 30 % B for 1 min, 30 to 80 % B in 4 min, and 80% B for 1 min. 4-HBA was detected at 280 nm.

8.1.5 Calculation of $\cdot\text{OH}$ -formation capacity

The concentration of $\cdot\text{OH}$ is calculated and based on the yield of 21.5 % of 4-HBA from the reaction between $\cdot\text{OH}$ and benzoic acid. The dose to radical concentration is then analyzed by linear regression to express the radical formation capacity as function of mass [e.g. nmol/mg].

8.1.6 Comments on use and applicability

The method is undoubtedly useful for measuring the concentration of particle-induced $\cdot\text{OH}$ -radicals. However, the system may be too simple to assess the radical formation capacity *in vivo* with much more complex (bio)chemistry. We are currently considering a modification, where the reaction is catalyzed with H_2O_2 , which is generated in phagolysosomes as part of the inflammatory response to particles. Moreover, other radicals may also be of great importance in inflammatory induction and genotoxicity. Hence, a wider suite of specific probes may be required in the future.

8.2 pH- and atmosphere-controlled stirred batch-reactor hydrochemical and biodurability tester

Yahia Kembouche and Keld Alstrup Jensen (NRCWE)

8.2.1 General description

This method has been developed to investigate the short-term biodurability and hydrochemical reactivity (acidity and redox activity and particle agglomeration/dissolution/precipitation dynamics) of NM and dissolved chemicals when incubated with synthetic lung-lining fluids under highly controlled pH-levels and atmospheric composition. The pH-reactivity is quantified by the amount of titrant required to maintain pH 7.4 in the slight CO_2 -enriched test atmosphere using a titrator for pH-control. The redox activity is measured by online using a redox potential electrode and is used as an integral measure for oxidative/reductive capacity of the particle. Finally, the average zeta-size is monitored on-line using flow-cell dynamic light scattering. This enables analysis of agglomeration/deagglomeration as well as dissolution/precipitation phenomena. The system can also be used for longer term tests, which requires a small modification of the instrumental SOPs.

8.2.2 Chemicals and Equipment

- Synthetic lung-lining fluid: Low-Ca Gambles solution = gs. (see Table 8.1 for composition)
- Atmosphere: Bubbled 3.75% CO_2 og 96.25% HEPA-filtered cleaned air
- Initial pH 7.4 adjustment agents: 7.7 M HCl (Analytical grade)
- pH-titrant: 1 M NaOH (Analytical grade)
- pH 7.4 calibration pH-buffer (e.g, IUPAC pH 7.413)

- pH 4.0 calibration pH-buffer (e.g, IUPAC pH 4.005)
- Redox buffers (e.g, Hamilton (DURACELL) +271 and +574 mV)
- Thermostatic water-bath (Gant W28) for controlling reactor temperature at: 37.4°C
- Gas-mixing and humidifier control unit
- pH electrode: PHC2401 (Radiometer Inc.)
- E_h electrode: MC3051 PT-9 (Radiometer Inc.)
- Two temperature electrodes: T201 (Radiometer Inc.)
- Titrator: TIM856 (Radiometer Inc.) installed with TIM85 vs. 1.6 or higher
- Multimeter for E_h monitoring: PHM240 (Radiometer Inc.)
- Reactor: 80 ml water-flow Pyrex glass reactor
- Aluminum foil for light-impermeable wrapping of the water-flow glass reactor
- Magnets for stirring
- Peristaltic pump (Titramaster)
- DLS: Malvern Zetasizer Nano ZS installed with Zetasizer software 5.1 or higher (Malvern Inc.)
- One computer to control the titramaster and log results from pH-measurements and titrant dosing
- One computer to log redox data sampled by the PHM240 and operate the Malvern DLS

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

8.2.3 Procedure

Test set-up

The test setup is illustrated in Figure 8.1. The flow-through test reactor is placed in a titrator and connected to the Gant W28 thermostatic bath. A Teflon tube is connected to a flow-controller where CO₂ air is mixed with purified and HEPA-filtered air and humidified by bubbling through Nanopure Diamond UV water prior to leading the test atmosphere into the flow-through reactor. The titrant is added to a titrant vessel and syringes and tubes are connected with the dosage tube placed in the test reactor (depth during test: 1 cm above the magnetic stirrer). A pH-meter is fixed in position in a suitable hole in the reactor cap and connected to the computer used to control the TIM856 titrator. The redox electrode is fixed in position in a suitable hole in the reactor cap and connected to a multimeter (PHM240). The printer port is connected to a computer with a RS232 data cable to record data using the terminal program. A temperature electrode is coupled each of the pH and E_h electrodes to enable correction for temperature fluctuations. Two latex tubes are placed in the reactor. The first is lead through the peristaltic pump and connected to the Malvern flow cuvette. The second tube is connected to the outlet of the flow cuvette and lead back to the reactor to complete sample circulation. At start-up, the test reactor is wrapped in aluminum foil to inhibit possible artefacts caused by photocatalytic reactions.

Preparing Gambles solution

The chemicals are dissolved in 2 L Nanopure water (Nanopure Diamond UV) and placed in an ultrasonic bath for 15 min. Thereafter, the solution is filtered through a 0.45µm sterile filter. The final solution is added 6ml of 4 % formaldehyde per 2 L Gamble's solution to avoid growth and placed in darkness in the refrigerator.

Specifications of Nanopure Diamond UV water:

The water purification system is designed to 4 stages of de-ionization combined with UV light-treatment.:

Resistivity: $\leq 18.2 \text{ M}\Omega\text{-cm}$ at 25°C

Pyrogens: $< 0.001 \text{ EU/ml}$

Total Organic Carbon: $< 3.0 \text{ ppb}$

Other: nuclease-free (RNase and DNase).

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

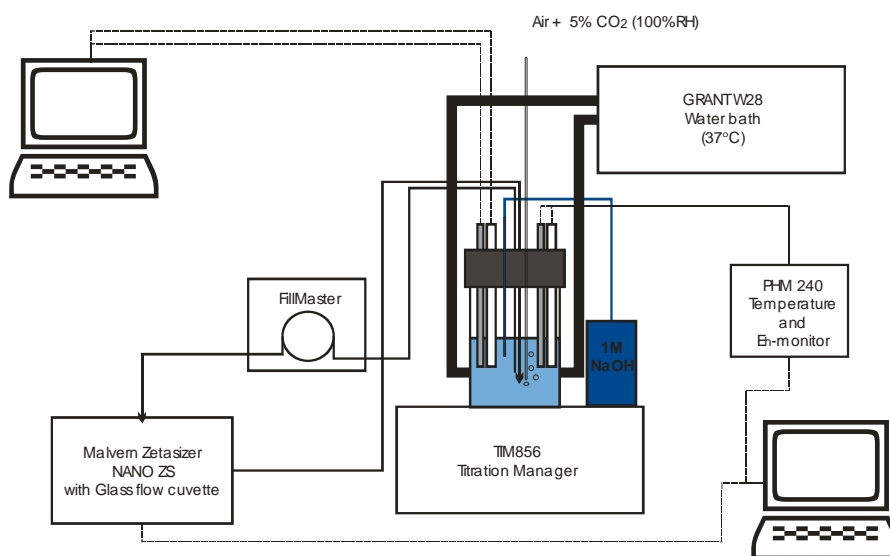


Figure 8.1. Principal set-up of the pH and atmosphere-controlled stirred batch reactor hydrochemical reactivity and biodurability tester

Performing a 24-hour test

- 1) Turn on the water-bath and adjust the temperature to 37.4°C
- 2) Turn on computers, DLS, Titramaster, Fillmaster and PHM240.
- 3) Connect the DLS flow-reactor to the latex tubes and circulate at least 10 ml Gambles solution through the tubes and flow-reactor for about 5-10 minutes using a continuous circulation program in the fillmaster.
- 4) Check that the titrator reagents are correct and that the syringe and tubings are free of air-bubbles. If air-bubbles are present, check for leaks and condition the system when it is verified that the system is tight.
- 5) In Titramaster, choose and export the suitable "application" for online control of pH, temperature to the TIM856
- 6) Choose method for logging redox potential and temperature at 5 min interval on PHM240
- 7) Start the Zetasizer software and program it for online measurement each 30 min using appropriate intensities and absorption for medium and particulate phase as well as dielectric constant and viscosity if the Gambles solution in the Measurement set-up. If properties of the Gambles solution

are unknown, use the parameters for water. The measurement temperature is 37°C and the equilibration time will always be 2 minutes.

8) Add 80ml low-Ca Gambles solution to the reaction vessel: $V_{gs}=80\text{ml}$).

9) Weigh out NM to achieve the target concentration in a 20 ml scintillation vial.

10) Withdraw 10ml from the V_{gs} and add this to the scintillation vial

11) Disperse the NM by shaking, ultrasound or ultrasonic probe depending on protocol.

In NANOGENOTOX, dispersions will be made following the conditions in the general NANOGENOTOX dispersion protocol, which is 16 min at 10% amplitude and 400 W in a ice-water using the 400 Watt Branson Sonifier S-450D (Branson Ultrasonics Corp., Danbury, CT, USA) equipped with a standard 13 mm disruptor horn (Model number: 101-147-037).

12) Start the terminal program on computer 2, to log the redox potential and associated temperature

13) Calibrate the redox electrode using one or two redox reference solutions (e.g., Hamilton (DURACELL +271 and +574 mV). Use 5 min test logging each 30 seconds to verify stability.

14) Calibrate the pH electrode using at least two standard such IUPAC pH 4.005 and 7.413 following the calibration SOP in the TIM85 leading to sensitivity above 98.5%.

15) Install the batch reactor with the 70 ml of V_{gs} in the TIM856 and mount all electrodes, temperature sensors, and tubes and add the magnetic stirrer.

15) Start magnetic stirring at 400 rpm using the interface of the TIM856

16) Adjust the pH of the Gambles solution to pH 7.4 using 7.7 M HCl.

17) Place the Teflon tube leading test atmosphere into the reactor into the Gambles solution and position it 1 cm above the magnetic stirrer and initiate bubbling with 150 ml/min CO_2 adjusted air (96.25 vol% air and 3.75% CO_2).

18) Empty the DLS flow-cuvette for Gambles solution "cleaning fluid"

19) Start the titrator application in TIM85 and redox monitoring each 5 minutes using the PHM240:

20) After 1 min, slowly add the test material dispersed in 10 ml Gambles solution into the reactor using a pipette.

21) Start the peristaltic pump and circulate 5 ml during 5 minutes

22) Make consecutively six DLS measurement manually, using automatic optimization of sub-runs and measurement time and note the settings for the subsequent periodic analysis

23) Program the peristaltic pump to circulate 5 ml per 5 minutes through the flow-cuvette every 30 minutes starting 20 minutes after the start of the experiment

24) Program the DLS to make a single size analysis every 30 minutes starting 27 minutes after start of the experiment. This allows stability and thermal calibration to establish in the DLS flow-cell.

25) Check the acidity of the dispersion. If the pH exceed 7.4 and continues to rise, abort the test and change titrant to 1 M HCl and start a new test from the beginning. If the reaction follows expectations, continue the experiment for 24 hours

26) At the end of the experiment (24-hour reaction)

27) End TIM85 application and save printouts and data

28) Immediately sample 10 μl with pipette and add this to a TEM grid (Lacey Carbon or Holey Carbon 200mesh Cu TEM grids).

29) Immediately withdraw 2 x 20 ml for ultracentrifugation: (Sorvall rotor SA-600, 16000 rpm i 20min, without de-acceleration; 20C).

30) Sample 20 ml from the reactor for measurement of viscosity at 37°C using the Vibracell Viscometer. This value may be used to correct the DLS measurements

31) Immediately withdraw 2 x 15 ml supernatant for elemental chemical analysis ICP-MS/AAS and filter it through a 0.45 μm filter or better.

32) Sample the centrifugation pellet for X-ray diffractometry.

8.2.4 Data treatment

All data data are synchronized and collected into one excel sheet.

The following data are collected:

- Time 1
- pH
- Temperature 1
- Added total volume of titrant
- Added differential volume of titrant
- Time 2
- Eh
- Temperature 2
- Zeta-size as well as size-distribution data
- Total concentration of leached/dissolved constituent elements
- Initial and final information on crystallinity and crystalline phases
- Information from electron microscopy

Monitoring E_h data are corrected for temperature deviations from the calibration temperature

E_h results may be used as monitored or re-calculated to determine the theoretical electron contraction in the test fluids according to the equation

$$E_h = -2,3RT/F \cdot \log[e^-]$$

The pH and Eh data can be plotted to evaluate the potential influence of the test NM on the local physiological conditions and the potential oxidative stress in the local region. Released elemental concentrations can be used to determine the biodurability of the test material.

8.2.5 Comments on use and applicability

The method is currently under establishment and reference can mainly be made to man-made vitrous fibers and asbestos, which may be considered useful benchmark materials. However, similar results may not be obtained as compared to historical tests due to highly improved test conditions.

Longer test durations may be required to investigate potential alteration of low to very-low soluble NM.

9. References

Chen, C et al. (2008) "Quantitative analysis of metal impurities in carbon nanotubes ...". *Anal. Chem.* 2008, 80, 9426-9434

de Bievre, P. "Isotope dilution mass spectrometry (IDMS) " in *Trace element analysis in biological specimen*, ed Elsevier, 1994

Brunauer, S., Emmett, P. H. & Teller, E. (1938) Adsorption of gases in multimolecular layers. *J. Am. Chem. Soc.* 60, pp. 309-319

Guiot C (2010) Dispersion protocols with BSA. Nanogenotox – WP4 – internal document. 7 p.

Hill R.J., (1986) *Journal of Applied Crystallography* 19, 10-18

ISO (2007a) Application of inductively coupled plasma mass spectrometry (ICP-MS): Standard ISO 17294-1

ISO (2007b) Application of inductively coupled plasma mass spectrometry (ICP-MS): Standard ISO 17294-2

Jacobsen NR, Pojano G, Wallin H and Jensen KA (2010). Nanomaterial dispersion protocol for toxicological studies in ENPRA. Internal ENPRA report. March 2010. National Research Centre for the Working Environment. 8 p.

Jarvis, K.E., Gray, A.L., Houk, R.S. (1992) "Handbook of inductively coupled plasma mass spectrometry" Blackie & Son Ltd

Jensen, K.A., Koponen, I.K., Clausen, P.A. and Schneider, T., 2009, Dustiness behaviour of loose and compacted Bentonite and organoclay powders: What is the difference in exposure risk? *Journal of Nanoparticle Research.* 11/1, 133-146.

Jung H., Guo B., Anastasio C. and Kennedy I.M. (2006) Quantitative measurements of the generation of hydroxyl radicals by soot particles in a surrogate lung fluid. *Atmospheric Environment* 40, 1043-1052.

Lambard, J., Lessieur P. and Zemb, Th. (1992) "A triple axis double crystal multiple reflection camera for ultra small angle X-ray scattering"; *JOURNAL DE PHYSIQUE I FRANCE*, 2 , 1191-1213

Né F. Né, I. Grillo, O. Taché, and T. Zemb. (2003) "From raw image to absolute intensity: Calibration of a guinier-mering camera with linear collimation"; *JOURNAL DE PHYSIQUE IV*, 10(P10), 403-413

OECD (2009) Preliminary Review of OECD Test Guidelines for their Applicability to Manufactured Nanomaterials. ENV/JM/MONO(2009)21, 71 pp.

Schneider T. and Jensen K.A., 2008, Combined single drop and rotating drum dustiness test of fine to nanosized powders using a small drum. *Annals of Occupational Hygiene.* 52/1, 23-34.

Spalla, O., Lyonnard, S., and Testard, F. (2003) "Analysis of the small-angle intensity scattered by a porous and granular medium"; *JOURNAL OF APPLIED CRYSTALLOGRAPHY*, 36, 338-347

Sebastian, K., Fellmann, J., Potter, R., Bauer, J., Searl, A., de Meringo, A., Maquin, B., de Reydellet, A., Jubb, G., Moore, M., Preininger, R., Zoitos, B., Boymel, P., Steanberg, T., Madsen, A. L., Guldberg, M.

2002. EURIMA test guideline: In-vitro acellular dissolution of man-made vitreous silicate fibres. Glass Sci. Technol. 75:263–270.

Taché, O. (2006) « Une architecture pour un système évolutif de contrôle commande d'expériences de physique », Engineer thesis, 2006, available at <http://iramis.cea.fr/sis2m/lions/tango/tango-ds/memoire.pdf>

Wright AK, Thompson MR (1975). "Hydrodynamic structure of bovine serum albumin determined by transient electric birefringence". Biophys. J. 15/2: 137–41.

Zemb, T. Zemb, O. Taché, F. Né, and O. Spalla (2003) "A high-sensitivity pinhole camera for soft condensed matter"; JOURNAL OF APPLIED CRYSTALLOGRAPHY, 36, 800-805.



The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

• Contact •

Website:

www.nanogenotox.eu

E-mail:

nanogenotox@anses.fr

Coordinator: French Agency for Food, Environmental and Occupational Health & Safety (ANSES)

27-31, avenue du Général Leclerc
94701 Maisons-Alfort Cedex
France



Partners

French Agency for Food, Environmental and Occupational Health Safety (France)	ANSES	
Federal Institute of Risk Assessment (Germany)	BfR	
French Atomic Energy Commission (France)	CEA	
Institute of Mineralogy and Crystallography (Bulgaria)	IMC-BAS	
Veterinary and Agrochemical Research Centre (Belgium)	CODA-CERVA	
Finnish Institute of Occupational Health (Finland)	FIOH	
Roumen Tsanev Institute of Molecular Biology Academy of Sciences (Bulgaria)	IMB-BAS	
Institut national de recherche et de sécurité (France)	INRS	
National Health Institute Doutor Ricardo Jorge (Portugal)	INSA	
Scientific Institute of Public Health (Belgium)	IPH	
Institut Pasteur of Lille (France)	IPL	
Istituto superiore di sanità (Italy)	ISS	
The Nofer institute of Occupational Medicine (Poland)	NIOM	
National Research Centre for the Working Environment (Denmark)	NRCWE	
National Institute for Public Health and the Environment (The Netherlands)	RIVM	
Universitat Autònoma de Barcelona (Spain)	UAB	

This document arises from the NANOGENOTOX Joint Action which has received funding from the European Union, in the framework of the Health Programme under Grant Agreement n°2009 21.

This publication reflects only the author's views and the Community is not liable for any use that may be made of the information contained therein.

