A common European approach to the regulatory testing of nanomaterials

NANoREG

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Improved measurement instruments, tools and methods (towards multi-metric, discriminating, etc.)

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1 Description of task 3.3

The goal of the task 3.3 is to generate improved information on occupational/consumer/environmental exposure to fill the identified knowledge gaps and to support regulation. The outputs of task 3.1 on the identified critical exposure scenarios on key value chains pointed out several data gaps that could be filled through field measurements.

In order to conduct a reproducible evaluation of the exposure to Engineered NanoMaterials (ENMs), several steps were accomplished within this task:

- Identification and selection of improved measurement instruments, tools and methods (towards multi-metric, discriminating, size classifying, and personal devices) including for air, water and soil. Preparation and evaluation of Standard Operating Procedures (SOPs).
- Identification and selection of companies where to perform occupational exposure measurements
- A solid measurement strategy was followed and evaluated (e.g. French approach, OECD tiered approach, …)
- Field measurement data and contextual information will be gathered, evaluated and stored in an harmonized way for further use

Deliverable D3.6 entitled “Improved measurement instruments, tools and methods” covers the first item. It will provide a description of several improved measurement instruments, tools and methods including for air, water and soil measurements.

Those tools were evaluated and compared to reference instruments. Improved sampling strategies were developed in cooperation with Task 3.2 (D3.3 and D3.7) to make the link between release and exposure. Some approaches towards conversion between metrics were evaluated. Attempts to discriminate background from actual environmental exposure were evaluated based on isotope analysis.

2 Description of work & main achievements

2.1 Summary

In the context of the evaluation of occupational / consumer / environmental exposure a wide variety of instruments tools and methods were selected and further studied to perform relevant measurements.

A total of 14 different instruments, tools and methods were selected in order to cover the three main compartments (air, water, and solid samples including soil) and different routes of exposure (inhalation and dermal exposure). The instruments tested investigate different principles and aim at providing portable monitoring solutions and/or techniques for specific cases. Those instruments, tools and methods encompasses from the most affordable and easy to implement portable devices (e.g. NANOBADGE sampler, tape stripping technique…) to the most sensitive, reliable and versatile instruments that requires highly qualified personnel (e.g. SAXS, cryogenic TEM …). Examples are given to explicit potentialities and limitations of those techniques.

State-of the-art direct reading instruments for occupational exposure assessment were directly used without further development in field measurements (D3.7) because some projects such as NanoGEM and nanoIndEx generated evaluation data and SOPs for those devices. However, in some cases conventional instruments such as the Electrical Low Pressure Impactor (ELPI) do not have available SOPs. Therefore, we investigated such instruments and SOPs were prepared accordingly.

In addition to atmospheric measurements for aerosol exposure assessment, biomonitoring tools were selected since they are essential to provide information to determine whether there is a real individual exposure situation.

The work performed in this task will benefit to other tasks and subtasks in NANoREG, ProSafe, other EU projects and beyond. The impact of this work will also be beneficial to the scientific community, regulators and stakeholders outside of the project since there is a strong need to reach consensus on tools and methods to assess exposure in practice along the whole value chain (workplace, consumer and environment). Information on the instruments, tools, methods, and exposure assessment strategies will allow regulators and industry to make appropriate choices to implement efficiently harmonized approaches for specific exposure situations.

2.2 Background of the task

The manufacturing and use of engineered nano-objects, agglomerates and aggregates (NOAA)[1] has been increasing rapidly over the recent years. NOAA are now mass-produced, used for a very wide range of applications and incorporated into mainstream consumer products (Keller et al. 2013). Besides the obvious benefits these materials bring, their potential adverse health and environmental effects, particularly caused by inhalation, are raising growing concerns (Oberdorster et al. 2005; Nel et al. 2006). The intrinsic ability of NOAA to reach and deposit in the deep alveolar region of the lungs suggests that their toxicity and fate may differ significantly depending on the particle size, shape and chemical composition (Kreyling et al. 2006; Vanwincle et al. 2009). Although the likelihood for exposure to NOAA by inhalation is higher for workers producing or handling these products at the industrial scale, end-users are also potentially exposed and the airborne release of NOAA in the environment cannot be excluded.(Seaton et al. 2010; Schulte et al. 2014; Pietroiusti et al. 2014). Since the hazards and the environmental impact are unknown, precautionary safety measures in the nanomaterial industry should ideally involve a continuous tracking of the personal exposure to airborne NOAA.

Qualitative and quantitative estimation of nanomaterial exposure is very complex as these materials have very low mass, can be highly dynamic in terms of particle aggregation or reactivity and co-exist with ambient particles in the same size range. There are currently no agreed standardized, validated and specific methods for measuring personal exposure to engineered nanomaterials. Furthermore, there are currently no validated models providing quantitative estimates of human (worker and general population) or environmental exposure.

In 2015, the OECD published a “Harmonized tiered approach to measure and assess the potential exposure to airborne emissions of engineered nano-objects and their agglomerates and aggregates at workplaces” (OECD, 2015). This three-tiered approach is based upon a systematic evaluation of previously proposed and used strategies, which mainly aim to deal with the problem that many of the instruments used for nanoparticle measurements are non-specific, i.e. they cannot distinguish the engineered nanoparticles from ambient nano-size particles.

In the first tier, information is gathered from the workplace, while in tier 2 some basic measurements are carried out to determine the potential for nanoparticle release in the workplace. Tier 3 consists of a detailed and comprehensive assessment to i) whether or not exposure to nano-objects has the potential to occur; ii) identify the level of exposure; and iii) determine the need for additional risk management steps. Figure 1 provides an overview of the OECD harmonized approach.

The aim consists in determining whether a process, or part of a process (operation), could potentially release nanoparticles that can be emitted to (indoor) air and that could eventually lead to potential occupational exposure (worker’s personal breathing zone).

While human exposure to MNMs may in principle occur during any stage of the material’s lifecycle, it is most likely in workplaces, where these materials are produced or handled in large quantities or over long periods of time. Inhalation is considered as the most critical uptake route, because the small particles are able to penetrate deep into the lung and deposit in the gas exchange region. Inhalation exposure to airborne nanomaterials therefore needs to be assessed in view of worker protection.

[1] In the literature, nano-objects, agglomerates and aggregates (NOAA) are also termed manufactured nanomaterials (MNMs) or engineered nanomaterials (ENMs). Although their exact definition may be slightly different, these terms are used synonymously in this document.

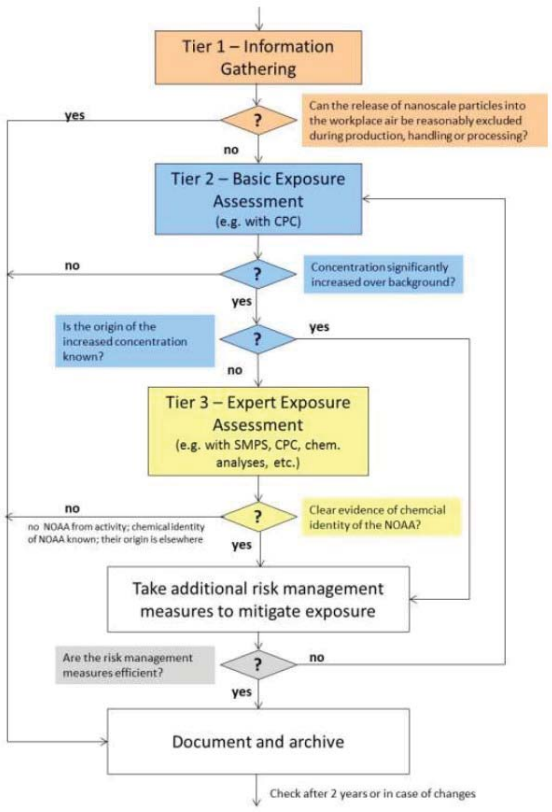

Figure 1: Flow chart of the tiered approach

Figure 1. Flow chart of the tiered approach (OECD, 2015)

Exposure to airborne particles can generally best be assessed by measuring the individual exposure in the personal breathing zone (PBZ) of an individual. The PBZ is defined as a 30 cm hemisphere around mouth and nose. Measurements in the PBZ require instruments that are small and lightweight. The individual exposure specifically to MNMs has not been assessable in the past due to the lack of suitable personal samplers and/or monitors. Instead, most studies related to exposure to MNMs have been carried out using either bulky static measurement equipment or not nanospecific personal samplers.

In recent years, novel samplers and monitors have been introduced that allow for an assessment of the more nanospecific personal exposure to airborne MNMs. Projects such as nanoGEM and nanoIndEx investigated on the accuracy, comparability and field applicability of these novel samplers and monitors (nanoIndEx, 2016).

Those two projects generated Standard Operating Procedures for the following instruments (nanoGEM, 2012; nanoIndEx, 2016).

Table 1. Standard Operating Procedures from nanoGEM and nanoIndEx projects concerning granulometers, counters and personal devices to characterize airborne NOAA

Instrument / Device	**SOP title**	**Particle size (nm) and concentration ranges**	**Remarks** (personal device, direct reading, offline …)
SMPS (TSI Model 3936)	Procedure of particle measurements with the Scanning Mobility Particle Sizer	3 - 1,000 0 - 10^7 #/cm^3	Direct reading transportable equipment
SMPS (Grimm 5403)	Procedure of particle measurements with the Sequential Mobility Particle Sizer	3 - 3,000 0 - 10^7 #/cm^3	Direct reading transportable equipment. Only available in German
FMPS (TSI Model 3091)	Procedure of particle measurements with the Fast Mobility Particle Sizer	6 - 500	Direct reading transportable equipment

NSAM (TSI Model 3550)	Procedure of particle surface determination with the Nanoparticle Surface Area Monitor	10 - 1,000 0 – 10,000 µm²/cm^3 (alveoli) 0 - 2,500 µm²/cm^3 (tracheobronchial)	Direct reading transportable equipment
Handheld CPC (TSI Model 3007)	Procedure of particle measurements with the Condensation Particle Counter Handheld	10 – 10,000	Direct reading portable equipment
APS (TSI Model 3321)	Procedure of particle measurements with the Aerodynamic Particle Sizer	500 – 20,000	Direct reading transportable equipment
NAS (TSI Model 3089)	Procedure of particle sampling with the Nanometer Aerosol Sampler	2 - 100 nm	Transportable sampler
MiniDiSC / DiSCmini (Testo)	Procedure of particle measurements with the Miniature Diffusion Size Classifier	10 - 300 10^3-10^6 #/cm^3	Direct reading personal monitor
nanoTracer (oxility)	Procedure of particle measurements with the nanoTracer	10 - 300 0-10^6 #/cm^3	Direct reading personal monitor
Partector (naneos)	Procedure of particle measurements with the partector	10 - 10,000 0 - 2*10^4 µm^2/cm^3	Direct reading personal monitor (and sampler,. TEM)
ESP (ESPnano 100)	Procedure of particle measurements with the Electrostatic Precipitator	20 – > 1,000	Personal sampler

In order to conduct a reproducible evaluation of the exposure to NOAAs several steps where accomplished through task 3.3. In particular, the identification and the selection of improved measurement instruments, tools and methods that are not currently available from other projects or literature. We focused our activities on personal devices but also on instruments that can provide information on several metrics for air, water and soil. A description of the improved measurement instruments, tools and methods with examples are given. Standard operating procedures were also prepared and can be found in the appendix section of this deliverable D3.6.

Along with Task 3.2, new data coming from scenario based simulations (to estimating release of nanoparticles from powders and NPs in matrices) and field studies were gathered. The analysis of this data (D3.7) allowed testing tools, method and instruments but also provides greater understanding in the linkage between determinants and exposure (Q11) and supports the development and validation of improved models (Q12).

A validated exposure assessment strategy (e.g. the OECD tiered approach) is beneficial to both industry and regulators. Task T3.3 aims to test the OECD tiered approach through a field studies programme in order to identify and overcome shortcomings and to improve the overall harmonized tiered approach to measure and assess the potential airborne emissions of engineered nano-objects and their agglomerates and aggregates at workplaces (Q12). However, this relies on having sufficient and appropriate field scenarios (D3.7).

Table 2. Task 3.3 related regulatory questions and needs

Related Q&Ns[2]	11. Exposure: *What are the main determinants for occupational and consumer exposure to MNM and what are the duration and type of exposure?*
	12. Exposure: *How should human and environmental exposure be assessed in practice (determining exposure scenario, quantify input parameters for models, assumptions and use of proxy indicators, background and uncertainty estimation)? Consider both measuring and specific modelling for nanomaterials and evaluate the needs for standardisation and validation.*
	13. Exposure: *Exposure and life cycle analysis: Which scenarios could denote potential exposure and what information do we have on them? Can we develop standardized and efficient testing procedures for estimating release of nanoparticles (NP) from powders and NPs in matrices? What are situations in which MNM exposure is expected to be negligible / high? Are the amount and the nature of releases of MNM similar to regular chemicals, when common recycling and end-of-pipe techniques are used?*

[2] Deliverable 1.1: table 5 on page 15 of the document 'NANoREG D1.1 2013-07-15 JRS plus annexes.pdf' in CIRCABC (Library > C-Consortium > 03 Deliverables uploaded to EC)

	How to minimise and structure LCA to avoid ending up with a '1:1 model of the world'? *In other words: what is the exposure probability throughout the different life cycle stages of the MNM: production process of the NM itself, releases during the production process of products in which MNM are used, waste treatment, consumer articles, wearing, abrasion, etc.? Do waste treatment / recycling processes lead to exposure to NMs that can be hazardous to health and environment? If so, are additional risk management measures required? Do the recycled product / residues lose some value /usefulness due to undesired characteristics?*

The impact of the work being done in this task will be beneficial first to the project and more particularly to the tasks 3.4 and 3.5. The exposure data generated (D3.7) will enable the validation of models and risk management measures. The impact of this task will also be beneficial to the scientific community, regulators and stakeholders outside of the project since there is a strong need to reach consensus on tools and methods to assess exposure in practice along the whole value chain (workplace, consumer and environment). This deliverable contributes to answer regulatory questions by providing means to assess occupational, consumer and environmental exposure. In particular; information on the instruments, tools, methods, and exposure assessment strategies will allow regulators and industry to make appropriate choices to implement efficiently harmonized approaches for specific exposure situations.

2.3 DESCRIPTION OF THE WORK CARRIED OUT: selection of improved measurement instruments, tools and methods

Nanomaterials (NMs) are primarily characterized by their size (1-100 nm), but this characterization is inadequate because NMs can be made from diverse materials with greatly varying properties. To predict how NMs will behave, we must instead classify them based on key parameters and biological interactions. To shift toward this new way of classifying NMs, a better fundamental understanding of the physico-chemical properties and biological interactions of NMs with proteins, cells, tissues, and organs as well as in the environment is needed. In addition, understanding these properties and interactions will help the scientific community to develop more predictive groups to support read-across approaches for hazard classification. Furthermore, determining clear classification parameters and analytical methods which are traceable from a metrological point of view to a common reference will improve data quality, thus allowing for cross-comparison of data and the development of quantitative structure–activity relationship (QSAR) models (Oomen et al., 2015).

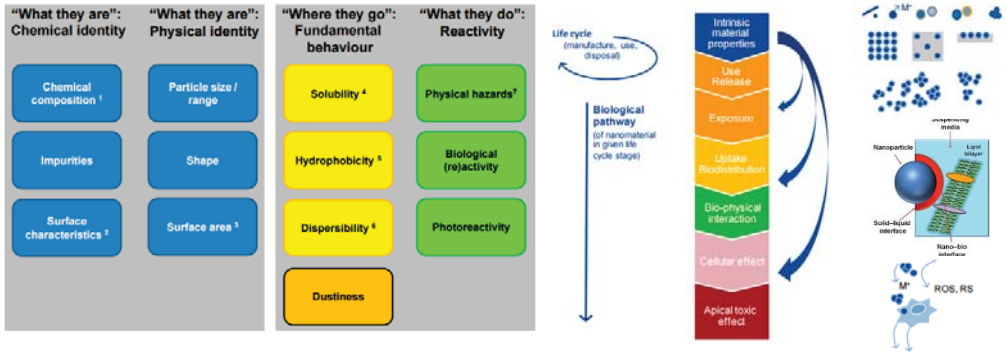

Figure 2. Frequently used parameters that can facilitate interpretation of nanoEHS data. Figure adapted from Oomens et al., 2015 and Nel et al., 2015.

Resulting from many discussions (Nel et al., 2015; OECD reports, 2012, 2014) on what constitutes a necessary set of physico-chemical parameters, a number of working groups (ISO, OECD, …) have listed frequently used parameters that can facilitate the interpretation of nanoEHS data, such as:

- "What they are": Synthetic identity, including chemical composition, crystal structure, surface coating, ligand or labelling molecules, functionalization and capping agents, impurities, all of which influence surface charge and reactivity.
- "What they are": Physical identity, including particle size, size distribution, surface area (which depends on particle size and porosity), surface roughness, shape and aspect ratio, all of which generally influence mobility and transport.
- "Where they go": Transport behaviour, which reflects characteristics of the nanoparticle that are (partly) influenced by the surrounding medium, such as solubility/dispersibility (rate of dissolution and equilibrium concentration, both size-related), surface charge, tendency to agglomerate, dustiness.
- "What they do": Reactivity, including physical hazards such as explosiveness and flammability, biological reactivity that describes the biomolecules that adsorb to the nanoparticles under specific conditions and the impact of these on the dispersion properties (e.g. redox or membranolytic activity) and photoreactivity.

The proper detection and characterization of nanomaterials is a critical pre-requirement for the safety assessment of the materials under study. The development of new and more robust methods and tools for the characterization of nanoparticles will directly be beneficial to the exposure assessment of nanomaterials the workers, the consumers and the environment.

The selection of instruments, tools and methods to be developed and evaluated during NANoREG were performed based on several requirements shared by the scientific communities but also pragmatic reasons.

Indeed, the instruments, tools and methods had to cover the various route of exposure to NOAAs. Primarily through inhalation, then through dermal contact and if possible through ingestion. That is the reason why, those instruments, tools and methods were classified based on the matrix to be evaluated:

- Air samples including aerosols (inhalation route of exposure; environmental releases …)
- Liquid samples including biological and environmental complex fluids (consumer and environmental exposure; oral route of exposure…)
- Hard samples including solids, soils and surfaces (dermal route of exposure; occupational / consumer / environmental exposure …)

Moreover, in the list of the selected instruments tools and methods, attention was given to the fact that some have to be easy-to-implement and affordable in order to facilitate their use for field measurements (e.g. personal samplers…). Of course, more complex and costly instruments that allow multi-parametric characterization and which requires highly qualified personnel were also selected when their potentialities were identified as remarkable by WP3 partners (e.g. SAXS, cryogenic TEM, X-ray computed tomography…). The pragmatic reasons to reduce the scope of the investigated techniques were the expertise of the partners involved in the consortium and their access to the selected instruments, tools and methods.

Personal devices

Scientifically sound investigations on the accuracy, comparability and field applicability of commercially available samplers and monitors had been evaluated during the project “Assessment of Individual Exposure to manufactured nanomaterials by means of personal monitors and samplers” (nanoIndEx). This project was running in parallel to NANoREG and recently ended. A guidance document was released in May 2016 (nanoIndEx Guidance document, 2016).

Personal monitors: to assess exposure to NOAA in air personal monitors that have the ability to quantify the aerosol in real time are available. To date, the only device that is small enough to be positioned directly in the respiratory sphere without hampering the operator's movements is the Partector from Naneos. This device is equipped with a unipolar charger by diffusion of ions for charging the sucked nanoparticles followed by a Faraday cage for determining the concentration expressed on the surface (Fierz et al., 2014). The other devices on the market, the DiSCmini (Matter Aerosol) and nanoTracer (Aerasense / Oxility) are larger and more expensive.

Personal samplers: in contrast to direct-reading personal monitors, personal samplers are devices that collect particles for subsequent analysis. Typical substrates used in personal samplers are filters for the analysis of the mass concentrations and/or chemical composition of the particles, and flat surfaces (e.g. Si wafer) or TEM grids for electron microscopic analysis of the particle size and morphology or – if coupled with energy dispersive x-ray fluorescence spectroscopy (EDX or EDS) – the chemical composition.

Complex technics and instruments

For example, electron microscopy (EM) is widely used to characterize airborne engineering nanomaterial and offers the capability to distinguish NP background airborne particles. Recently, several sampling methods have been developed for EM analysis in order to get semi-quantitative methods (Lyyränen et al., 2009; R’mili et al., 2011; Thayer et al., 2011). However these methods are not robust and have to be developed, by establishing collection efficiencies (for both SEM- and TEM-samplers) and improving automated image analysis techniques. Moreover EM technics are limited to size and shape observation, not really suitable for NP characterization in complex matrices (environmental and biological compartment), expensive and not easily reachable for SME’s. Samples preparation is a key point and new technics like Cryogenic preparation is emerging to fill the gap and allowing observations of NP in liquid and biologic media.

Indeed, Cryo-TEM is a revolutionary in situ analytical platform that allows scientists and engineers to image materials and biological samples in a frozen state, directly within the TEM. Samples and processes that previously required deposition on substrates (and presented aggregation), or could not be imaged in their native environment, could now be studied and observed in an amorphous frozen liquid and at high resolutions. From hydrated materials such as inks and gels, to biological materials that include whole cells and macromolecules such as proteins, liposomes and viruses, cryogenic TEM could allow to load samples in a

controlled environment and image within the TEM, and provide compositional analysis capability through EDS and EELS.

The Small Angle X-rays Scattering (SAXS) technique seems to be very promising to provide information on the morphology, size and distribution of nanoparticles in different media. This technic is based on the principle scatter measurement the X-ray small angle (for SAXS Small Angle X-ray Scattering). The structure and morphology of the sample will influence the distribution and intensity of the scattered radiation. The signal collected at small angles (SAXS) provides information on the morphology, size and distribution. The technique has been used for nanoparticle characterization for many years, but was long regarded as somewhat complicated and expensive. However, new developments in instrumentation and software are making the technique potent for nanomaterials characterization for samples in very different forms (powder, dispersion, matrices) and with more compact and affordable instruments.

Laser Induced Breakdown Spectroscopy (LIBS) technology is another candidate for nanoparticle detection in situ in real time or semi real time. Applied in air or on substrate, this technique does not require sampling preparation and allows measuring to the entire list of atomic species.

Consequently, a total of 14 different instruments, tools and methods were selected and evaluated by WP3 partners. The corresponding SOPs were prepared in order to cover the three main compartments (soil, air, water) and different routes of exposure (inhalation and dermal exposure). The instruments tested investigated different principles and aimed at providing portable monitoring solutions and/or advanced techniques for specific cases. In addition to atmospheric measurements for aerosol exposure assessment, biomonitoring tools were selected since they are essential to provide information to determine whether there is a real individual exposure situation.

Table 3. Selected instruments, tools and methods to assess nanoparticle parameters (size, shape…) depending on the compartments: air, liquid/complex matrices and surfaces/hard samples.

<table>
<tr><th>Air / Aerosols</th><th>Water / suspensions</th><th>Surface / Soil / hard samples</th></tr>
<tr><td>Laser Induced Breakdown Spectroscopy (INERIS)</td><td>Single particle - Inductively Coupled Plasma Mass Spectrometry (VN)</td><td rowspan="3">X-ray computed tomography (CEREGE)</td></tr>
<tr><td>Mini Particle Sampler (INERIS)</td><td rowspan="2">Asymmetric-Flow Field Flow Fractionation-Inductively Coupled Plasma Mass Spectrometry (CEA, VN, NMBU)</td></tr>
<tr><td>NANOBADGE Sampler (CEA)</td></tr>
<tr><td>Electrical Low Pressure Impactor (INRS) & translated NanoGEM SOPs</td><td rowspan="3">Cryogenic – Transmission Electron Microscopy (CEA)</td><td rowspan="3">Small Angle X-rays Scattering (CEREGE)</td></tr>
<tr><td>Nasal paper flag (CEA)</td></tr>
<tr><td>Exhaled breath condensate (CEA)</td></tr>
<tr><td colspan="3">Surface swab (ITENE) & Tape stripping technique (LTH)</td></tr>
<tr><td colspan="3">Isotopic labelling approaches (NMBU)</td></tr>
</table>

2.4 RESULTS: Description and evaluation of the selected improved measurement instruments, tools and methods

2.4.1 Air compartment: indoor and outdoor aerosol measurements

2.4.1.1 Mini Particle Sampler (MPS)

The MiniParticleSampler (MPS) is an easy-to-use, low-cost and portable sampler of particles for TEM analysis. This sampling technique is based on aerosol filtration by porous grid, as proposed by Lyyränen (2009), and has been previously described and experimentally and theoretically assessed (R'mili et al., Aerosol Science and Technology, 2013). This novel tool can very quickly sample nanoparticles on a TEM grid that can be later analyzed with a TEM microscope. In this report, the protocol of this technique is described and illustrated for a laboratory environment as well as for a pilot scale pharmaceutical unit.

Transmission electron microscopy (TEM) coupled with energy-dispersive X-ray (EDX) offers a very comprehensive tool for individual particle analysis allowing the determination of size, morphology, specific surface, and elemental composition. This information is needed in aerosol studies, especially in the field of nanomaterials. However, observations with TEM require a controlled sampling on an adapted analysis support, namely TEM grid. Techniques allowing sampling on TEM grids are of great interest to aerosol analysis. Indeed, sample preparation is not required, thereby gaining time and avoiding a risk for the sample to be altered.

Developed at INERIS (R'mili 2013), the MPS (cf. Figure 1) is the particle collection technique presented here. The MPS is based on filtration through one class of TEM-dedicated supports, namely TEM porous grids. It enables the aerosol sampling on a TEM (Transmission Electron Microscope) grid for further analysis.

This technique has been shown to be robust and easily usable for various kinds of environment (Industrial, laboratory, environmental/occupational sampling).

Various examples of the MPS use are available in the literature, in various contexts. Below are described a few detailed examples of applications.

Characterization of an aerosol release from CNT powder agitation

The study reported here (Le Bihan 2014) relates to the risk of suspension of inhalable particles upon production and/or use of powders constituted of nano-objects. The powder chosen for this study was Graphistrength C100 (ARKEMA), a multiwalled carbon nanotube. Its agitation is carried out with a vortex shaker system at 1500 rpm. Various instruments are connected to the aerosol outlet, e.g. the MPS (Figure 3).

Thanks to the MPS, it has been demonstrated that the agitation of the powder leads to an aerosol divided into four families, from isolated fibres to micronic pellets (Figure 4).

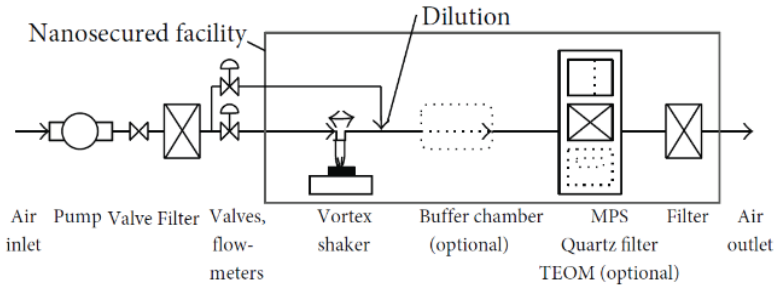

Figure 3. Experimental set-up.

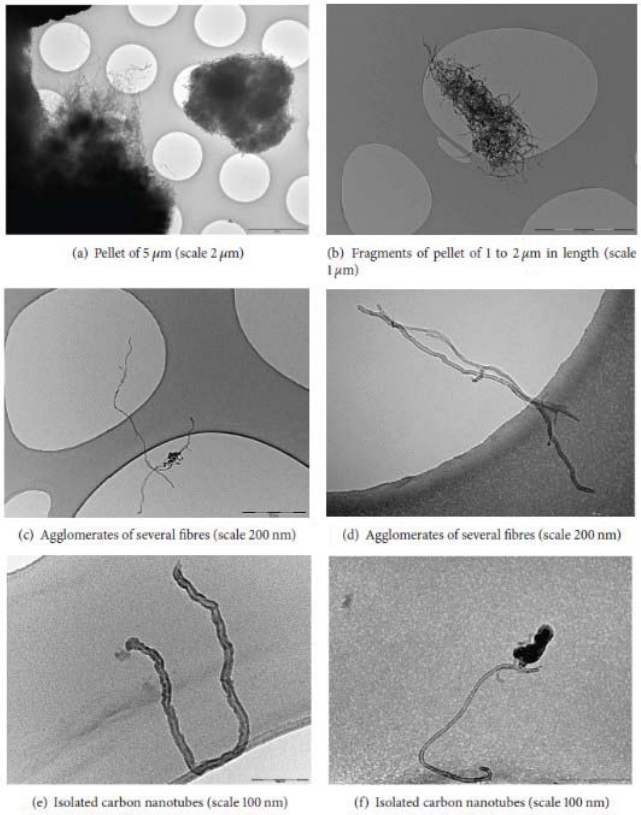

Figure 4. TEM pictures of aerosolized particles (1500 rpm). (Le Bihan 2014).

Exposure assessment at the workplace

The study reported here (Bressot 2015) takes place in experimental research on the development of nano-devices containing a drug for inflammatory 2 disorder treatment (NANOFOL Eu Project). This article provides recommendations for nanosafety based on a measurement campaign which aimed at identifying exposure risks with respect to two specific phases of the product's lifecycle, i.e. production of the device and its waste management. The nanoparticle's presence both in air as well as in liquid phase was studied. Thanks to the MPS, background particles and emitted particles have been characterized. In that specific case, it has been demonstrated that no emissions of engineered nanomaterial were detected during the production period.

Similar field studies have been carried out with the MPS in the frame of the SANOWORK EU project.

The SOP relative to the use of the MPS provided in the annex 1 of the document and is available online through the CIRCABC Library > C-NANoREG results > NANoREG developed_improved SOPs and Methods > WP3 developed_improved SOPs and Methods > SOPs related to D3.6.

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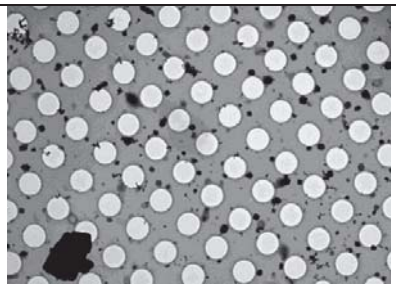
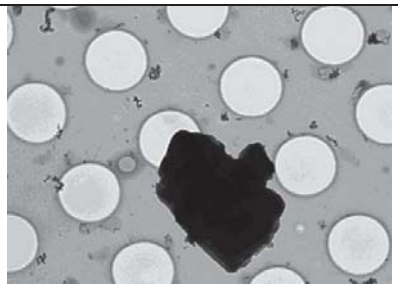
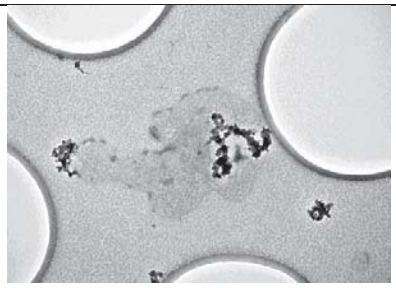
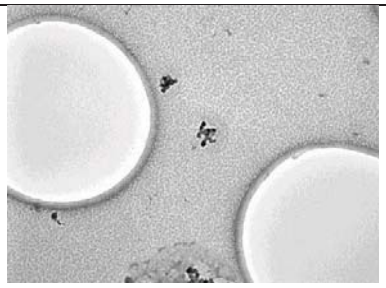
<tr><td>  </td><td>  </td></tr>
<tr><td>Overview of the sampled particles from the background</td><td>Typical cement-type debris present in the ambient air</td></tr>
<tr><td>  </td><td>  </td></tr>
<tr><td>Typical TEM image examples of soot and oil droplets (main compound carbon and iron)</td><td>Typical TEM image examples of soot (main compound carbon) reaped during the airborne particles collection while the worker filters the liquid suspensions</td></tr>
</table>

Figure 5. TEM images (Bressot 2015).

The MPS is dedicated to the study of aerosol, and more precisely aerosol morphology and elemental composition. The purpose of the MPS is to sample the aerosol particles, leading to their analysis by transmission electron microscopy (TEM) and energy-dispersive X-ray (EDX). As a result, the domain of application is the characterization of aerosol, in any field of aerosol science. Most of the ISI papers citing the MPS are focused on nanosafety or development of new nanomaterials. However, some papers are dedicated to exhaust and non-exhaust emissions from cars, etc.

Despite various advantages that have been described earlier some limitations could be mentioned. Up-to-now, the MPS leads to qualitative data. In case of high skilled microscopist and/or well-known applications, it can provide semi-quantitative data. The MPS can be used both for intentional and unintentional particles, with a real differentiation based on morphology and elemental composition of the particles. The flow rate and sampling time have to be defined carefully, with the support of the corresponding SOP. In case of high concentration or if long term sampling (>30 min) is needed, dilution of aerosol can be carried out.

2.4.1.2 Laser-induced breakdown spectroscopy (LIBS)

Laser-Induced Breakdown Spectroscopy (or LIBS) is a spectroscopic atomic emission technique dedicated to fast in-situ elementary analyses of unknown samples. Applied to air-containing particles measurement, this technique consists of focusing a high power infrared laser pulse in air in order to generate plasma at the focal point. Inside the plasma, temperature and electronic density reach high values which are sufficient to dissociate, vaporise and ionise the sampled particles. Light emitted from the plasma is then analyzed with spectrometer and the obtained emission spectrum gives qualitative and quantitative information about atomic composition of particles trapped by the plasma. The following figure (Figure 6) summarizes the principle of the technique.

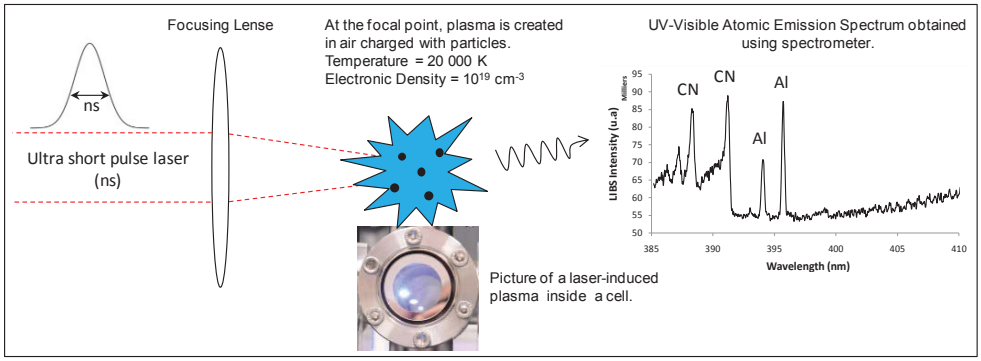


Figure 6. LIBS technology principle.

INERIS uses this tool to perform on-line chemical measurement of airborne particles at workplace or nearby industrial environment. Measurement strategy aims to detect manufactured NOAA (nano-objects aggregates and agglomerates) by means of their elementary chemical composition. Such a strategy differentiates nanoparticles of interest from high background aerosols.

In the last few years, in the frame of a PhD work, INERIS has studied feasibility of this approach and fundamental processes related to analytical LIBS signal. Results have shown that the LIBS technique can measure the elementary composition of particles without any size dependence between 60 and 5000 nm. Calibration procedures can be performed to measure concentration of atomic species of air charged with particles. More recently, a prototype has been developed for in-situ measurements. This tool was especially designed to be the most compact as possible in order to be easily operated on the field.

Figure 7. LIBS technology dedicated to air analysis and especially designed in a 19 inches format.

The detection of Manufactured Nanoparticles (MNs) at the workplace is an important issue because of their high potential toxicological risk related to nanoparticle inhalation and the difficulty of their measurement. Classical tools such as Size Mobility Particle Scanning (SMPS), Condensation Particle Counters (CPC), Optical Particle Sizer (OPS), Low Pressure Cascade Impactor devices or Surface Aerosol Monitors can perform real time measurements of size distribution, number concentration or surface area of particles at the workplace, but nevertheless several reasons limit the reliability of these measurement methods. First, a previous study showed that the distribution of particles may vary with distance to sources due to bi-modal agglomeration. In this work, the author explained that the time of life of nanoparticles released is very short due to the high probability of collision and their agglomeration with larger particles coming from the background. Second, in most cases, it is very difficult to extract engineered nanoparticles from the background particles because of the high number of natural nanoparticles.

For these reasons, manufactured nanoparticles can be detected by taking into account other parameters than their size, their surface or their number concentration, such as their chemical composition or their shape. Most

of the analytical techniques available for MNs detection rest on the use of a sampling step before chemical analysis. Different sampling techniques allow collecting particles such as electrostatic precipitator, thermal precipitator and filtration. Chemical analysis of these samples can be done using mass spectrometry or X ray fluorescence spectrometry, morphological studies using electron microscopy (SEM, TEM) or single particle chemical analysis using Dispersive X-ray analysis (EDX). Regarding TEM-EDX analysis, INERIS has developed the Mini Particle Sampler (MPS) which consist to sample directly particles on TEM grid by filtration. This approach is really interesting to understand the shape and the state of agglomeration and of the MNs released. However, it cannot be used as a quantitative technique.

All these methods are time integrated techniques because they require a sampling step. By developing LIBS technology for airborne particles measurement, INERIS wanted to perform in situ on-line measurement. Indeed, LIBS can measure chemical composition of airborne particles without any sample step because plasma is directly generated in air charged with particles. This analytical technique is unique because it is on line and evolution of atomic species concentration as a function of time can be measured. Moreover, analytical response of LIBS does not depend of the size of particles. In other words, this technique can measure with the same sensitivity mass concentration of nano-object, agglomerates and aggregates.

Limitation, threshold and detection limits

The main limitation of this technique is due to the fact that it is a tool of micro analysis. Indeed, the plasma volume from which emission light is collected is as small as 10^{-3} cm^3. While absolute detection limits of LIBS are between 1 fg and 1 pg as a function of atomic species, relative detection limits expressed in term of mass concentration becomes 1 $\mu g/m^3$ and 1 mg/m^3 respectively, due to the small size of the sampling volume. Regarding small particles, such as nano-objects, limits of detection are achieved if high numbers of particles are in the sampling volume. The particles bigger than a few hundreds of nanometers, such as agglomerates and aggregates, can be individually detected by LIBS because the absolute limit of detection is achieved inside the sampling volume. Detection limits are reported in the following table for particle smaller than 100 nm and for different atomic species:

Table 4. LIBS LOD ($\mu g/m^3$) of different type of nanoparticle (<100 nm) among the most commonly used.

Atomic species	Emission line (nm)	LOD ($\mu g/m^3$)
Al	396.15	300
Ag	328.06	41
Ca	393.36	7
Ce	417.80	350
Cu	324.16	40
Cd	508.12	1200
Mg	279.55	30
Fe	440.47	200
Zn	481.75	300
Ti	334.94	300

Although this instrument is operational for in-situ measurements, developments are still in progress at INERIS to improve sensitivity of the technique. These developments consist of increasing the number of particle inside the plasma volume using particle concentrator. Two methods are applied to achieve this goal. The first consists of using a concentrator of particles and the second is based on the using of air focusing nozzle to improve probability of plasma-particles interaction.

Typical use in workplace environment

Four typical experiments using LIBS as a tool for the measurements of manufactured nanoparticles are reported here. Two of them concern workplace surveillance. The others report the measurements performed in the vicinity of an industrial site and monitoring of a toxicological experiment.

A first measurement campaign has been performed in workplace during a milling process which consisted of incorporating Silicon Carbide nanoparticles into Aluminium particles under extraction hood. LIBS measurement was focused on Aluminium and Silicon monitoring. The instrument was placed nearby the workplace and outside the ventilation hood in order to evaluate the efficiency of the safety barriers. Aluminium microparticles have been detected by LIBS during different steps of the process. During 60 minutes, 11 series of 1000 LIBS spectra, centred on the aluminium ray (396.15 nm), were recorded, i.e. a total of 11000 spectra corresponding to as many as laser shots. The concentration of aluminium particles, suspended in the air, was deduced with the help of a statistical analysis of the spectra. This method consists of isolating the spectra with an aluminium ray. This way, a rate is obtained which corresponds to the number of laser shots that sampled an aluminium particle. This rate is called the Particle Sampling Rate or PSR and it is expressed as a percentage (%). Then the selected spectra are averaged. The ray intensity obtained, this way, is compared with a sampling line so as to deduce the mass concentration. This concentration is then multiplied by the PSR to calculate the true concentration in aluminium particles. With this procedure, mass concentration of aluminum micro particles has been evaluated (Figure 8), and it oscillates between 40 and 1200 µg/m3 as a function of time.

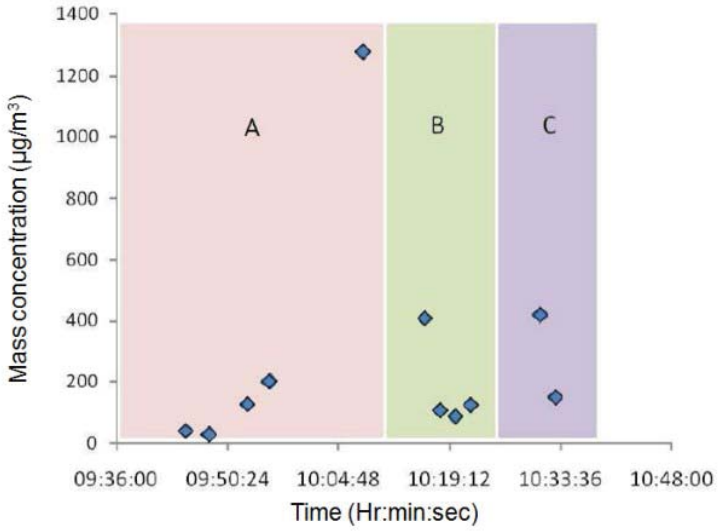


Figure 8. Temporal evolution of mass concentration of aluminum particles - A. when the hood is aspirating - B. when the hood is turned off - C. when the hood is aspirating at the end of the operations.

Regarding Silicon nanoparticles detection, the number concentration of individual nanoparticles was too small to be detected by LIBS. Nevertheless, LIBS was able to detect few microparticles of silicon, indicating that a part of silicon was released under agglomerate form. An example of a spectrum containing silicon is presented in Figure 9.

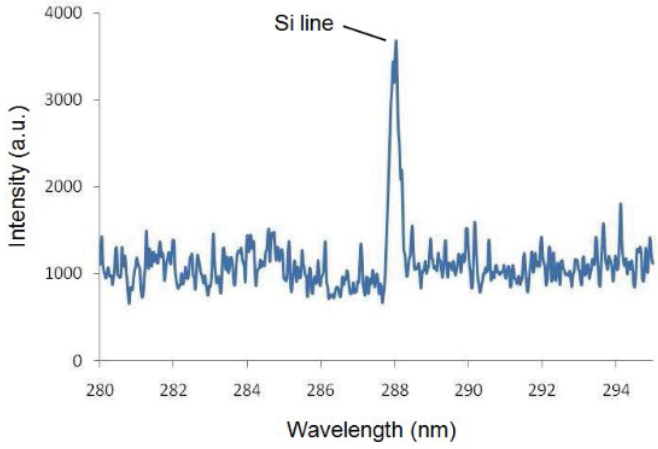


Figure 9. Example of detection of a particle containing silicon when monitoring operations in workplace.

The second reported experiment was dedicated to measurement of aerosolized Titanium nanoparticles incorporated in paint. Painting operations have been simulated in our laboratory. Direct sampling of particles has been performed near the operator in order to evaluate the worker exposition. 110 LIBS spectra were recorded during a time of a few minutes. Each spectrum was the average of 20 individual spectra corresponding to as many as laser shots. Contrary to the previous experiments, spectra were averaged because it was evident that nanoparticles were not agglomerated and because concentration of paint particles was too high to be detected individually by LIBS. Recording has been started before pulverisation operations and stopped after. Real time monitoring of titanium is presented in Figure 10.

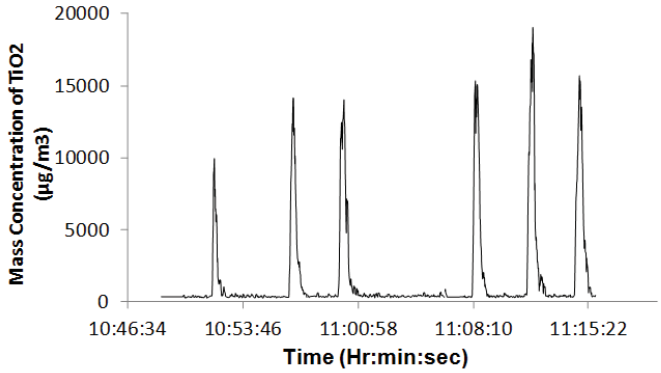


Figure 10. Real time monitoring of mass concentration of titanium measured by LIBS when pulverizing paint
Results showed that LIBS can monitor changes in the concentration of Titanium. On the average, a concentration of 6 mg/m^3 of titanium dioxide was measured by LIBS during pulverisation.

A third experiment has been performed in the vicinity of a manufacturing site of TiO_2 nanoparticles in order to know the potential release of manufactured nanoparticles outside of the industrial site. The sampling point was located in ambient air, downwind to the industrial site at 50 meters of the different potential emission areas (exhaust stacks and storage building). LIBS measurements were performed during several days. Several thousands of individual spectra were recorded. As the same way as in the first reported experiment, Titanium spectra have been isolated (Figure 11) and PSR has been calculated.

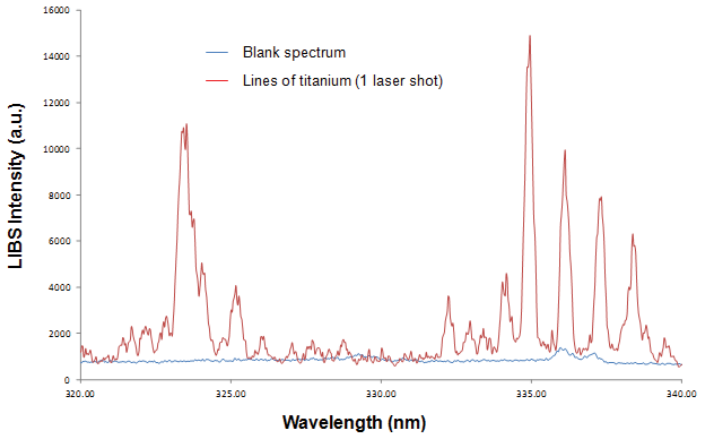


Figure 11. Example of LIBS spectrum displaying titanium lines.

A few number of big agglomerates have been detected by LIBS. The number of events (PSR) was small but LIBS signal of individual laser shots was quite important. The results showed that large agglomerates were emitted from the industrial site at a very low number concentration. Such an event is difficult to observe with other on-line techniques because of the presence of high level of background anthropogenic and natural particles. Indeed, number concentration measurement devices or granulometer cannot differentiate Titanium from another type of particles. Moreover, the increase of concentration due to nanoparticle emission is sometimes too low to be detected.

This last example of typical use of LIBS technology concerns the monitoring of toxicological experiments. Cells have been disposed in a device specifically designed for exposition studies. The titanium dioxide nanoparticles, having size of 25 nm, have been suspended in distilled water and then generated by liquid atomiser coupled with a dryer device. LIBS have been used to measure, as a function of time, the mass concentration of Titanium generated during the entire experiment duration. The sampling point was located in the tube which transports particles from the generator to the cell exposition chamber. Each LIBS spectra obtained is the average of 30 plasma corresponding to so many laser shots. Spectra are averaged because aerosols were composed of a high number concentration of particles smaller than 200 nm. Finally, 600 spectra have been recorded continuously during three hours. One of these spectra is presented in Figure 12.

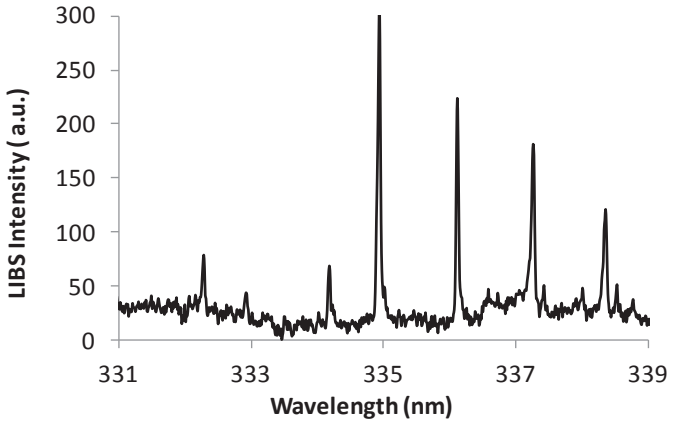


Figure 12. Example of LIBS spectrum displaying titanium lines.

Mass concentrations have been measured as a function of time (Figure 13) and values were around 2 mg/m^3.

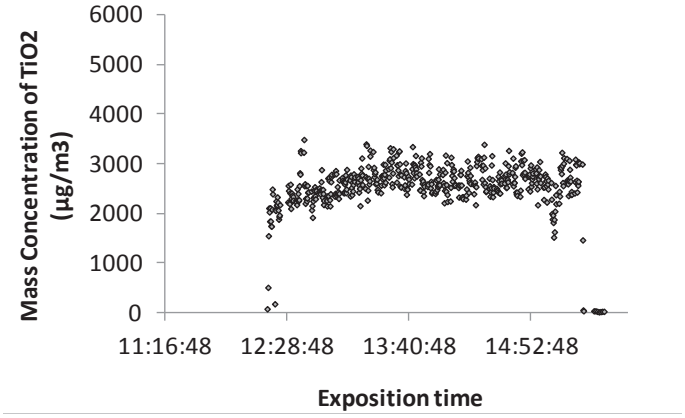


Figure 13. Real time monitoring of the mass concentration of Ti measured by LIBS when exposing cells to NP.

LIBS is really interesting for monitoring applications because it is the only technique which can measure mass concentration in real time without any need of prior information on the (fractal) shape or the density of particles. The SOP relative to the use of the LIBS technique is provided in the annex 2 of the document and is available online through the CIRCABC Library > C-NANoREG results > NANoREG developed_improved SOPs and Methods > WP3 developed_improved SOPs and Methods > SOPs related to D3.6.

2.4.1.3 NANOBADGE Sampler

The NANOBADGE (NanoInspect, Alcen group, France and French Alternative Energies and Atomic Energy Commission - CEA) is a lightweight battery-operated portable device which collects airborne particles in the breathing zone of the worker. The sampler is connected to a cassette, whose filter analyzed offline by X-ray fluorescence spectroscopy (XRF) is providing a cumulative mass-based quantification of the chemical elements present on the filters. The measurement of the engineered nanoparticles concentration by their constitutive element using XRF represents a very powerful strategy, because it is a way to get rid of the existing high and fluctuating background level of natural and anthropogenic nanoparticles. Moreover it is a non-destructive analytical technique, meaning that the same sample can be characterized further with other techniques such as scanning electron microscopy (SEM). The instrument is provided with filter units (single use) in individual zip bags and personal ID badge (personal use, one for each person operating the sampler). The filter unit is a sealed cassette containing a polycarbonate track-etched membrane to collect particles and equipped with a RFID chip to store data (sampling time, date, flow rate, errors, worker ID, sample ID, …). Track-etched membranes allows particles collection for subsequent analysis by XRF (elemental composition and concentration) and SEM-EDS (particle size, morphology and chemical identification). The sampler allows collection of nanoparticles, their aggregates and agglomerates. It can be equipped with two different pre-separators to remove coarse particles (impactors for respirable fraction - d_{50}=4µm or PM2.5) that were not evaluated in this study.

Figure 14. The NANOBADGE sampler (2015 version).

After sampling the cassettes are extracted from the NANOBADGE and sent directly to NanoInspect or CEA for analysis and subsequent data restitution (e.g. elemental mass concentration in the breathing zone averaged over the total sampling time).

The table below indicates weights and volumes of two version of the NANOBADGE sampler. The 2013 version is operated with a flowrate of 0.6 L/min while the 2015 version is operated at 1 L/min. For both versions, the flow rate is kept in the range of ± 5% over more than 10 hours. The 2013 version of the NANOBADGE sampler was evaluated in the project nanoIndEx and will be referred herein as 'NANOBADGE'.

	NANOBADGE 2013	NANOBADGE 2015
Weight (g)	150	255
Volume ($cm^{3)}$ (length x height x depth)	156 10.6x6.4x2.3	312 14.0x7.2x3.1

The SOP relative to the use of the NANOBADGE sampler is provided in the annex 3 of the document.

Accuracy & Comparability

The NANOBADGE filters are analyzed by X-ray fluorescence spectroscopy (XRF) providing a cumulative mass-based quantification of the chemical elements present on the filters. Thus the sampler provides the mass concentration in the breathing zone averaged over the total sampling time. The quantification by mass of the elements deposited on the filters requires that the XRF spectrometer is calibrated, which was done using a previously reported methodology (Motellier et al., 2011). In short, sets of filters of increasing particle loading are generated by sampling controlled aerosols (e.g. ZnO, TiO_2, …). The filters are then analyzed by XRF, followed by dissolution of the particles for elemental quantification by ICP-MS. The plot of the normalized XRF intensity versus the mass determined by ICP-MS yields the calibration curves for the different elements studied, used to convert the X-ray fluorescence intensity to mass. The limits of detection (LOD) for the following elements have been determined for the NanoInspect XRF as shown in Table 5.

Table 5. Limits of detection of the NANOBADGE using XRF analysis (NanoInspect XRF, optimized Zoffset, 0.1° angle, 200 sec acquisition)

	LOD (ng/filter)
Al	100.2
Si	20.1
Ti	2.6
Ca	6.8
Zn	1.5

To further illustrate the validity of the sampler for personal exposure assessments, the LOD for ZnO and TiO_2 have been determined using another instrument the Rigaku Nanohunter XRF that was used at CEA for the project nanoIndEx (Faure et al., 2016). The European Agency for Safety and Health at Work distinguishes long-term and acute exposure, the former being a repeated exposure averaged over working shifts of 8 h and the latter a peak exposure averaged over 15 min (ECHA, 2012). Thus the LOD have been converted to aerosol mass concentrations for a full shift based on the latest recommended exposure levels (REL) of the National Institute for Occupational Safety and Health (NIOSH, 2011). The minimum sampling time required to detect an exposure at or above the REL has been calculated. As shown in Table 6, the limits of detection are much lower than the REL for the two oxides considered in this study. The detection of peak exposure is also possible, since a few seconds of sampling at or above the REL are sufficient to exceed the LOD. Since the LOD are several orders of magnitude smaller than the current REL, the NANOBADGE sampler can already accommodate tougher regulation, should the exposure levels be lowered in the future.

Table 6. Comparison between the recommended exposure levels (REL) published by the NIOSH and the limits of detection (LOD) of the NANOBADGE sampler for shift and acute exposure (Rigaku NanoHunter XRF, optimized Zoffset, 0.75° angle, 200 sec acquisition).

	REL for ultrafine dust from NIOSH (µg/m^3)	LOD (ng/filter)	LOD (µg/m^3) for 8 h of sampling	Minimum sampling time at the REL
ZnO	5000	30 ±20%	0.1 ±25%	< 1 min
TiO_2	300	12 ±25%	0.04 ±30%	< 1 min

The highly-sensitive XRF technique yields the elemental composition of the collected particles with sensitivity in the order of a few tens of nanograms per filter and consequently could be used either over a full shift (e.g. 8h) or during short operations (e.g. 15 min) to detect acute exposure events. The main drawback observed is that the sensitivity of this analytical technique is decreasing dramatically for light elements (Z<13) and consequently carbon-based particles cannot be analyzed with this technique.

Several measurement campaigns were organized during the course of the project at IUTA, IGF and CEA on monodisperse, polydisperse, compact and agglomerated particles. Those measurements allowed us to evaluate the NANOBADGE sampler in various conditions with different aerosols (size distribution, morphology, chemical composition …) and against different granulometers, counters and monitors.

The example shown below illustrates the performance of the NANOBADGE compared to a scanning mobility particle sizer (SMPS) by carrying out simultaneous measurements on test aerosols of ZnO. The effective density and shape of the particles present in the test aerosols were determined experimentally using a tandem DMA-ELPI setup (Kelly et al., 1992) to compare number-based data obtained with the SMPS with mass-based data obtained with the NANOBADGE.

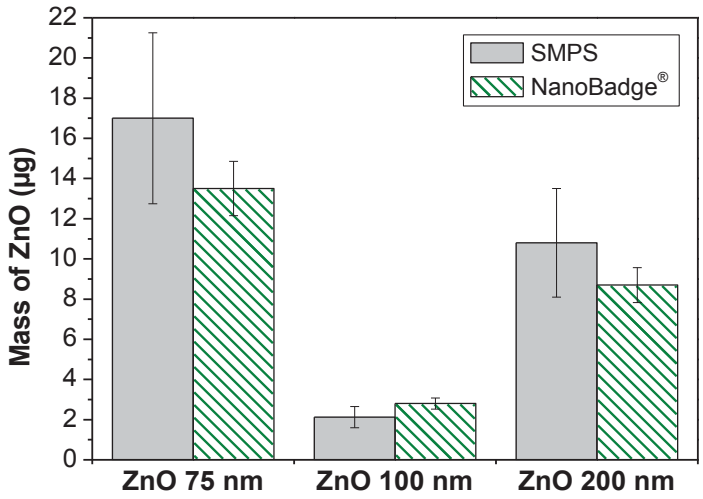


Figure 15. Mass of ZnO calculated from the SMPS data and mass of ZnO measured by XRF analysis of the NANOBADGE filters (calculated from the mass of Zn). An effective density of 2.2 was used.

The sampler has been evaluated and validated up to a size of 200 nm using several aerosols of ZnO and TiO_2 particles. The agreement between the SMPS and the NANOBADGE sampler was within ±25% on all test aerosols for which the effective density was determined (Faure et al., 2016).

This study highlights the fact that the density of the particles in aerosols is of great importance to compare electrical-mobility-based results to mass-based measurements. When aerosols are monodisperse with perfectly spherical non-agglomerated particles, results from SMPS, CPC and to a larger extent electrometer might be easily converted to mass. However in case of more complex aerosols (i.e. polydisperse or agglomerated) the effective density of the agglomerates has to be precisely known in order to reduce the deviation between monitors and samplers. Therefore, metric conversion will not be performed on data

generated during field measurements. Qualitative evaluation of events from direct reading instruments and cumulative mass-based quantification of the chemical elements present on the NANOBADGE filters could be of high value for the occupational exposure assessment.

Specific case of carbon-based aerosols

The NANOBADGE cassette was adapted to allow sampling carbon-based aerosols on quartz filter for subsequent analysis in a thermal-optical analyzer (Lab OC-EC Aerosol Analyzer from Sunset Laboratory). Soot particles were generated by spark generator and by diesel engines and were successfully collected by the sampler on freshly fired quartz filters. The mass of elemental carbon deposited on the filters has been determined by thermal-optical analysis. The low mass of elemental carbon on the filter, combined with contamination by organic compounds when mounting the filters, made it difficult to draw reliable conclusions on the results obtained. Nevertheless a proof-of-concept has been obtained and the preliminary results suggested that with some technical improvements (e.g. new ways to mount the filters and to sample a representative piece of filter …) the NANOBADGE sampler could provide quantitative analysis of elemental carbon (“black” carbon).

Field applicability

The NANOBADGE is equipped with a USB rechargeable battery that procure more than 10 hours of continuous sampling. The sampling will not start unless the charge remaining in the battery is sufficient for 8 hours of operation. A single on-off switch makes the device robust and very simple to use. Unintentional switching off is unlikely. The sampling time and the sampled volume is automatically logged into the RFID tag located in the sampling unit. Additional information such as temperature, pressure, humidity, worker’s position and contextual information can help to identify the location of unexpected emission sources or climatic conditions that could be problematic for the device operation in specific cases. Therefore it is recommended to write down those information separately.

Figure 16. The NANOBADGE worn during field measurements.

The NANOBADGE is equipped with red/green lights and an alarm sound to warn the user for any malfunction (e.g. inlet clogged, discharged battery …). The encountered errors are logged in the filter unit memory. There is no maintenance required on the user side, apart from loading the battery and keeping track of the filter units. If the outside of the instrument is contaminated with dust, it can be cleaned using wet wipes. The device is recognized to be comfortable, securely fastenable and does not restrict the mobility of the user. However, in quiet workplaces the device is perceived by some users as noisy and producing annoying vibrations.

The NANOBADGE is personal sampler dedicated to the airborne NOAA characterization in order to assess occupational exposure by providing information on the elemental composition, size distribution and morphology of the collected material. The purpose of the NANOBADGE is to sample airborne particles in the personal breathing zone of workers, leading to their characterization by XRF and SEM-EDS. Therefore this sampler could provide more directly mass concentration shift averages with the advantage to enable the

identification of specific morphological or chemical features. As a result, the domain of application is the characterization of airborne particles and the assessment of exposure in occupational settings.

Despite various advantages that have been described earlier some limitations could be mentioned. Indeed, there might be a certain conflict between monitoring (using DiSCmini or Partector for instance) and sampling if very short activities are to be investigated, as limits of detection of certain analytical methods may require longer sampling times than monitoring periods. The SOP relative to the use of this personal sampler is provided in the annex 3 of the document and is available online through the CIRCABC Library > C-NANoREG results > NANoREG developed_improved SOPs and Methods > WP3 developed_improved SOPs and Methods > SOPs related to D3.6.

2.4.1.4 Counters and granulometers (ELPI and nanoGEM equipments)

Counters and granulometers presented in Table 1 are necessary for a Tier 3 exposure assessment according to the OECD harmonized tiered approach and were used during field measurement campaigns. Projects such as nanoGEM and nanoIndEx prepared several SOPs for the most common counters and granulometers. Nevertheless, some SOPs were available only in German and some others were missing.

The Standard Operation Procedure relative to the SMPS (GRIMM Model 5403) was translated in English and is reported in Annex 13. The scope of this standard operation procedure is the data acquisition and backup as well as the quality control of the measurement data obtained with this instrument for the determination of the inhalative exposure to nanoscale product materials and ultra-fine aerosols in the workplace.

The procedure for setting up and for making a measurement with the Electrical Low Pressure Impactor (Classical ELPI®, Dekati) was missing from other projects besides the fact this instrument is routinely used. Consequently, this SOP was prepared and can be found in Annex 4. In addition, this SOP describes the data treatment and the routine instrumentation maintenance. Both SOPs are also available online through the CIRCABC Library > C-NANoREG results > NANoREG developed_improved SOPs and Methods > WP3 developed_improved SOPs and Methods > SOPs related to D3.6.

2.4.1.5 Nasal paper flag

The assessment of external exposure to nanoparticles thanks to air sampling is essential, but this type of measurement does not take into account the use of individual protective devices, and individual parameters such as absorption, distribution and elimination. This is why, it is admitted that biomonitoring is the best approach to assess individual exposure to occupational and environmental toxicants. Concerning nanoparticles, there is now a general agreement for the necessity to develop research studies on the establishment of biomonitoring. Pending the results of toxicological studies, a first step in this research area is the determination of biomarkers of exposure in order to identify the situations of exposure.

In general, biomonitoring is based on blood and urine analyses, but in the case of insoluble nanoparticles, toxicokinetics data would tend to prove that very few nanoparticles would be found in those matrices following inhalation. This is why other methods are to be explored. A test able to verify the non-exposure to nanoaerosols seems to be of great interest, especially at work places where workers might be exposed to nanomaterials. Therefore, nasal sampling is proposed for the evaluation of potential inhalation to nanoparticles. This type of collection is not quantitative but its use has already shown its interest in the field of radiomonitoring to alpha and gamma emissions. Whatever the method used for nasal sampling, i.e. tissues, swabs or flags, it helps quickly determine if there was or not inhalation and the magnitude of exposure. Among the various collection methods, the technique of the nasal flag (piece of absorbent paper rubbed in the nostrils) is easy to implement and is better to improve sensitivity since the amount of support is minimized. The nasal flag is used in medical laboratories conducting examinations of radiotoxicology, such as the LBM of CEA Grenoble, for end of shift workers or in the case of incidental situations. The use of the nasal flag was

also developed by the CEA for individual monitoring of workers exposed to beryllium powder at work and metallurgical operations.

The objective is therefore to develop a method that allows the sampling and the analysis of nanoparticles deposited in the nose. Ideally, this method has to be fast and convenient in order to give a quick indication on the existence, or not, of an inhalation situation.

Microscopic analyses allow nanoparticles identification according to their shape, size, aggregation state, and even their composition using EDX additional technique, but such analyses are time-consuming and hardly adequate for routine measurements. Total reflection X-ray fluorescence (TXRF) allows elemental analysis of surfaces without fastidious pretreatment procedures, but the analysis is not sensitive when performed on a paper surface. That is why, chemical analyses, such as atomic adsorption spectrometry or inductively coupled plasma mass spectrometry, seem the best option for nasal flag analysis. These methods require the digestion of the nasal flag prior to analysis. Since all nanoparticles need different digestion protocols, we have chosen to set the digestion protocol on the digestion of the flag and not on the digestion of the nanoparticles. Since the digestion is not complete for all nanoparticles, the result is semi-quantitative and gives an information on the level of contamination more than on the exact measurement of exposure.

The method has been developed essentially for ZnO and Al_2O_3 nanoparticles. In the figure below, we can see the ICP-MS calibration curve obtained for ZnO nanoparticles deposited on nasal flags in comparison with a calibration curve of ZnO nanoparticles alone. The nasal flag brings no additional significant background for the analysis of ZnO nanoparticles, and the global analysis method is linear.

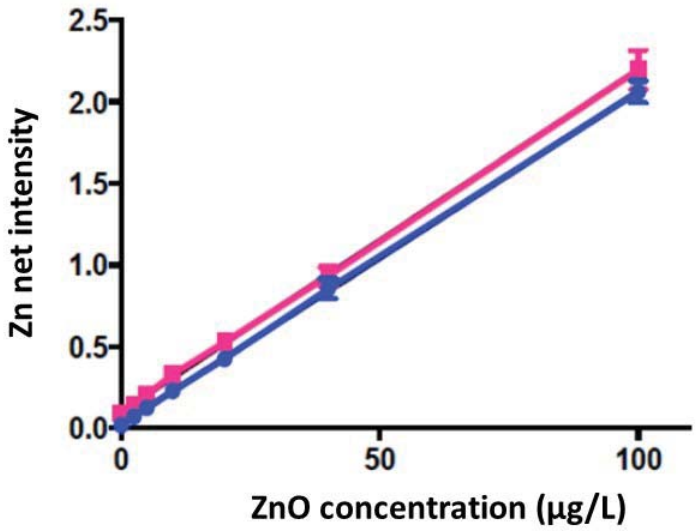


Figure 17. Calibration curve for ZnO nanoparticles deposited on nasal flags followed by digestion and ICP-MS analysis (red curve), in comparison with ZnO calibration curve analyzed with ICP-MS (blue curve).

The method of the nasal flag has been tested in a pilot study on workers of a manufacture of metallic nanoparticles. The results were confidential. The SOP relative to the use of this method is provided in the annex 5 of the document and is available online through the CIRCABC Library > C-NANoREG results > NANoREG developed_improved SOPs and Methods > WP3 developed_improved SOPs and Methods > SOPs related to D3.6.

The Nasal paper flag is a human biomonitoring tool to determine biomarkers of exposure in order to assess personal exposure to occupational and environmental toxicants. As a result, its domain of application are the occupational health surveillance and human biomonitoring.

Despite various advantages that have been described earlier some limitations could be mentioned. Indeed, the current method gives no information on the particulate contents (only an elemental information). Moreover, due to the ICP-MS technology, the carbonaceous particles cannot be evaluated through this method.

2.4.1.6 Exhaled breath condensate

The assessment of external exposure thanks to air sampling is essential, but this type of measurement does not take into account the use of individual protective devices, and individual parameters such as absorption, distribution and elimination. Biomonitoring is the best approach to assess individual exposure to occupational and environmental toxicants. In general, biomonitoring is based on blood and urine analyses, but in the case of insoluble nanoparticles, toxicokinetics data would tend to prove that very few nanoparticles, or derivatives, would be found in those matrices following inhalation. This is why other methods have to be explored, and in this regard, exhaled breath condensate (EBC) seems promising. EBC is a totally non-invasive respiratory sampling that can be used in the field of occupational health. It contains small molecules emanating from the respiratory tract and potentially reflects pulmonary pathobiology. Both biomarkers of exposure and effect can be found in this matrix.

Biomarkers of exposure

The best biomarkers of exposure for metallic (or metal oxides) nanoparticles are the nanoparticles themselves or associated elements. That is why we have worked on determining with ICP-MS the basal concentrations in EBC of 17 elements that might be constitutive of engineered nanoparticles (Zn, Al, Ti, Co, Cu, Zr, Ni, Cr, Ga, In, Pt, Mn, Fe, Se, Cd, Ge, Be) in non-exposed volunteers. Thanks to these levels we are able to determine potential elevations of these elements in the case of nanoparticle exposures.

In addition to multi-elemental analysis of EBC, we are working on the observation of EBC deposits with SEM and TEM. As an example, Figure 18 shows SEM images of particles found in exhaled cigarette smoke condensate.

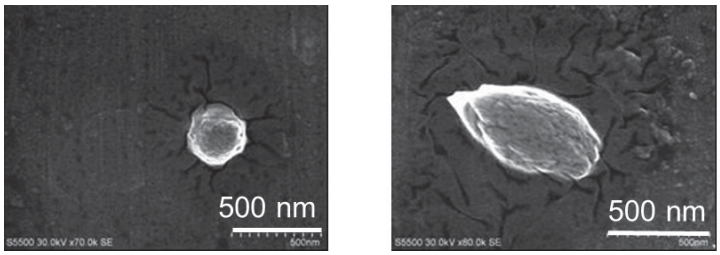


Figure 18. SEM images of particles found in exhaled cigarette smoke condensate.

Biomarkers of effect

A common pathway of toxicity that has been depicted for nanoparticles is inflammation. That is why we have developed the measurement of cytokines in EBC. Thanks to a multiplex assay we are able to analyze 29 inflammatory markers (Il-1b, Il-1ra, Il-2, Il-4, Il-5, Il-6, Il-7, Il-9, Il-10, Il-12, Il-13, Il-15, Il-17a), chemokines (Il-8, eotaxin, RANTES, IP-10, MCP-1, MIP-1a, MIP-1b) and others (TNF alpha, IFN-g, G-CSF, GM-CSF, PDGF, FGF, VEGF, ICAM-1, VCAM-1). Since the levels are very low in EBC, we have developed the assay after concentration of EBC samples by freeze-drying. Basal levels in volunteers have been determined although near detection limits.

The SOP relative to the use of this method is provided in the annex 6 of the document and is available online through the CIRCABC Library > C-NANoREG results > NANoREG developed_improved SOPs and Methods > WP3 developed_improved SOPs and Methods > SOPs related to D3.6.

The EBC is a human biomonitoring tool to determine biomarkers of exposure and biomarkers of effect in order to assess personal exposure to occupational and environmental toxicants. As a result, its domain of application are the occupational health surveillance and human biomonitoring. Despite various advantages

that have been described earlier some limitations could be mentioned. Indeed, the biomarkers of effect are not specific to nanoparticles therefore no specific biomarker to ENMs is available currently. Moreover, due to the ICP-MS technology, the carbonaceous particles cannot be evaluated through this method.

2.4.2 Water compartment: suspensions measurements

2.4.2.1 Asymetric field field flow fractionation technique (AF4)

This analytical chain FFF (Field Flow Fractionation) is a technique of liquid chromatography. FFF is coupled to an array of detectors comprising a UV-Vis (UV-visible spectrometer), a MALLS (static light scattering), a DLS (dynamic light scattering), a DRI (refractometer) and an ICP-MS that can also be used separately.

The chain provides a lot of information on particles in suspension. The particles may be inorganic or organic and of size between 1 nm and 10 microns (with some adjustments).

This technique allows among others the environmental monitoring, and personal exposure measurements by being able to characterize complex matrices such as environmental waters, biological fluids.

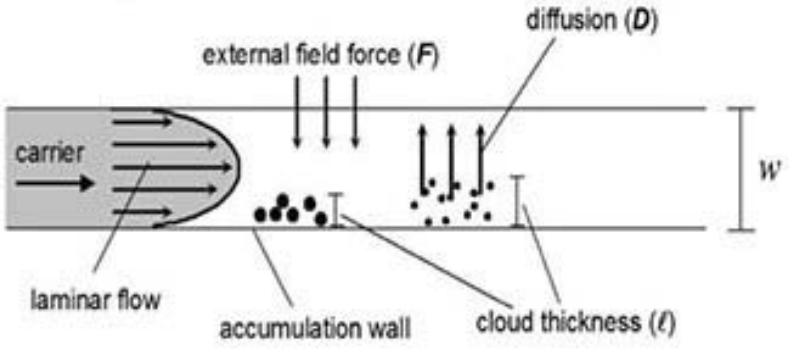


Figure 19. Principle of the Field Flow Fractionation.

The various detectors coupled with the fractionation step provides very rich information such as:

- Size information [FFF-MALLS (gyration diameter) / DLS (hydrodynamic diameter)]
- Concentration [FFF-DRI/UV-Vis]
- Elemental composition [FFF-ICPMS]
- Molar mass [FFF-MALLS/DRI]
- Shape [FFF-MALLS/DLS/DRI]

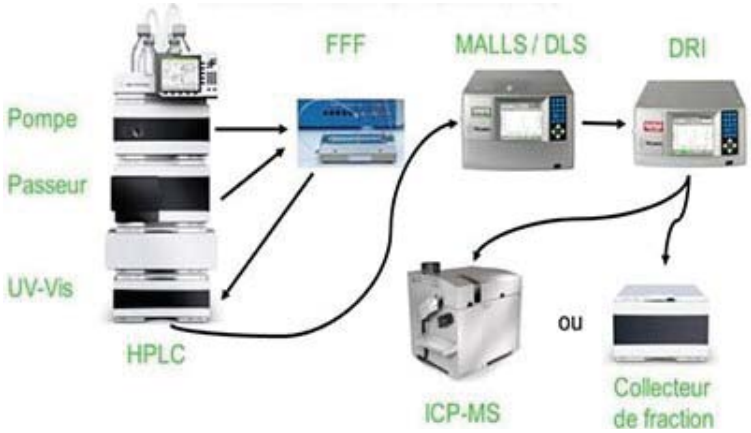


Figure 20. The different components of the analytical chain

The SOP relative to the use of the analytical chain for the characterization of silver nanoparticles in suspension is provided in annex 7 of the document and is available online through the CIRCABC Library > C-NANoREG results > NANoREG developed_improved SOPs and Methods > WP3 developed_improved SOPs and Methods > SOPs related to D3.6.

2.4.2.2 Cryogenic transmission electron microscopy (Cryo-TEM)

The electron transmission microscopy (TEM) allows the observation of atomic stacking at small-scale (nanoparticles), these nanoparticles can be of different compositions and dispersed in various media i.e. organic, inorganic, biological, or functionalized materials.

The instrument evaluated during the project is the analytical TEM Field Emission Gun (FEG) 200 KV Osiris from Tecnai. It allows the characterisation of nanoparticles based on the following techniques:

- X-ray spectroscopy analysis (EDX = energy dispersive X-ray spectroscopy).
- by energy loss analysis (EELS = Electron Energy Loss Spectroscopy) or energy filtered (EF-TEM = Energy Filtered Transmission Electron Microscopy): acquisition by point analysis, along a line or mapping.
- imaging by transmission microscopy (TEM = Transmission Electron Microscopy), high resolution (HRTEM = High Resolution TEM), STEM (Scanning Transmission Electron Microscopy), tomographic 3D (= acquisition of satellite pictures series).

This instrument allows observations of the surface and in transmission: morphology, crystallographic structure with the possibility to image the atomic scale (resolution 1 ≈ 1.4 Å) and also 3 D visualizations of objects.

EDX, EELS, EF-TEM provides information on the elemental composition: this identification allows coupling with other visualization techniques to locate the different atomic species in relation to each other (resolution ≈ 2-3 Å).

The methodology to prepare frozen aqueous samples was developed in order to characterize them using the CEA cryogenic TEM (Figure 21).

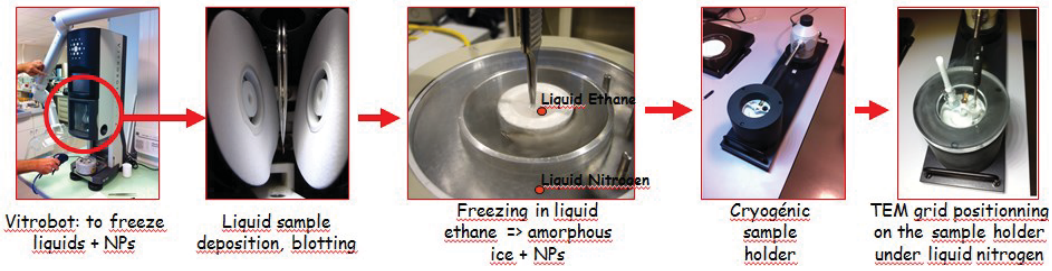


Figure 21. Sample preparation using Quantifoil TEM grids

The example presented below is related to the characterization of Ag nanoparticles (NM300K). The same suspension provided by JRC was characterized using conventional TEM (Figure 22 and Figure 23) and cryo-TEM (Figure 24).

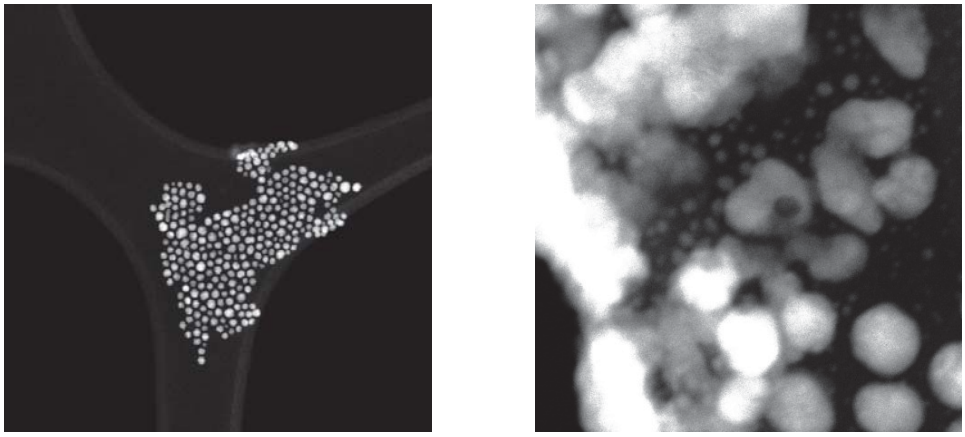


Figure 22. TEM images of NM 300K particles using conventional TEM (left 10 nm particles, right higher magnification with 3nm grain)

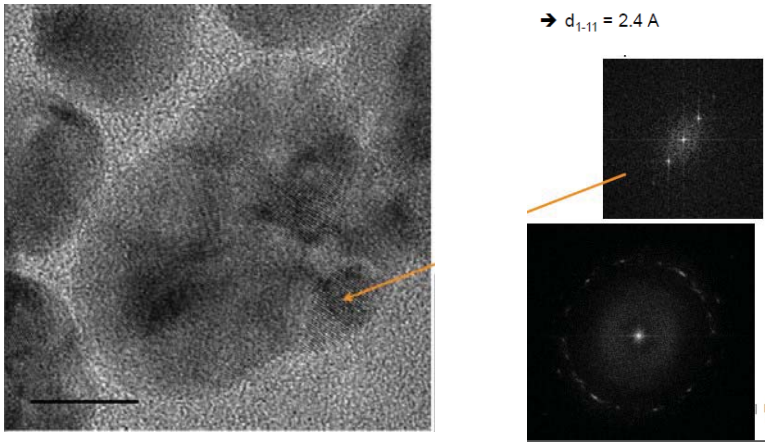
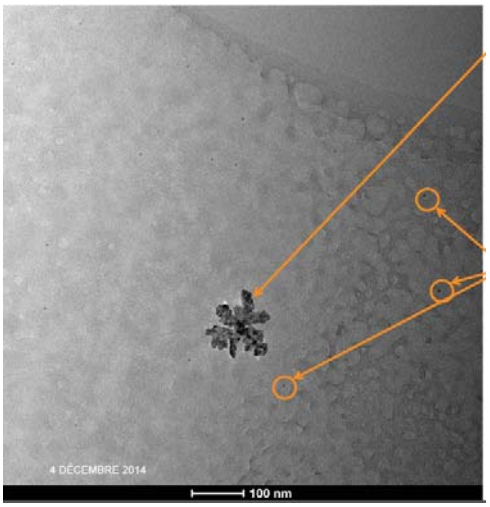


Figure 23. High resolution conventional TEM showing grain boundaries (NM300K).

Characterization using conventional TEM shows 10 nm spherical particles made of Ag (EDS). At higher magnification, a substructure around 3 nm is observed (multigrain particles). Using cryo-TEM it seems that most of the particles consist of primary particles of approximately 3 nm. Few large aggregates were also found.



Few large aggregates

Numerous small single particles of about 3 nm

Figure 24. Characterization of frozen aqueous sample (NM300K) using the CEA cryogenic TEM.

This characterization method provides information related to the aggregation state of particles in suspension. Its domain of application is the characterization of suspensions including complex matrices such as environmental waters and biological fluids. The main limitation that could be mentioned is that it is an expensive, non-routine technique that requires skilled personnel and a consequent sample preparation time. The SOP relative to this instrument is provided in the annex 8 of the document in order to allow the preparation and the observation of cryogenised samples. The SOP is also available online through the CIRCABC Library > C-NANoREG results > NANoREG developed_improved SOPs and Methods > WP3 developed_improved SOPs and Methods > SOPs related to D3.6.

2.4.3 Other characterization techniques for surfaces, soft and hard samples

2.4.3.1 X-ray computed tomography

Principle

Conventional 2D radiography reproduces a shade 2D of a 3D object. The intensity of X-rays passing through an object is modulated based on the absorption capacity of the different parts of the object irradiated. The absorption capacity depends on the thickness, density and chemical composition of the object characterized.

The X-ray computed tomography is an imaging technique in three dimensions (3D), invented in 1972 by Godfrey N. Hounsfield, which earned him the Nobel Prize in Medicine. The RX tomography allows reconstruction (visualization) of the interior of a solid object from X-rays 2D radiographies.

X-ray computed microtomography

The instrument is a MicroXCT-400 sold by the company Zeiss-Xradia. This unit has a scintillator turret and optical lenses (x0.4, x4, x10, x20, x40) to add to the geometric magnification of the object, an optical magnification. Lens used, the spatial resolution varies from about 50 microns to less than 1 micron with a voxel size (3D pixels) of up to 0.3 micron in the latter case. The detector is a CCD camera of 2K x 2K pixels.

Its motorized stage of rotation (360 °) and translations (X, Y, Z) is designed to accommodate samples of up to 100 mm diameter and a weight of 15 kg, which allows the analysis of samples in specific environment. The microXCT-400 has an X-ray source (anode W, 59keV) up to a maximum voltage of 140 kV. The power of the X-ray generator is adjustable to a maximum of 10W.

The acquisition time for a volume varies depending on the spatial resolution of less than 30 min at low power for over 24 hours with the objective x40.

The specificity of this device compared to other micro-tomography RX is to combine geometric magnification (conventionally used in X-ray micro-tomography) to an optical magnification (with its nosepiece), allowing to dispose of high resolution for source-sample distance up to 45 mm. Various sample environments can be considered (eg. In-situ analysis in soil columns ...) and a multi-scale approach can be performed on the same sample without having to reduce its size.

X-ray computed nanotomography

The nano-tomography instrument is a UltraXRM L200-Zeiss-Xradia. This instrument has unique characteristics on the market and similar to those obtained by the devices developed on synchrotrons.

Two spatial resolution at the nanoscale are available: 50 and 150 nm (HRS LFOV and fashion respectively) leading to voxels size of 16 and 64 nm. This spatial resolution, single-wide laboratory scanners RX is achieved through the development of optical devices, Fresnel lenses, for focusing the X-rays transmitted by the sample.

The interest of having those two instruments through the EquipEx NanoID project is that they offer a wide range of spatial resolutions and allows to scan a wide scale observation for the same sample. A sample can be analyzed initially by micro-tomography RX, for 3D viewing with a wide field of view and a micrometer spatial

resolution. It can then be seen in 3D with nanometer resolution over a volume of interest previously identified without any modification or new specific preparation.

The SOP relative to this technique adapted to biological tissues and cells is provided in the annex 9 of the document and is also available online through the CIRCABC Library > C-NANoREG results > NANoREG developed_improved SOPs and Methods > WP3 developed_improved SOPs and Methods > SOPs related to D3.6.

The domain of application of the X-ray computed tomography is to provide semi-quantitative 3D chemical mapping for solids or frozen liquids (100 ppm concentration). The main limitations that could be mentioned are the following. It is an expensive, non-routine technique that requires skilled personnel. It requires sufficient electron contrast with the matrix/solvent and in some cases the resolution might be limited. Moreover, a critical parameter is the sample stability over data acquisition period (e.g. drying, deforming).

2.4.3.2 Small Angle X-ray Scattering: SAXS

Size, shape and most importantly agglomeration/aggregation level of nano-particles in suspension remain challenging measurement. Light scattering techniques exhibit unique features that enable to determine not only the size of the sub-unit but also the structure of the larger agglomerates.

Method

SAXS is a technique that examines the structural features of objects between a few Å and ca. 200 nm in size, i.e. it covers nearly 3 orders of magnitude. As opposed to XRD, SAXS is also applicable to non-crystalline objects. The incident X-ray beam is scattered by the electrons of the sample thus producing a scattering pattern from which parameters such as size, shape and crystallinity can be determined.

The SOP relative to the characterization of ENMs by SAXS is provided in the annex 10 of the document and is also available online through the CIRCABC Library > C-NANoREG results > NANoREG developed_improved SOPs and Methods > WP3 developed_improved SOPs and Methods > SOPs related to D3.6.

The domain of application of the SAXS method is to provide information on size, shape, aggregation state and structure of aggregates. Moreover this technique is also able to provide with information on specific surface area, concentration and speciation. The applicability of this technique is large:

• Physical state: solids, liquid suspensions, aerosols.

• Concentration: dependent on X-ray source; 10^{12}-10^{15} particles/cm^3 for an X-ray flux of 10^9-10^{10} photons/mm^2/sec yield usable data.

The main limitations that could be mentioned are the following. It is an expensive, non-routine technique that requires skilled personnel. It requires sufficient electron contrast with the matrix/solvent and it is non element specific. Moreover, it has a limited applicability for very heterogeneous samples in size and chemical composition.

2.4.3.3 Surface swab and tape stripping technique

The emission of airborne NOAA during production and handling of nanomaterials can contaminate not only workplace atmosphere, but also surfaces. NOAA may be released into the working atmosphere by several sources (sonication, mixing and agitation) and, after a period of time, be deposited into the surfaces contributing to the dermal exposure. However, these compounds are able to be resuspended into the working environment as a consequence several actions such as the cleaning of the surfaces with a brush or the air streams generated by the movement of the workers, resulting in a secondary inhalation. In general terms, a visual evaluation of the amount of dust present on a surface may give an idea about the amount of dirt accumulated. Nevertheless, the degree of surface contamination and the effectiveness of cleaning cannot be assessed objectively only by visual inspection. Thus, it is necessary to develop a monitoring method in order to quantify the NOAA concentration on surfaces where nanomaterials are handled.

Apart from air quality measurements with on-line devices, such as particle counters and off-line systems, as deposition filters, surface swabs are used for sampling surfaces. Any potential contaminant or cleaning

residue present in irregular surfaces, hard-to-reach areas or heated and porous surfaces can be eluted from the swab and analysed down to trace levels using standard analytical techniques such as Transmission Electron microscopy (TEM), high performance liquid chromatography (HPLC), gas chromatography or Total Organic Carbon (TOC) tests.

Environmental monitoring encompasses testing of the equipment, cleanrooms and people in order to:

- Monitor surface contamination and cleaning efficiency in cleanrooms, laminar flow cabinets and isolators, among others,
- study the fate of airborne nanomaterials (nature of particulate deposition, spray drift),
- ensure that the SOPs maintain the cleanroom environment within the correct limits,
- analyse physico-chemical properties and microbiological content of surfaces and other monitoring questions.

Analysis can be routinely performed on a range of organic and inorganic air contaminants and a wide variety of microbiological contaminants. The swab consists, basically, in a wet swab which is rubbed for the desired surface in order to collect the particles that have been deposited there. However, this technique has the disadvantage of not covering all the surface area. Therefore, the zone to perform the test must be chosen with care in order to allow a representative sampling.

This technique must comply some requisites in order to offer a reliable analysis:

- ✓ *Minimal extractable interferences*: Background is the amount of contaminant on a swab measured by the analytical technique after testing has been performed according to the analytical protocol before sampling. Blank contribution from the swab must be minimal.
- ✓ *Ultra-low particles and fibres*: It is critical that the swabbing material leave the swabbed Surface free from particles which would further contaminate the surface.
- ✓ *Solvent compatibility:* the substance to eluate the swab must not interfere, contaminate or destroy the sampled materials.
- ✓ *High recovery rates:* Recovery means the percentage of contaminant actually measured by the analytical technique when the swab is spiked with a known quantity of that species.

Methodology:

Two different methodologies were tested:

- To collect a tape sample, ordinary clear transparent adhesive tape (Staples Europe B.V., the Netherlands) was used with a width of 15 mm, length of ~15 cm, with ends folded. Single tape samples were collected from each surface location. The sticky surface of the tape was pressed against the workplace surface to be sampled, and rubbed lightly to assure adhesion to dust deposited on the surface. Then, the tape was pulled off with a fluent and decisive movement, and placed with the sticky side down on a new sheet of plastic film and labelled. The plastic film was placed in a new plastic cover for storage until analysis. A new pair of nitrile gloves was used for each collected tape sample. Also, field blanks were collected by placing the piece of tape on the plastic sheet. The nature of the sampled surfaces is of importance and should be documented. The tape samples were qualitatively analyses by scanning electron microscopy.

- Polyester swabs with stick handle. These are of smaller size and not wearable, to cover specific areas such as corners, edges or inner surfaces of objects. The most widely used sort of this kind sampling swab is made of laundered polyester knit fabric (Fig. 1), since this material offers the lowest levels of releasable particles, the highest recovery and the lowest background when analysed by TOC technique. Also, the substance to eluate the swab must not interfere, contaminate or destroy the sampled materials. Some products compatible with polyester

Polyester Swab Compatible Solvents	
Acetic Acid	Hydrogen Peroxide
Acetone	Isopropyl Alcohol
Acetonitrile	Methanol
Ammonia, Anhydrous	Methyl Ethyl Ketone
Aniline	Methylene Chloride
Benzene	Nitrobenzene
Carbon Tetrachloride	Perchloroethylene
Chloroform	Phosphoric Acid 85%
Cyclohexane	Sodium Hydroxide 36%
Ethyl Acetate	Toluene
Ethyl Alcohol	Trichloroethylene
Ethyl Chloride	Vinyl Acetate
Formic Acid	Water
Hexane	Xylene

swabs are shown in at the right.

Following OSHA recommendations, the size of the surface area to be wiped is a 10 x 10 cm square, since the 100 cm^2 approximates the surface area of a worker's palm in order to approximate the amount of contaminant that could be transferred upon contact. A common rule to estimate an acceptable value for surface contamination in work areas is based on comparison of this information with the value of the occupational exposure limit (OEL, $\mu g/m^3$).

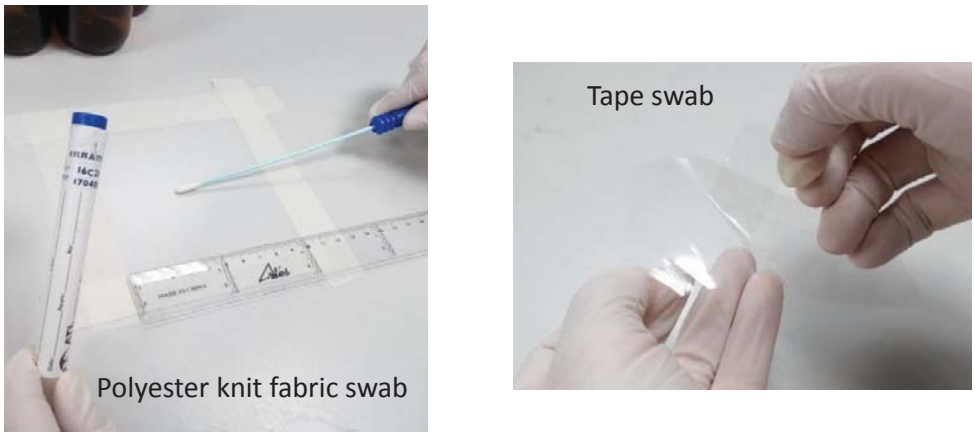


Figure 25: Types of surface swabs

Results:

With the method it is possible to determine if NOAA are present at workplace surfaces during or after a process. Surface materials suitable to be tape sampled can be made of e.g. metal, plastic, coated concrete, laminate, and coated wood. In the scope of the study, surfaces to be applied are surfaces of laboratory benches and personal protective equipment.

Figure 26: Surface swabs placed on the surface of a protective suit (left) and on a Half mask (right).

In order to extract the nanoparticles captured from the material of the swab for further characterisation, it must be sonicated during approx. 10 mins in a solution of 2 ml of pure water. A drop of the liquid resultant is placed onto a thin carbon grid and evaporated under vacuum, to prepare a sample of up to 1µg to be characterized

by Transmission Electron Microscope (TEM), able to measure ranges between 0,1 nm to 2 µm providing high resolution images which enables information of particle shape, size and size distribution characterization, plus visualization of the degree of aggregation/agglomeration, crystalline phase and chemical analysis.

Conclusions:

Swab / tape sampling has demonstrated to be useful tools to determine surface contamination by NOAA at workplaces. It can be used for assessing if the NOAA, after being released to the air by processes such as sonication, agitation, pouring or mixing, are deposited in determined surfaces in the workplace, being potential sources of secondary inhalation and dermal exposures. This methodology is complementary to air sampling due to the NOAA deposited in the surfaces that may be released to the working atmosphere by several factors such as cleaning activities or the movement of the workers. Together, these methods (air and surface sampling) provide a better overview of the hygienic situation in workplaces where nanomaterials are handled. After sampling, a characterization of the NOAA deposited in the surfaces can be carried out by TEM in order to characterize their size distribution or shape, and if there exists any relation between these parameters and the tendency to deposition.

Further description of the surface swab and tape stripping methods along with their SOPs are respectively provided in the annexes 11 and 12 of this document and are also available online through the CIRCABC Library > C-NANoREG results > NANoREG developed_improved SOPs and Methods > WP3 developed_improved SOPs and Methods > SOPs related to D3.6.

References:

- Swabs for Cleaning Validation in Pharmaceutical Manufacturing, Technotes by Texwipe, Vol. XI, No. 1, Feb. 2011
- Choosing the Correct Swab for Cleaning Validation, K. Miscioscio, CLEANROOMS, The Texwipe Company, Jan. 1997
- Environmental Monitoring - Maintaining a Clean Room, P. Tierney, R. Burke, B. O'Donnell and J. McAteer, Pharmaceutical Processing, 2010
- USP <797> Environmental Monitoring Program Design and Application, J. P. Manfrida, Ph.D., EMLab P&K Analyst, The Environmental Reporter, Vol. 8, Issue 2, 2010
- How to Succeed in the Search for Nothing: Effective Swabbing Techniques for Cleaning Validation, H. Siegerman, W. Hollands, M. Strauss, ITW Texwipe Feb. 2006
- OSHA Technical Manual (OTM), U.S. Department of Labor, Occupational Safety & Health Administration. https://www.osha.gov/dts/osta/otm/otm_extended_toc.html

2.4.4 Isotope analysis method to discriminate background from actual environmental exposure

The major challenge in studying nanoparticles is to detect their presence and distinguish them from natural nanoparticles and the large variety of amorphous materials present in environmental media. Available tools used to trace NPs are currently based on chemical signatures that may be recognized in clean systems. While useful and sufficient in the context of in vitro experiments and many aspects on human toxicity of NPs, different tools are needed when dealing with heterogeneous and complex matrices like soil, sediments and tissues, as well as the liquid media used in aquatic exposure studies. Hence the main focus of this part of the project has been on the development of radionuclide labelling methods and protocols that can be applied in such matrices. The main objective has been to modify techniques for application in environmental media, focusing on sample preparation, method protocols and detection limits for selected NANoREG Nanomaterials.

Radioisotope labelling can be used as an aid to the detection and localisation of NP in environmental media, as well as kinetic studies of stability and bioavailability. The unparalleled detection limits, mean that technique can be used to study bioavailability at environmentally relevant concentrations, avoiding the risk of saturation that can arise when high concentrations of NP are needed to ensure detection in the exposed organisms.

To date two main methods have been applied for radionuclide labelling: neutron activation (NA) of dry powders and labelling during wet preparation. Neutron activation is the preferred method for NANoREG materials, since the characterised materials can be labelled directly, with minimum change in physical or chemical properties. Provided the irradiation times are short, and neutron fluxes low enough the resultant specific activity (ca. 1 radioactive nuclide per 10,000-10,000 atom) is still high enough to allow fg levels of detection without any discernible chemical change. NMBU has access to a research reactor and certified

laboratories for radioisotope work. The radiolabelling techniques have been previously demonstrated to be practicable for a variety of NP and fate studies (Oughton et al., 2008, Coutris et al., 2012).

The present work has focused on the application of radioisotope labelling to support work on characterisation of ENP exposure solutions, and in particular, the stability and change in chemical speciation during exposure. In this respect a number of studies have already been carried out on radiolabelled particles that are of relevant to the NANoREG consortium (Ag, Ce, Sn). Radiolabelled NANoREG particles have been produced and discussions initiated discussions with partners as to how we can best apply the techniques for the support of NANoREG objectives.

An example of the types of studies that can be carried out is given in Figure 1. This shows sequential extraction studies on the stability of Ce and Sn NPs in different types of soils, and change in speciation and binding. The results indicate that despite a very low solubility of these NPs, there can be an increase in the bioaccessible fraction of Ce and Sn over time (Carbone et al, in prep).

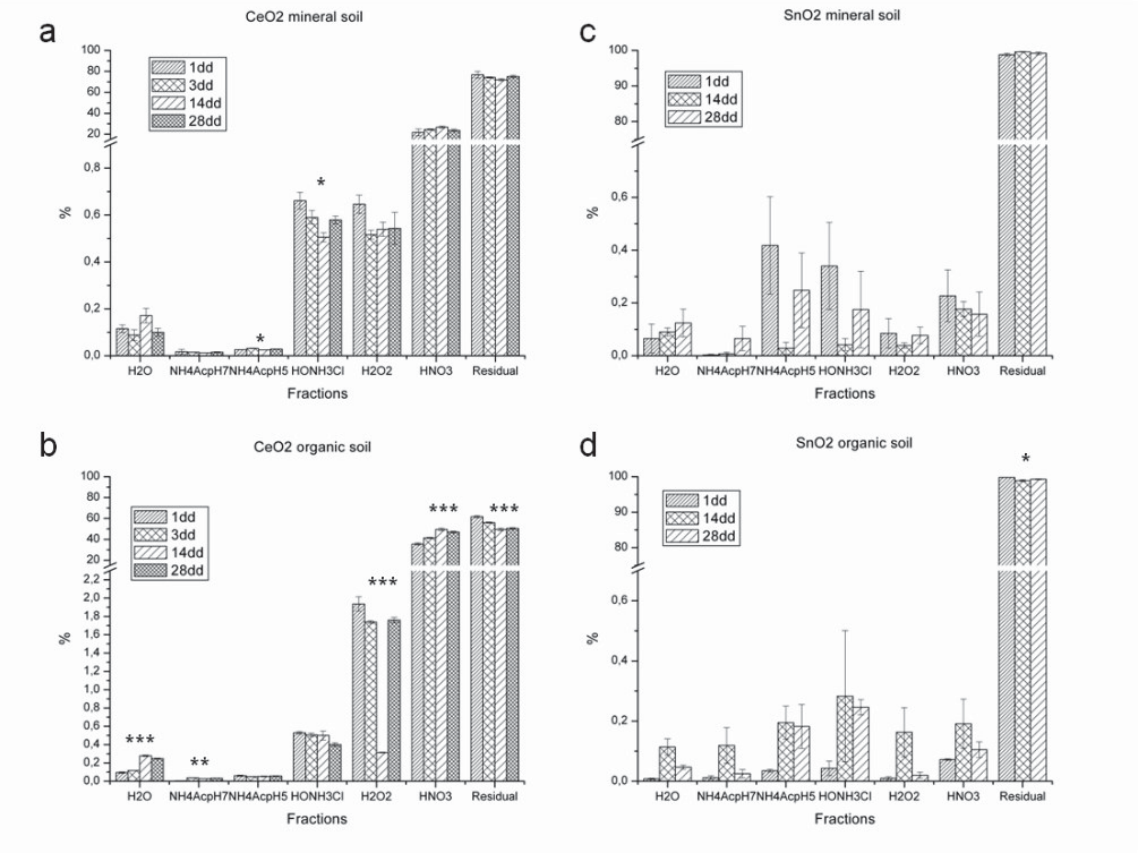


Figure 27. Relative distribution (%) of ^{141}Ce and ^{113}Sn in sequential extraction fractions of the mineral (a, c) and organic (b, d) soil spiked with radiolabeled Ce NPs (a, b) and Sn NPs (c,d). Results are means, error bars are SD (n=3). Significance of ANOVA: ***=$P \leq 0.001$; **=$P \leq 0.01$; *=$P \leq 0.05$ according to the HSD test (Carbone et al., in prep). NOTE SPLIT IN SCALE FOR X-AXIS.

2.5 Evaluation and conclusions

In the context of the evaluation of occupational / consumer / environmental exposure a wide variety of improved instruments, tools and methods were selected and evaluated by WP3 partners.

A total of 14 different techniques were selected in order to cover the three main compartments (air, water, and solid samples including soil) and different routes of exposure (inhalation and dermal exposure). The instruments tested investigate different principles and aim at providing portable monitoring solutions and/or techniques for specific cases. Those instruments, tools and methods encompasses from the most affordable

and easy to implement portable devices (e.g. MPS and NANOBADGE samplers, tape stripping and surface swab methods…) to the most sensitive, reliable and versatile instruments that requires highly qualified personnel (e.g. SAXS, cryogenic TEM …). Examples are given to explicit the domain of applicability, potentialities and limitations of those techniques.

Table 3. Selected instruments, tools and methods to assess nanoparticle parameters (size, shape…) depending on the compartments: air, liquid/complex matrices and surfaces/hard samples.

<table>
<tr><th>Air / Aerosols</th><th>Water / suspensions</th><th>Surface / Soil / hard samples</th></tr>
<tr><td>Laser Induced Breakdown Spectroscopy (INERIS)</td><td>~~Single particle - Inductively Coupled Plasma Mass Spectrometry (VN)~~</td><td rowspan="3">X-ray computed tomography (CEREGE)</td></tr>
<tr><td>Mini Particle Sampler (INERIS)</td><td rowspan="2">Asymmetric-Flow Field Flow Fractionation-Inductively Coupled Plasma Mass Spectrometry (CEA)</td></tr>
<tr><td>NANOBADGE Sampler (CEA)</td></tr>
<tr><td>Electrical Low Pressure Impactor (INRS) & translated NanoGEM SOPs</td><td rowspan="3">Cryogenic – Transmission Electron Microscopy (CEA)</td><td rowspan="3">Small Angle X-rays Scattering (CEREGE)</td></tr>
<tr><td>Nasal paper flag (CEA)</td></tr>
<tr><td>Exhaled breath condensate (CEA)</td></tr>
<tr><td colspan="3">Surface swab (ITENE) & Tape stripping technique (LTH)</td></tr>
<tr><td colspan="3">Isotopic labelling approaches (NMBU)</td></tr>
</table>

State-of the-art direct reading instruments for occupational exposure assessment were directly used without further development in field measurements (D3.7) because some projects such as NanoGEM and nanoIndEx generated evaluation data and SOPs for those devices. However, in some cases SOPs from conventional instruments such as the Electrical Low Pressure Impactor (ELPI) are not freely available. Therefore, we investigated such instruments and SOPs were prepared accordingly.

In the case of personal devices to assess occupational exposure, several low-cost and easy-to-use samplers and biomonitoring tools were investigated.

The Mini Particle Sampler (MPS) allows the characterization of aerosol in any field of aerosol science. Its purpose is to sample the aerosol particles onto a transmission electron microscopy grid to characterize NOAA, and more precisely aerosol morphology and elemental composition through energy-dispersive X-ray analysis. Despite various advantages that have been described earlier some limitations could be mentioned. Up-to-now, the MPS leads to qualitative data. In case of high skilled microscopist and/or well-known applications, it can provide semi-quantitative data as long as the flow rate and sampling time are defined carefully. In case of high concentration or if long term sampling (>30 min) is needed, dilution of aerosol can be carried out.

The NANOBADGE is personal sampler dedicated to the airborne NOAA characterization in order to assess occupational exposure by providing information on the elemental composition, size distribution and morphology of the collected material. Its purpose is to sample airborne particles in the personal breathing zone of workers, leading to their characterization by XRF and SEM-EDS. Therefore this sampler could provide more directly mass concentration shift averages with the advantage to enable the identification of specific morphological or chemical features. Despite various advantages that have been described earlier, some limitations could be mentioned. Indeed, there might be a certain conflict between monitoring (using DiSCmini, NanoTracer or Partector for instance) and sampling if very short activities are to be investigated, as limits of detection of certain analytical methods may require longer sampling times than monitoring periods.

The surface swab method and the tape stripping technique have demonstrated to be useful tools to determine surface contamination by NOAA at workplaces. They can be used for assessing if the NOAA, after being released to the air by processes such as sonication, agitation, pouring or mixing, are deposited in determined surfaces in the workplace, being potential sources of secondary inhalation and dermal exposures. This

methodology is complementary to air sampling due to the NOAA deposited in the surfaces that may be released to the working atmosphere by several factors such as cleaning activities or the movement of the workers. Together, these methods (air and surface sampling) provide a better overview of the hygienic situation in workplaces where nanomaterials are handled. After sampling, a characterization of the NOAA deposited in the surfaces can be carried out by TEM in order to characterize their size distribution or shape, and if there exists any relation between these parameters and the tendency to deposition. In order to preserve the integrity of clean surfaces and to assign semi-quantitatively the origin of contamination, it is recommended to perform surface analysis on a regular basis along with proper periodic surface cleaning.

Used in a coherent strategy, those easy-to-use personal devices (MPS, NANOBADGE, surface sampler …) are able to provide a fine picture of the potential release sources and emissions of NOAA that could lead to exposure (through dermal and inhalation routes) in occupational settings. Nevertheless, depending on the analytical technique associated to the sampling method, information on the particle identity might be partial (shape, morphology, chemical composition …)

In addition to atmospheric measurements for aerosol exposure assessment, biomonitoring tools were selected since they are essential to provide information to determine whether there is a real individual exposure situation. The Nasal paper flag and the Exhaled Breath Condensate methods are human biomonitoring tools to determine biomarkers of exposure and biomarkers of effects in order to assess personal exposure to occupational and environmental toxicants. They could therefore be used for occupational health surveillance and human biomonitoring. Despite various advantages that have been described earlier some limitations could be mentioned. Indeed, the current method gives no information on the particulate contents (only an elemental information is provided after complete digestion). Moreover, due to the ICP-MS technology, the carbonaceous particles cannot be evaluated through this method. Finally, the biomarkers of effect are not specific to nanoparticles therefore no specific biomarker to ENMs is available currently.

More sensitive, reliable and versatile instruments that requires highly qualified personnel were also investigated since they could provide valuable information on potential exposure.

The Field Flow Fractionation analytical chain (FFF) is a technique of liquid chromatography. FFF is coupled to an array of detectors comprising a UV-Vis (UV-visible spectrometer), a MALLS (static light scattering), a DLS (dynamic light scattering), a DRI (refractometer) and an ICP-MS that allow an in-depth physical chemical characterization of the particles. This technique allows among others the environmental monitoring, and personal exposure measurements by being able to characterize complex matrices such as environmental waters or biological fluids. The main limitation of the method is the time needed to set-up the right operational parameters (flowrates, membranes, ionic strength etc.) in order to allow a complete separation of the different components of the mixture. Moreover, depending on the type of complex matrices studied, a specific preparation step is required (e.g. selective digestion, colloidal des-agglomeration and stabilisation etc.). Indeed, in some circumstances FFF might not be able to distinguish loose agglomerates from aggregates compared to sp-ICPMS or SEM analysis.

The SAXS method provides information on size, shape, aggregation state and structure of aggregates. This technique is also able to provide with information on specific surface area, concentration and speciation. This technique could be applied to samples with various physical states (i.e. solids, liquid suspensions, aerosols…). The main limitations that could be mentioned are the following: it is an expensive, non-routine technique that requires skilled personnel; moreover, it requires sufficient electron contrast with the matrix/solvent and it is non element specific; finally, it has a limited applicability for very heterogeneous samples in size and chemical composition.

The X-ray computed tomography is a technique that provides semi-quantitative 3D chemical mapping for solids or frozen liquids (up to 100 ppm concentration). The limitations related to this technique is that it is an expensive, non-routine technique that requires skilled personnel. It requires sufficient electron contrast with the matrix/solvent and in some cases the resolution might be limited. Finally, its main limitation is due to the long data acquisition period during which the sample has to remain stable (e.g. drying, deforming that can cause modifications).

Cryogenic transmission electron microscopy (Cryo-TEM) is an *in situ* analytical technique that allows imaging materials and biological samples in a frozen state, directly within the TEM. Samples and processes that previously required deposition on substrates (and presented aggregation), or could not be imaged in their native environment, could now be studied and observed in an amorphous frozen liquid and at high resolutions. From hydrated materials such as inks and gels, to biological materials that include whole cells and macromolecules such as proteins, liposomes and viruses, cryogenic TEM could allow to load samples in a controlled environment and image within the TEM, and provide compositional analysis capability through EDS and EELS. This characterization method provides information related to the aggregation state of particles in suspension. Its domain of application is the characterization of suspensions including complex matrices such as environmental waters and biological fluids. The main limitation that could be mentioned is that it is an expensive, non-routine technique that requires skilled personnel and a consequent sample preparation time.

Laser Induced Breakdown Spectroscopy (LIBS) is another candidate for *in situ* nanoparticle *detection* in real time or semi real time. LIBS is really interesting for monitoring applications because it is the only technique which can measure mass concentration in real time without any need of prior information on the (fractal) shape or the density of particles. Applied in air or on a substrate, this technique does not require sampling preparation and allows measuring to the entire list of atomic species. INERIS uses this tool to perform on-line chemical measurement of airborne particles at workplace or nearby industrial environment. LIBS can measure chemical composition of airborne particles without any sampling step because plasma is directly generated in air charged with particles. This analytical technique is unique because it is on line and evolution of atomic species concentration as a function of time can be measured. Moreover, analytical response of LIBS does not depend of the size of particles. In other words, this technique can measure with the same sensitivity mass concentration of NOAA. The main limitation of this technique is due to the fact that it is a tool of micro analysis. While absolute detection limits of LIBS are between 1 fg and 1 pg as a function of atomic species, relative detection limits expressed in term of mass concentration becomes 1 $\mu g/m^3$ and 1 mg/m^3 respectively, due to the small size of the sampling volume. Regarding small particles, such as nano-objects, limits of detection are achieved if high numbers of particles are in the sampling volume. The particles bigger than a few hundreds of nanometers, such as agglomerates and aggregates, can be individually detected by LIBS because the absolute limit of detection is achieved inside the sampling volume.

The major challenge in studying engineered nanoparticles is to detect their presence and distinguish them from natural and anthropogenic particles and the large variety of amorphous materials present in environmental media. Radioisotope labelling can be used as an aid to the detection and localisation of nanoparticles in environmental media, as well as kinetic studies of stability and bioavailability. The unparalleled detection limits, mean that technique can be used to study bioavailability at environmentally relevant concentrations, avoiding the risk of saturation that can arise when high concentrations of nanoparticles are needed to ensure detection in the exposed organisms. However, the major bottleneck is due to the important cost of the equipment as well as the time required for sample preparation.

The impact of the work being done in this task is beneficial to other tasks and subtasks in NANoREG (particularly to the tasks 3.4 and 3.5 in which the exposure data generated -D3.7- enable the validation of models and risk management measures), ProSafe, other EU projects and beyond. The impact of this task will also be beneficial to the scientific community, regulators and stakeholders outside of the project since there is a strong need to reach consensus on tools and methods to assess exposure in practice along the whole value chain (workplace, consumer and environment). This deliverable contributes to answer regulatory questions by providing means to assess occupational, consumer and environmental exposure. In particular, information on the instruments, tools, methods, and exposure assessment strategies will allow regulators and industry to make appropriate choices to implement efficiently harmonized approaches for specific exposure situations.

2.6 Data Management

Standard Operating Procedures relative to the improved measurement instruments, tools and methods were developed and uploaded to CIRCABC Library > C-NANoREG results > NANoREG developed_improved SOPs and Methods > WP3 developed_improved SOPs and Methods > SOPs related to D3.6. Moreover, those SOPs are reported in the NANoREG toolbox.

Data generated during field measurements and simulated approaches are reported in D3.7. Field measurements datasets were entered by organizing the necessary information including contextual information within Excel templates adapted to the NECID database. It is worth remembering that it was decided by the NANOREG MC that exposure dataset will not be included within the NANOREG database. However datasets are temporarily stored at the CEA (using a local NECID database) and can be available upon request.

3 Deviations from the work plan

The deliverable 3.6 entitled “Improved measurement instruments, tools and methods” was initially due by December, 2015. However, due to missing contribution on specific methods and instruments by several contributors, a significant delay was experienced. It shall be noted that the delay should not affect the review of NANoREG deliverables by the PROSAFE Task force in preparation of the OECD meeting in November since several draft of the deliverable 3.6 were distributed to WP3 and the project office during the year 2016.

Most of partner’s contribution and SOPs were received by July 2016. However, despite several reminders by the lead beneficiary and under the supervision of WP leaders some partners did not provided the contribution initially planned on time. In November 2016, all contributions were received except the one relative to the single particle - Inductively Coupled Plasma Mass Spectrometry.

4 References

Amodeo T., Dutouquet C., Le Bihan O., Attoui M., Frejafon E. (2009) On-line determination of nanometric and sub-micrometric particle physicochemical characteristics using spectral imaging-aided Laser-Induced Breakdown Spectroscopy coupled with a Scanning Mobility Particle Sizer. Spectrochimica Acta Part B-Atomic Spectroscopy. 64(10): p. 1141-1152.

Coutris C, Joner EJ, Oughton DH. Aging and soil organic matter content affect the fate of silver nanoparticles in soil. Science of The Total Environment. 2012;420:327-33

Oughton, D.H., Hertel-Aas, T., Pellicer, E., Mendoza, E., Joner, EJ (2008) Neutron activation of engineered nanoparticles as a tool for tracing their environmental fate and uptake in organisms. Environmental Toxicology and Chemistry 27, 1883-1887.

Coutris, C., Hertel Aas, T., Lapied, E., Joner, E.J. Oughton, D.H. 2011. Bioavailability of Cobalt and Silver Nanoparticles to the Earthworm Eisenia fetida. Nanotoxicology, 6: 186-195

Bouillard J., Badr R’Mili, Daniel Moranviller, Alexis Vignes, Olivier Le Bihan, Aurelien Ustache, Joao A. S. Bomfim, Emeric Frejafon, Dominique Fleury, Nanosafety by design: risks from nanocomposite/nanowaste Combustion, J Nanopart Res (2013) 15:1519 DOI 10.1007/s11051-013-1519-3

Bressot C., Shandilya N., Nogueira E. S da Costa , Cavaco-Paulo A., Morgeneyer M., Le Bihan O., Aguerre-Chariol O., Exposure assessment based recommendations to improve nanosafety at nano-liposome production sites, Journal of Nanomaterials, September 2015.

ECHA, 2012 - European Chemical Agency. Guidance on information requirements and chemical safety assessment Chapter R.8: Characterisation of dose [concentration]-response for human health; ECHA-2010-G-19-EN; 2012; p 186.

Faure, B., Dozol, H., Brouard, C., Guiot, A., Clavaguera, S. (2016): Evaluation of a personal sampler for mass-based measurement of airborne nanoparticles, submitted to Journal of Aerosol Science.

Fierz M., Meier D., Steigmeier P., Burtscher H. (2014), Aerosol Measurement by Induced Currents. Aerosol Science and Technology 48(4): 350-357.

Hedmer M, Isaxon C, Ludvigsson L, Nilsson PT, Rissler J, Gudmundsson A, Bohgard M, Messing ME, Tinnerberg H, Pagels JH. Workplace measurements of semiconductor nanowires at a small-scale producer. SENN2015, 12-15 April 2015, Helsingfors, Finland.

Hedmer M, Ludvigsson L, Isaxon C, Nilsson P, Skaug V, Bohgard M, Pagels JH, Messing ME, Tinnerberg H. Detection of carbon nanotubes and carbon nanodiscs at workplace surfaces in a small-scale producer. Ann Occup Hyg 2015; 59: 836.

Keller, A. A.; McFerran, S.; Lazareva, A.; Suh, S. Global life cycle releases of engineered nanomaterials. J. Nanoparticle Res. 2013, 15, 1692.

Kelly, W. P.; McMurry, P. H. Measurement of Particle Density by Inertial Classification of Differential Mobility Analyzer–Generated Monodisperse Aerosols. Aerosol Sci. Technol. 1992, 17, 199–212.

Kreyling, W. G.; Semmler-Behnke, M.; Möller, W. Ultrafine particle-lung interactions: does size matter? J. Aerosol Med. 2006, 19, 74–83.

Le Bihan O.L.C., Ustache A., Bernard D., Aguerre-Charriol O., Morgeneyer M., Experimental study of the aerosolization from a carbon nanotubes bulk by a vortex shaker, Journal of Nanomaterials, 2014.

Lyyränen, J., Backman, U., Tapper, U., Auvinen, A., and Jokiniemi, J. (2009). A Size Selective Nanoparticle Collection Device Based on Diffusion and Thermophoresis. J. Phys. Conf. Ser., 170:012001.

Morgeneyer M., Shandilya N., Chen Y. M., Le Bihan O., Use of a modified Taber abrasion apparatus for investigating the complete stress state during abrasion and in-process wear particle aerosol generation, Chemical Engineering Research and Design, 2015, 93: p. 251-256.

Motellier, S.; Lhaute, K.; Guiot, A.; Golanski, L.; Geoffroy, C.; Tardif, F. Direct quantification of airborne nanoparticles composition by TXRF after collection on filters. J. Phys. Conf. Ser. 2011, 304, 012009.

NanoGEM Project, Standard Operation Procedures for assessing exposure to nanomaterials, following a tiered approach, 2012.

NanoIndEx Project, Assessement of Personal Exposure to Airborne Nanomaterials, A guidance document, 2016.

Nel, A.; Xia, T.; Mädler, L.; Li, N. Toxic potential of materials at the nanolevel. Science 2006, 311, 622–627.

NIOSH. Current Intelligence Bulletin 63: Occupational Exposure to Titanium Dioxide; 2011–160; 2011; p 120

Oberdorster, G.; Oberdorster, E.; Oberdorster, J. NANOTOXICOLOGY: An Emerging Discipline Evolving from Studies of Ultrafine Particles. Environ. Health Perspect. 2005, 113, 823–840.

OECD, “HARMONIZED TIERED APPROACH TO MEASURE AND ASSESS THE POTENTIAL EXPOSURE TO AIRBORNE EMISSIONS OF ENGINEERED NANO-OBJECTS AND THEIR AGGLOMERATES AND AGGREGATES AT WORKPLACES Env/jm/mono(2015)19,” 2015.

Oomen A.G., Bleeker E.A.J., Bos P.M.J., van Broekhuizen F., Gottardo S., Groenewold M., Hristozov D., Hund-Rinke K., Irfan M-A., Marcomini A., Peijnenburg W.J.G.M., Rasmussen K., Sanchez Jimenez A., Scott-Fordsmand J., van Tongeren M., Wiench K., Wohlleben W., Landsiedel R. (2015) Grouping and Read-Across Approaches for Risk Assessment of NanomaterialsInt. J. Environ. Res. Public. Health, 12, 13415-13434.

Nel A.E., Parak W.J., Chan W.C.W., Xia T., Hersam M.C., Brinker, C.J., Zink J.I., Pinkerton K.E., Baer D.R., Weiss, P.S. (2015) Where Are We Heading in Nanotechnology Environmental Health and Safety and Materials Characterization? ACS Nano, 9 (6), 5627–5630.

OECD reports - ENV/JM/MONO(2012)8 & ENV/JM/MONO(2014)15 ; JRC report – EUR 25404 EN

Ounoughene G., Le Bihan O., Chivas-Joly C., Motzkus C., Longuet C., Debray B., Joubert A., Le Coq L., Lopez-Cuesta J., "Behavior and fate of Halloysite nanotubes (HNTs) when incinerating PA6/HNTs nanocomposite", Environmental Science and Technology 03/2015; 49(9):5450–5457.

Pietroiusti, A.; Magrini, A. Engineered nanoparticles at the workplace: current knowledge about workers’ risk. Occup. Med. (Lond). 2014, 64, 319–330.

Pitch, J. (1964). Impaction of Aerosol Particles in the Neighbourhood of a Circular Hole. Collect. Czech. Chem. Commun., 29:2223–2227.

R'Mili B., Dutouquet C., Sirven J.B., Aguerre-Chariol O., Frejafon E. (2011) Analysis of particle release using LIBS (laser-induced breakdown spectroscopy) and TEM (transmission electron microscopy) samplers when handling CNT (carbon nanotube) powders. Journal of Nanopart Res, 13(2): p. 563 – 577.

R'mili Badr, Le Bihan O., Dutouquet Christophe, Aguerre-Chariol Olivier, Frejafon Emeric, Particle Sampling by TEM Grid Filtration, Aerosol Science and Technology, 04/2013; 47(7).

Rubow, K. L., and Liu, B. Y. H. (1986). Characteristics of Membrane Filters for Particle Collection, in Fluid Filtration: Gas. Vol. 1 (ASTM Special Technical Publication). ASTM, Philadelphia, PA, p. 975.

Schulte, P. A.; Geraci, C. L.; Murashov, V.; Kuempel, E. D.; Zumwalde, R. D.; Castranova, V.; Hoover, M. D.; Hodson, L.; Martinez, K. F. Occupational safety and health criteria for responsible development of nanotechnology. J. Nanopart. Res. 2014, 16, 2153.

Seaton, A.; Tran, L.; Aitken, R.; Donaldson, K. Nanoparticles, human health hazard and regulation. J. R. Soc. Interface 2010, 7 Suppl 1, S119–29.

Shandilya N., Le Bihan O., Bressot C., Morgeneyer M., Emission of Titanium Dioxide Nanoparticles from Building Materials to the Environment by Wear and Weather, Environmental Science and Technology, January 15, 2015.

Shandilya N., Le Bihan O., Bressot C., Morgeneyer M., Evaluation of the Particle Aerosolization from n-TiO2 Photocatalytic Nanocoatings under Abrasion, Journal of Nanomaterials, Volume 2014, Article ID 185080, 11 pages.

Shandilya N., Le Bihan O., Morgeneyer M., A review on the study of the generation of (nano-) particles aerosols during the mechanical solicitation of materials, Journal of Nanomaterials, vol. 2014, Article ID 289108, 16 pages, 2014.

Shandilya N., Le Bihan O., Morgeneyer M., Effect of the normal load on the release of aerosol wear particles during abrasion, Tribology Letters, Tribology Letters 08/2014; 55(2):227-234.

Shandilya N., Morgeneyer M., Le Bihan O., First development to model aerosol emission from solid surfaces subjected to mechanical stresses: I. Development and results, Journal of Aerosol Science 07/2015; 89:43-57 2015.

Thayer D., Koehler K.A., Marchese A., Volckens J. (2011) A Personal, Thermophoretic Sampler for Airborne Nanoparticles, Aerosol Sci. Technol. 45: 734-740.

Vanwinkle, B. A.; de Mesy Bentley, K. L.; Malecki, J. M.; Gunter, K. K.; Evans, I. M.; Elder, A.; Finkelstein, J. N.; Oberdörster, G.; Gunter, T. E. Nanoparticle (NP) uptake by type I alveolar epithelial cells and their oxidant stress response. Nanotoxicology 2009, 3, 307–318.

5 List of abbreviations

AgNP	Silver nanoparticles
AgNP10, AgNP20…	Silver nanoparticles of nominal diameter of 10 nm, 20 nm…
AF4	Asymmetric Flow Field Flow Fractionation
Cryo-TEM	Cryogenic Transmission Electron Microscopy
DLS	Dynamic Light Scattering
DRI	Differential Refractometer
FFF	Field Flow Fractionation
HNO_3	Nitric acid
EBC	Exhaled Breath Condensate
EDS (EDX)	Energy Dispersive X-ray Spectrometry
EELS	Electron Energy Loss Spectroscopy
ELPI	Electrical Low Pressure Impactor
ENMs	Engineered NanoMaterials
ICP-MS	Inductively coupled plasma mass spectrometry
ISTD	Internal standard
LIBS	Laser Induced Breakdown Spectroscopy
LOD	Limit of detection
MPS	Mini Particle Sampler
MALLS	Multi Angle Laser Light Scattering
NOAA	Nano-Objects, their aggregates and agglomerates
NaOH	Sodium hydroxide
PBZ	Personal Breathing Zone
RI	Refractive Index
SAXS	Small Angle X-rays Scattering
SOP	Standard Operating Procedure
TEM	Transmission Electron Microscopy
UV/VIS	Ultraviolet/Visible
XRF	X-ray Fluorescence spectrometry

Annex 1: MPS Standard Operating Procedure

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Aim(s) or Objective(s) of SOP
The present operating document aims to facilitate the use of MPS. It indicates how to use a Mini Particle Sampler (MPS), which is an easy-to-use, low-cost and portable sampler of particles for TEM analysis. This sampling technique based on aerosol filtration by porous grid has been previously described and experimentally and theoretically assessed (R'mili et al., Aerosol Science and Technology, 2013).

Scope and required operating conditions

This novel tool can very quickly sample nanoparticles on a TEM grid that can be later analyzed with a TEM microscope. In this report, the protocol of this technique is described and illustrated for a laboratory environment as well as for a pilot scale pharmaceutical unit.

Transmission electron microscopy (TEM) coupled with energy-dispersive X-ray (EDX) offers a very comprehensive tool for individual particle analysis allowing the determination of size, morphology, specific surface, and elemental composition. This information is needed in aerosol studies, especially in the field of nanomaterials. However, observations with TEM require a controlled sampling on an adapted analysis support, namely TEM grid. Techniques allowing sampling on TEM grids are of great interest to aerosol analysis. Indeed, sample preparation is not required, thereby gaining time and avoiding a risk for the sample to be altered.

Developed at INERIS (R'mili 2013), the MPS (Figure 28) is the particle collection technique presented here. The MPS is based on filtration through one class of TEM-dedicated supports, namely TEM porous grids. It enables the aerosol sampling on a TEM (Transmission Electron Microscope) grid for further analysis.

Figure 28. Mini Particle Sampler - MPS.

The test method, described below, is based on the experience gained during the development (R'mili et al., 2013) and use of MPS in occupational Health (R'mili 2011, Fleury 2012, Bressot 2015), combustion and incineration (Bouillard 2013, Ounoughene 2015), emission from nanomaterials (dustiness, ageing) (Le Bihan 2014, Shandilya 2014-2015).

Field of application

The sampling on the TEM grid enables a TEM morphologic and elementary analysis of the sampled particles. The MPS has a multitude of applications e.g. Professional hygiene, Industrial combustions and emissions (in-stack or diffused), Indoor air, ambient air, and possibly be used in series with a DMA to sample monodisperse aerosol.

Concerned people

Concerned people by this Guideline are persons in charge of aerosol sampling for TEM analysis purposes, in the frame of the studies conducted on exposure assessment, risk analysis, powder and material release, etc.

Materials and equipment needed

Principle and experimental design

The aim of the experiment is to collect the aerosol particles on a TEM grid for their observation and analysis. The MPS is a filter holder (Figure 29) connected to a pump (Figure 30).

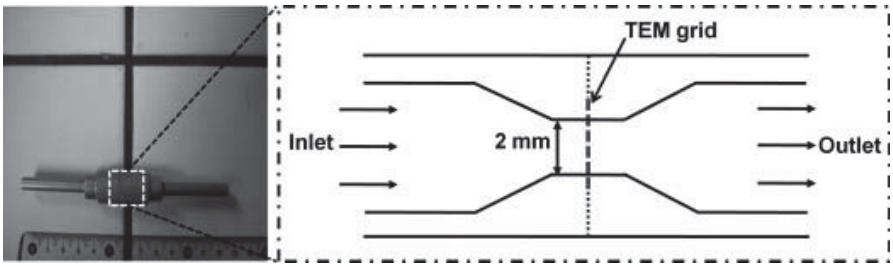

Figure 29. Concept diagram of the filter holder (R'mili 2013).

Figure 30.

Theory

The TEM grids selected are comparable to a microporous membrane. Given their geometric characteristics, the "capillary tube model" was chosen (Rubow and Liu 1986) –Figure 31-, an approach originally started by Pich (1964). The collection efficiency by filtration of a membrane depends on many physical mechanisms. In the absence of any external field of energy other than gravity, the most important ones are inertial impaction, interception, and Brownian diffusion (Rubow and Liu 1986)- Figure 31.

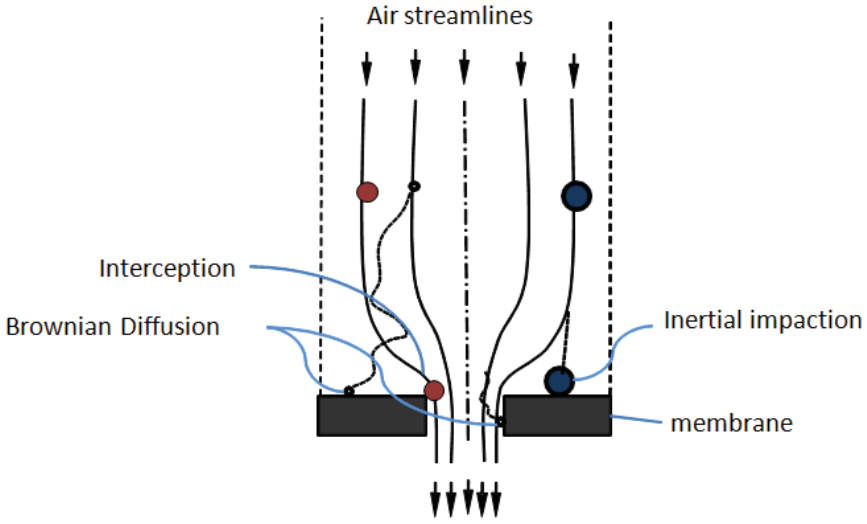

Figure 31. Capillary tube model (Rubow & Liu 1981).

Sampling efficiency

Figure 32 presents the collection efficiency of TEM grids “Quantifoil 1.2/1.3. The values presented are an average of results of at least three tests.

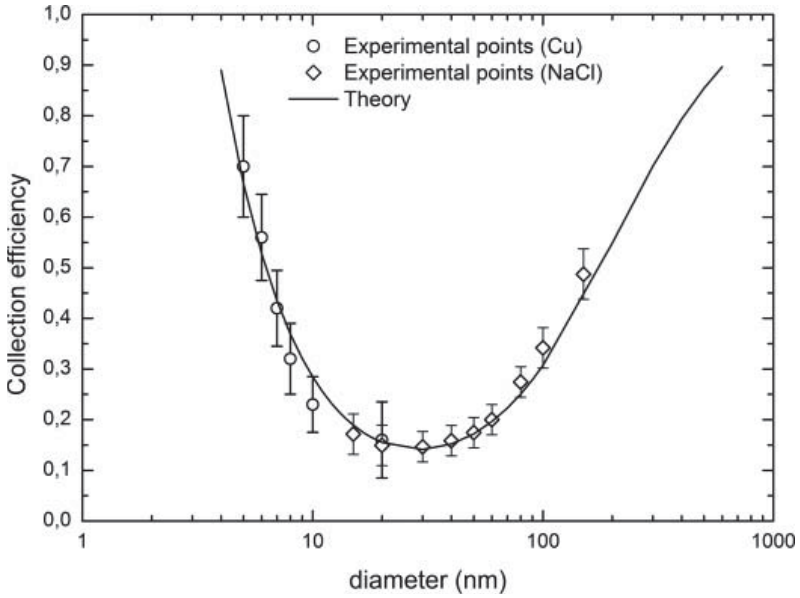

Figure 32. Particle collection efficiency by size for the Quantifoil 1.2/1.3 TEM grid, with a flow rate of 0.3 lpm.

A theoretical estimate is also presented and detailed in Figure 33. A very good theory–experiment convergence is observed in the entire 5 nm–150 nm range. A minimum collection efficiency is obtained for particles of a diameter ranging between 20 nm and 40 nm (diameter of electrical mobility). This minimum efficiency is evaluated at approx. 15%. This diameter range corresponds, in our conditions (sampling speed equal to 1.6 $m \cdot s^{-1}$ and characteristics of TEM grids studied), to the particles least sensitive to the different collection mechanisms. The collection efficiency is all the more important as the diameter of the particles reaches the area 20–40 nm, simultaneously toward the higher or lower diameters. The relative standard deviation of the experiment remains lower than or equal to 10%, which shows a very good reproducibility of the collection system.

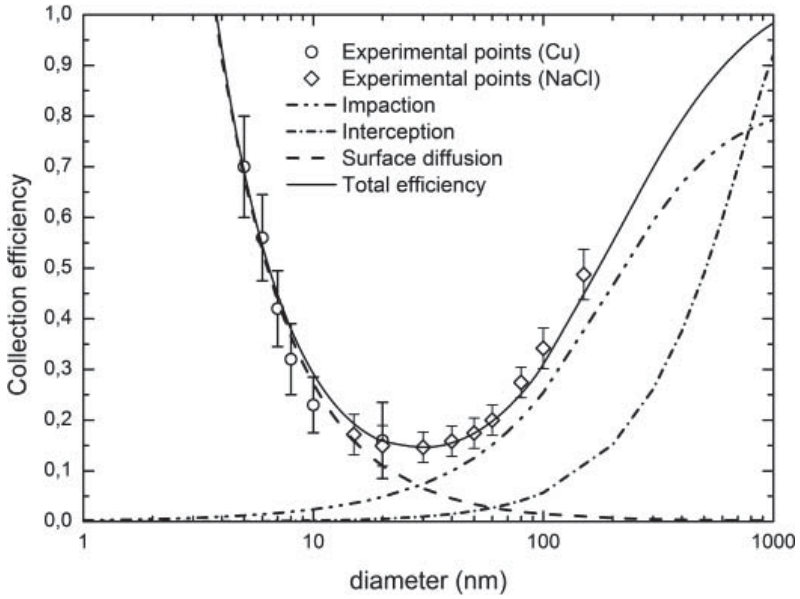

Figure 33. Comparison of experimental and theoretical approaches to assessing collection efficiency of the Quantifoil 1.2/1.3 TEM grid.

Efficiency versus air flow rate

Sampling conditions are different regarding the context. For instance, the average number concentration in a clean room is very low compared to the number concentration in combustion fumes. The challenge is thus to sample a sufficient number of particles on the grid, but not too much, so as to keep independent the collected particles (analysis by microscopy). As a consequence, larger volumes of air have to be sampled in for clean rooms with respect to combustion fumes.

Sampling time is also important. For instance, with a flow rate of 0.3 lpm, a minimum of 30 minutes could be considered in the first case (clean rooms) , while only a few seconds of sampling time would be necessary for the second case (combustion fumes). Sampling time is not the only way to adapt the MPS to the field conditions. The sampling flow can also be considered. An assessment of the impact of sampling flow set-up has been calculated (Figure 34). The efficiency curve moves horizontally, with a minimum sampling efficiency diameter of about 10 nm at 1.5 lpm to 50 nm at 0.1 lpm.

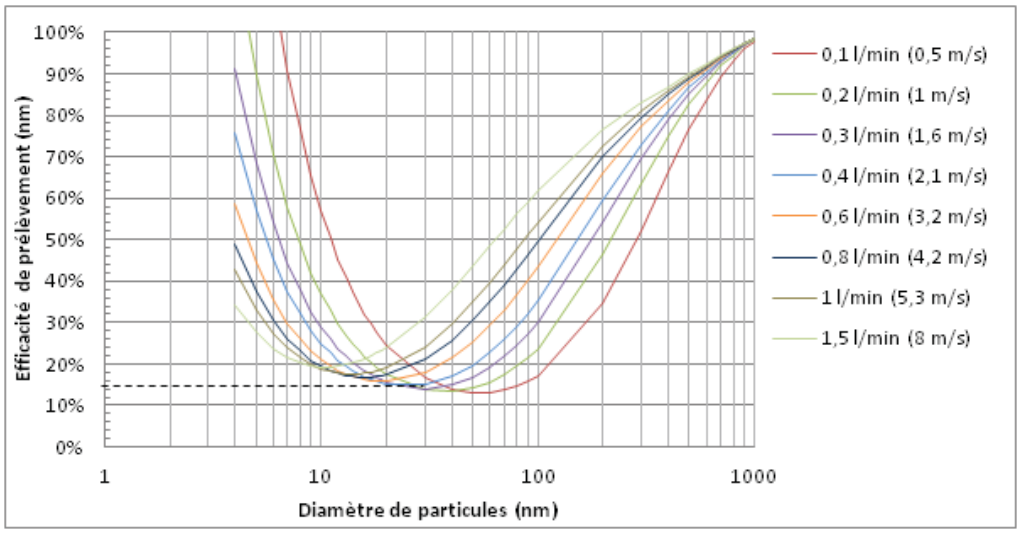

Figure 34. Numerical modeling of the sampling efficiency of a TEM grid «Quantifoil 1,2/1,3» for different flows.

Equipments

The MPS technique requires the use of various tools, listed below and presented in Figure 35 to Figure 41:

- ✓ MPS (Figure 28)
- ✓ Tube of diameter 1/4 inch
- ✓ Gilian Pump LFS 113 or equivalent (Figure 35)
- ✓ TEM grid (Figure 36)
- ✓ Sealing joints for TEM GRID (Figure 38)
- ✓ Balston small and blue filter (Figure 41)
- ✓ Pincer for the handling of the grids and joints (Figure 40)

Figure 35. Gilian Pump LFS 113.

Figure 36. TEM Grid box.

Figure 37. Joint box.

Figure 38. TEM grid.

Figure 39. Copper joint.

Figure 40. Pincer.

Figure 41. Small and blue Balston filter.

Here are some examples of products used at INERIS:

- ✓ Tube of diameter 1/4 inch
- ✓ TEM grid box Quantifoil (Figure 36) ref: S143-3 / Agar Scientific
- ✓ Sealing joint box for the grid (Figure 37) ref: G2620C / Agar Scientific
- ✓ Balston small and blue filter (Figure 41) ref: 922-05-BQ / EIF

Setup of experiments

Preparation of MPS

Cleaning

- Unscrew the two parts of the MPS and immerse them in a beaker containing ethanol.
- Plunge the beaker in an ultrasonic bath for 15 min.
- Let the two parts dry inside a hood for a night or dry using filtered compressed air.

In the absence of compressed air, if the cleaning is required onsite between two samplings, it is possible to remove the excess of ethanol by connecting the MPS to a pump and positioning the blue filter 'upstream' to avoid contamination (drying under filtered air flow), see Figure 42.

Figure 42. Drying without contamination.

Conserve the cleaned MPS in a sachet to keep it away from the dust and indicate its state (for example: Cleaned on 10/12/13 by LMe)

MPS loading with a TEM grid

The steps to follow to load the TEM grid in the MPS are:

1. Position the lower part of the MPS vertically (Figure 43)

2. Take out the TEM grid from its box by grasping an edge with a pincer and put it in the slot provided in the lower part of the MPS. The shining side must be above (Figure 44 and Figure 45).
3. Take the copper sealing joint and put it over the grid (Figure 47 and Figure 48)
4. Close back the MPS keeping the lower part fixed and slowly screwing the upper part in a clockwise direction. Plug the two ends (Figure 46).

Figure 43. Lower part of the MPS.

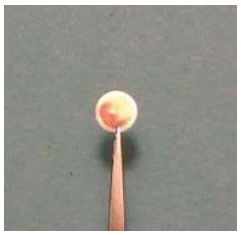

Figure 44. TEM grid shining side.

Figure 45. TEM grid dark side.

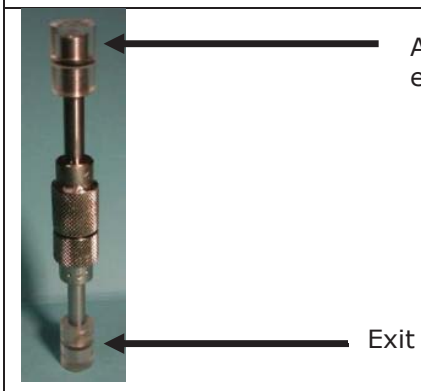

Figure 46. Closed MPS.

Figure 47. Sealing joint over the grid.

Figure 48. TEM grid in the slot.

Aerosol sampling

Flow rate verification and adjustment

Some flow verification has to be carried out prior to sampling:

1. Connect the prepared MPS (attention to the flow direction) to the pump LFS 113.
2. Connect a blue filter at the entry of the MPS
3. Connect then the flow meter and verify that the flow rate is 0.3 l/min.
4. Adjust if necessary (Figure 49)

Figure 49. Verification using TSI flowmeter.

Sampling operation

The steps to follow for aerosol sampling are:

1. Connect a short tube to the pump,
2. then the blue filter
3. and a long tube with the MPS at the end
4. Turn ON the pump

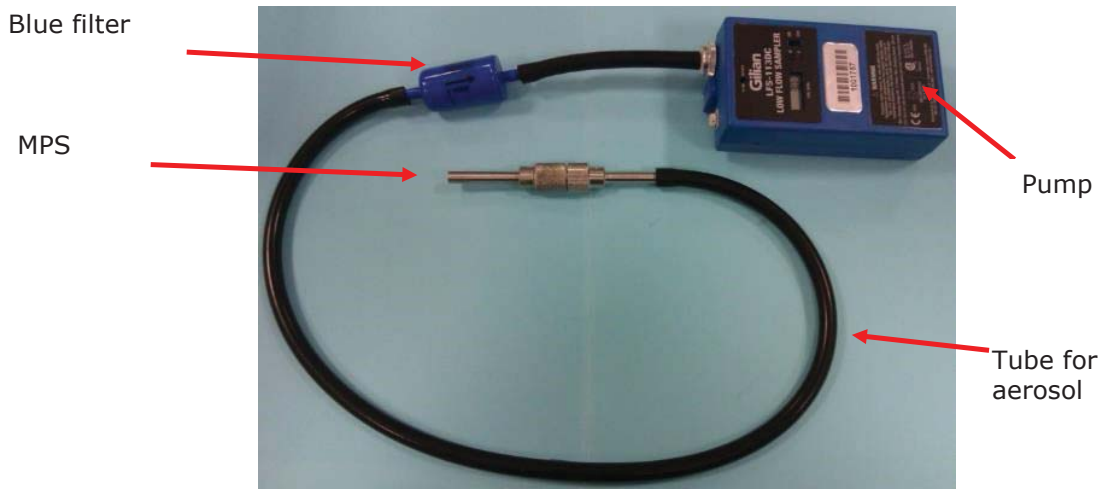

Figure 50. A complete system for the sampling.

The black tube, used especially for the aerosols, is not obligatory to use for the sampling.

Blue filter helps to protect the pump from dust.

Figure 51. Sampling over a person.

MPS verification

At the reception of a new MPS, it is necessary to proceed by cleaning, verify the compliance (leak proof, sampler aspect) and attributing a number before the use.

For the cleaning, proceed as suggested in 2.6.1.

To verify the absence of leakage,

- Equip the MPS with a grid and a copper sealing joint
- Connect a blue filter at the MPS entry
- Connect the entire system to the entry of the reference CPC 3375 whose flow rate is fixed at 0.3 l/min (Figure 52).

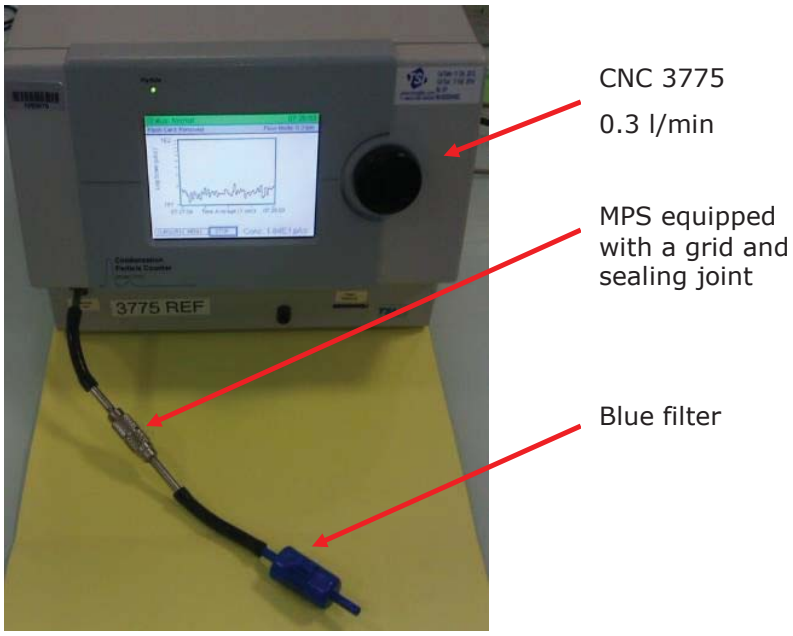

Figure 52. Leakage test.

Proceed to saving the data on the particle counting during a period of 2 min. This should remain lower than 1 pt/cm^3. If the number concentration is higher, then check the MPS if it is tightened enough and restart the counting. If the problem persists, there is a leak between the two parts of the MPS. It should not be used.

Sampling time

The sampling time is an important parameter to consider for providing good samples to the microscopist. It is recommended to each user of MPS to feed-back his own experience from his specific sampling conditions.

Such an approach is illustrated on Figure 53 optimized sampling time has been plotted regarding the related total number concentration provided by a CPC in the submicronic range. This feed-back is based mostly on studies conducted by INERIS at a flow rate of 0.3 lpm in various context such as measurement at the workplace (Bressot 2015), dustiness testing (Le Bihan 2014), mechanical solicitation of nanomaterials (Shandilya 2014-2015, Morgeneyer 2015), incineration of nanomaterials (Ounoughene 2015), etc.

As a result, the operator can assess the order of magnitude of the sampling time by using the total number concentration measured jointly in real time. For instance, a number concentration of about 10.000 p.cm^{-3} leads to a sampling time a bit larger than 300s (Figure 53).

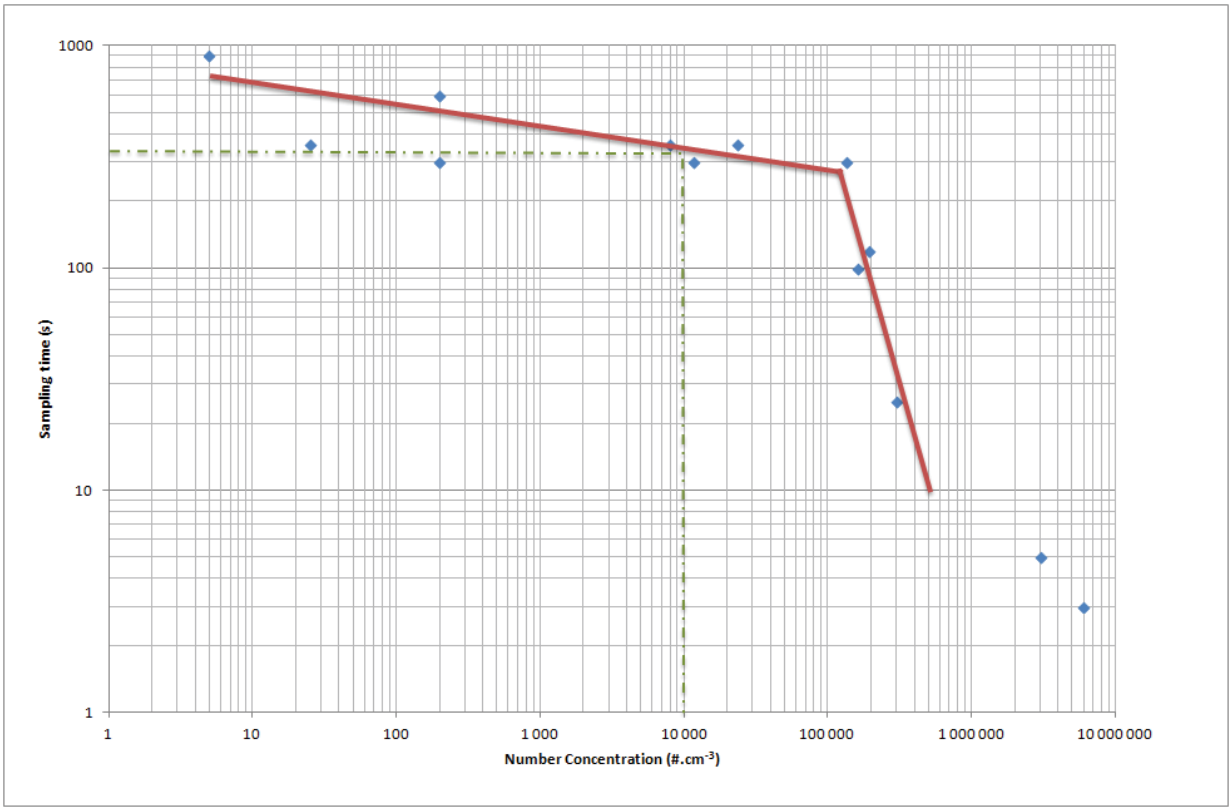

Figure 53. Example of the estimation of the sampling time.

Annex 2: LIBS Standard Operating Procedure

Protocol of use of LIBS to detect nano-objects their aggregates and their agglomerates

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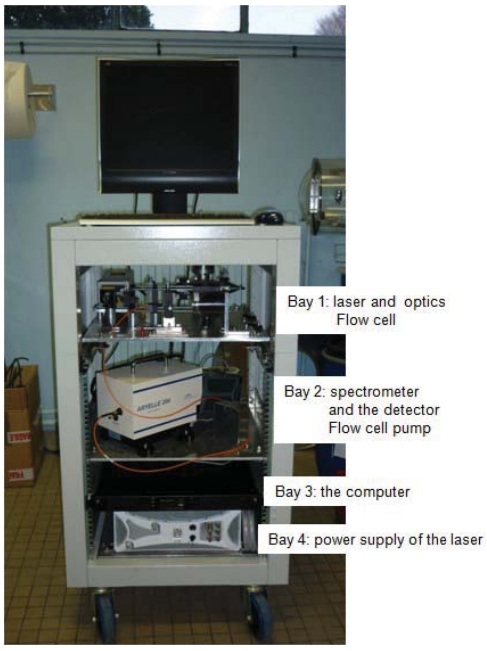
Aim(s) or Objective(s) of SOP
Having presented the context and the advantages of LIBS over other techniques, this section is intended to explain the necessary requirements to make the INERIS LIBS system work. Thus, all the components constituting the LIBS analyzer are presented and described. The procedure to start the system and make the alignment is described as well. Quantitative LIBS measurements require a calibrating step and post data treatment. Calibrating LIBS requires the use of aerosols generator of ultrafine particles and device which can measure mass concentration.

A first scheme of this protocol of implementation of LIBS is described below.

Scope and required operating conditions

Description of the INERIS LIBS system

The components of the LIBS system are embedded in a 19 inch rack with four bays with the following arrangement (see Figure 54):



- Bay 1: the optics (laser focusing and plasma light collection) and the flow cell
- Bay 2: the spectrometer, the ICCD and a pump
- Bay 3: a computer
- Bay 4: the power supply of the laser

All the components are connected to one another by extension cords. A diagram displaying all the connections is presented in Figure 55. The circuits of the cooling fluid and that of light following its collection are presented on Figure 56.

Figure 54. The INERIS LIBS instrument

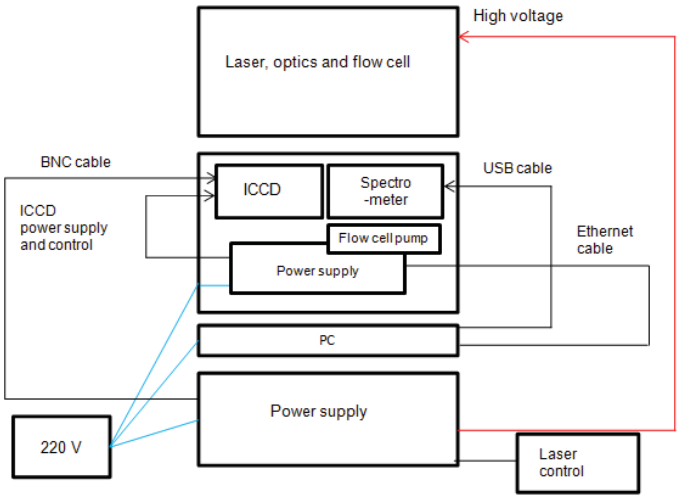


Figure 55. Electric circuit of the LIBS system

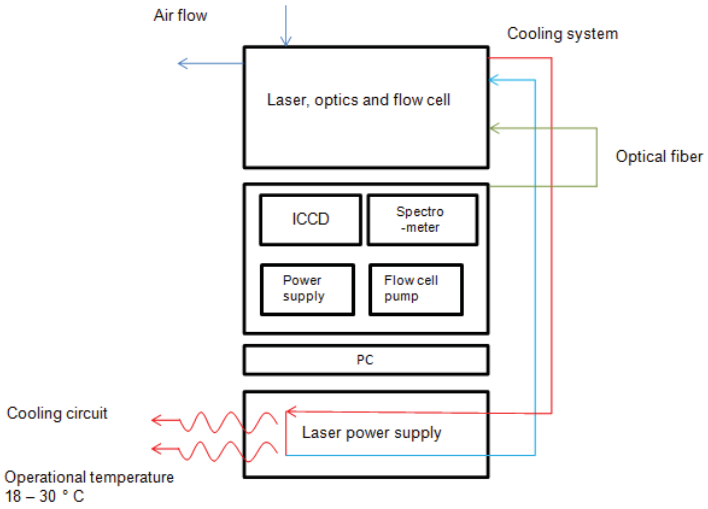


Figure 56. Optical path and cooling fluid circuit

Materials and equipment needed

List of the components

The LIBS system comprises:

- The laser: NdYag Q-switched laser Brio model (Manufacturer Quantel). Maximum laser energy 100 mJ, pulse duration 5 ns and a wavelength of 1064 nm
- The spectrometer: Echelle spectrometer (Manufacturer LTB) wavelength range 200-600 nm, resolution $\Delta\lambda / \lambda = 0.05$
- The ICCD camera: Pimax 3-1024i-SB-SG-P43 model (Manufacturer PRINCETON)

Turning the instrument on

The procedure to turn the LIBS system on is described step by step below

Step 1 The laser

A key situated on the power supply of the laser is to be turned. The pump of the cooling system is then turned on as well as the control panel. There is then a warming up time of ten minutes before switching on the laser itself.
The laser frequency is set to 20 Hz by the manufacturer. It is not to be altered. The energy must be at its maximum that is to say 100 mJ (QS-FI delay = 140 µs on the control panel).
More details as to the laser settings may be found in the laser manual.

Step 2 The ICCD, the spectrometer and the computer

The power supply of the ICCD camera is to be switched on before the computer is turned on. The switch button is located on the back panel of the power supply. The ICCD camera and the spectrometer must be connected to the computer using two cables

- An Ethernet cable connecting the ICCD camera and the computer
- A USB cable to control the spectrometer

A BNC cable links the laser and the camera to synchronize signal recording with the laser shots. It is to link the “Q-switch out” of the laser to the “external trigger” of the ICCD camera. The optical fiber must be connected to the spectrometer. The computer is then to be turned on.
The Software “Sophi” controlling the spectrometer is to be launched.

Step 3: The pump of the flow cell

The pump allowing the aerosol to flow through the flow cell must be switched on. One must check that a filter has been positioned upstream the pump to protect it.
The flowrate within the flow cell is to be set with values ranging from 1 to 10 lpm. It is adjusted with a valve positioned upstream the pump.

Optimization of the LIBS signal

The optimization of the LIBS signal rests on three main parameters:

- Laser focusing to achieve an intense, stable plasma
- Plasma light collection
- Temporal parameters: time delays and integration time

Laser focusing and plasma light collection are advanced settings requiring personnel trained to operate class IV lasers (class IV referring to the most dangerous lasers). They are to be performed once when setting up lenses and mirrors on the optical bench. Laser focusing and plasma light collection optical paths are presented in Figure 57.

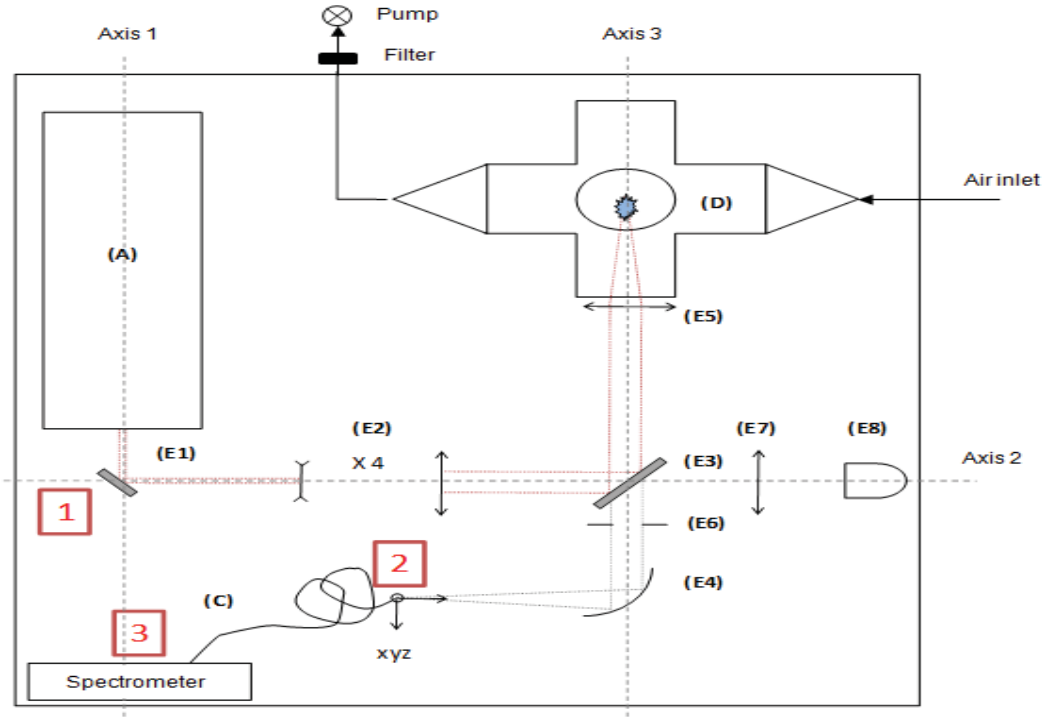


Figure 57. Adjustments of the optics

Alignment of the optics

The E1 mirror is centered on axis 1 and 2, the latter two being perpendicular to each other. The axis normal to the E1 mirror makes a 45° angle with axis 1 and axis 2. The angle of the mirror may be set using adjustment screws.

The E2 telescope is made of two lenses (divergent and convergent lenses with focusing lengths of -25 mm and 100 mm respectively). Their centers are positioned on the axis 1. The distance between these two lenses is to be set with accuracy. To this end, a lens with a 500 mm focal length (E7) is placed behind the dichroic mirror (E3). A camera is positioned at the focal point of the 500 mm focal length lens (E7). The optimization of the telescope is achieved by minimizing the diameter of the focal spot imaged with the camera.

The dichroic mirror (E3) is centered on axis 2 and 3, the latter two being perpendicular to each other.

The axis of the focusing lens (E5) is aligned with that of the laser. The focusing lens position is set so as to ignite the plasma in the center of the flow cell.

The laser-induced plasma is ignited within the flow cell (D). The axis (axis 3) centered on and perpendicular to the flow cell windows is to be aligned with that of the laser beam.

A diaphragm (E6) is to be positioned on the optical bench. Its axis is to be aligned with that of the laser according to axis 3.

An ellipsoidal mirror (E4) allows collecting the plasma light and focusing it on the entrance of the optical fiber put on XYZ translation plates. The optical axis of the mirror makes an angle of 45° with axis 3.

Eventually, the core of the optical fiber (c) is positioned right at the ellipsoidal mirror focal point.

Once all these settings are made, refinements may be made gaining access to optical elements indicated by the numbers 1, 2 and 3 on the drawing.

Refining the adjustments

Refinement 1: focusing the laser beam in the cell

The focusing of the laser pulses is to be optimized with the adjustment screws of the E1 mirror. These allow changing the angle between the axis of the laser beam and that of the focusing lens. The focusing is optimized when the laser beam passes through the centers of the telescope, of the dichroic mirror, of the focusing lens and of the flow cell window.

Refinement 2: focusing the plasma light onto the fiber entrance

In order to optimize plasma light collection, the optical fiber is placed on XYZ translation stages for precise positioning along all these axes.

Refinement setting 3: temporal settings

The laser-induced plasma has a transient nature. It is not a continuous plasma discharge neither in space nor in time. The plasma lifetime is of about a few tens of microseconds and its dimensions of around a few millimeters at full expansion. This has a consequence on the detection. In order to get the best signal-to-noise ratio when recording LIBS spectra, one must use in most cases detectors (such as Intensified CCD cameras) with time resolution of a few nanoseconds. This is the consequence of the properties of the laser induced plasma. In the early times of its ignition, it is far too luminous for spectra to be recorded. The lines of analytical interest are not visible, masked by a strong continuum radiation.

Thus, spectra are recorded with a precise time delay and integration time or gate width. The time delay represents the duration elapsed between laser firing and the beginning of plasma spectrum recording. The integration time refers to the duration of the spectrum recording.

Once all these settings are done, a protective housing is positioned to protect the optics. Thence, only the XYZ translation stages remain accessible to the operator in the course of the LIBS experiments.

Table 7 presents the temporal settings optimizing the recording of lines of a given element. These results are valid for the LIBS system described in this document (laser wavelength, energy and pulse duration of 1064 nm, 100 mJ and 5 ns respectively). The detection limits displayed in the table were assessed from spectra recorded by accumulating 200 laser pulses with a gain camera of 150 (The gain of the ICCD camera represents the signal amplification to be applied depending on the mass concentration of the probed element ("the lower the concentration, the higher the gain" is the basic principle to keep in mind when it comes to the gain).

Table 7. Line wavelength, ionization degree, time delay, integration time and the corresponding limit of detection (LoD)

Atomic species	Emission line (nm)	Ionization degree	Time delay (µs)	Integration time (µs) (Gate width)	LoD (µg/m^3)
Al	396.15	I	20	50	300
Ag	328.06	I	20	50	41
Ca	393.36	II	15	50	7
Ce	417.8	I	15	50	350
Cu	324.16	I	25	50	40

C	247,85	I	3	10	
Cd	508.12	I	15	50	1200
Mg	279.55	II	7	50	30
Fe	440.47	I	15	50	<200
Zn	481.75	I	15	50	300

Calibration

The LIBS system necessitates to be calibrated. The calibration bench consists of (see Figure 58):

- An aerosol generator to generate ultrafine particles
- A TEOM (Tapered Element Oscillating Microbalance) allowing determining the mass concentration of a given element or an enriched filter to be subsequently analyzed by ICP-AES
- The LIBS system described in this document

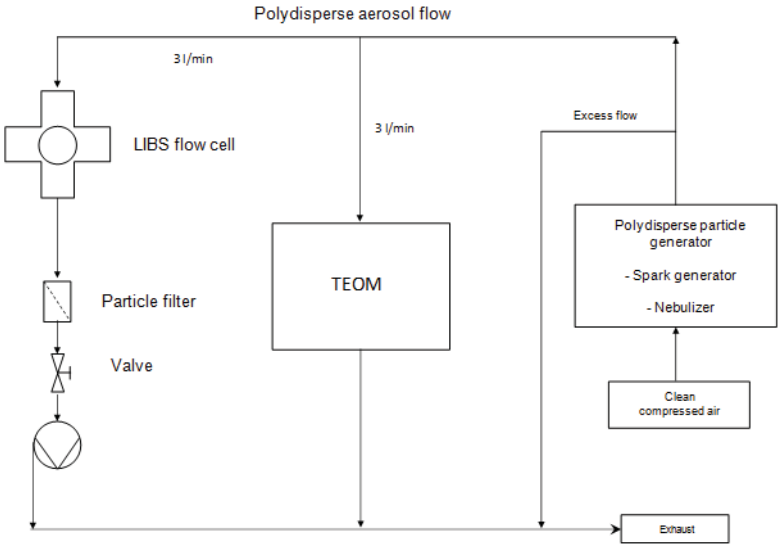


Figure 58. Experimental setup for calibration.

The principle of the calibration is as follows. Aerosol of a given element is generated with different mass concentrations. These are then related to the recorded spectra. The LIBS signal is therefore expressed as a function of mass concentrations.

The aerosol generator

The aerosol generator in use at INERIS is a TSI 3076 nebulizer. It generates micrometric and submicrometric sized particles using solutions of a given chemical element. These particles may be in solid or liquid state. The size distribution may be adjusted by modifying the solution concentration. The principle of this generator is presented in Figure 59.

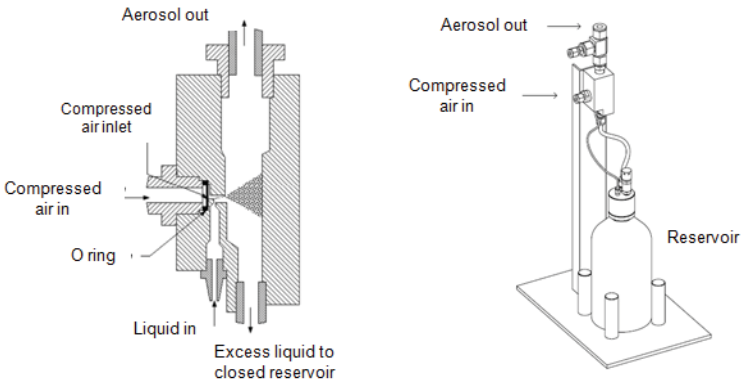


Figure 59. Particle nebulizer (displayed on the left) showing how the nebulization is achieved (right).

Using the atomizer, material intended to be generated in aerosol form is dissolved in water (for instance sodium or potassium chloride) and poured into a 1-liter bottle. Two tubes connect the solution contained within the atomizer to the bottle cap: the first from which the solution is drawn from the bottle and the second evacuating excess liquid. Aerosol flow is yielded subsequently to the interaction between a high-velocity clean air jet colliding perpendicularly with the solution being drawn. The aerosol is then flowed through a dryer before entering the LIBS flow cell. Such system produces particles with sizes ranging from a few nanometers to 1 µm with a possibility to adjust granulometry by varying the solution concentration.

Thus, the produced aerosols have a composition identical to that of the compound dissolved in a solution. The mass concentration of the aerosol (µg/m^3) is proportional to the concentration of the dissolved solution (g/l).

The TEOM

The TEOM allows measuring mass concentrations of particles suspended in the air. The mass concentration value indicated by this instrument corresponds to an average value calculated over 120 seconds each two second. Pressure variations at the inlet end of this instrument are a real hindrance to its functioning. Indeed, it is ideally operated at ambient air measurements for which it has been designed. As a consequence, one is to pay attention to evacuate the excess air flow so as to avoid malfunction of the TEOM. Overpressuring it may lead to overestimating the mass concentration. It should be noted that this instrument may also be sensitive to vibrations. It should not be positioned right onto the floor.

Data recording

This section aims at indicating the procedures and settings to be applied when analyzing particle in the air and recording the LIBS signal or line intensities using the SOPHI software. This software designed by LTB (Laser Technik Berlin) allows acquiring LIBS spectra by controlling the Aryelle spectrometer and the PIMAX3 (Princeton) ICCD camera. When analyzing particles using LIBS, two methods may be used:

- Conditional analysis
- Ensemble averaging

These two recording modes are presented below.

Ensemble averaging and conditional analysis

When analyzing a solid or scanning a filter each laser shot interacts with the analyte. Under these conditions, LIBS recordings usually consist of systematically adding the signal originating from all laser shots in one final

single resulting spectrum. These accumulations allow obtaining satisfying signal-to-background ratio. This corresponds to ensemble averaging.

The laser-induced plasma has a transient nature. In addition, the discrete nature of the particles and the stochastic character of LIBS analysis may lead to the use of an alternative recording method, depending on particle size and mass concentration. A laser-induced spark does not systematically sample a particle leading inevitably to signal absence in the spectrum. Thus, there is a necessity to record individual spectra corresponding to one unique laser shot, and therefore one unique laser-induced plasma spark. The ratio of the number of spectra having sampled one particle (spectra therefore considered as positive) to that of the total amount of laser shots fired yields the hit rate which when expressed as a percentage denominates as the particle sampling rate (PSR). Spectra free of signal are discarded prior to the addition leading to the final spectrum. The latter is obtained by adding the intensities of all the positive spectra and multiplying the resulting sum by the hit rate. This method is termed conditional analysis.
Thus depending on particle size and mass concentration, these two recording modes may be used. The conditional analysis is resorted to when the PSR is found beneath 20 %. Ensemble averaging is utilized otherwise.
As indicated above, the Sophi software allows adjusting the parameters of spectrum detection and recording spectra resorting to conditional analysis or ensemble averaging.

Launching the Sophi software

The ICCD camera is to be turned on

The computer is to be turned on which automatically leads to the launching of the software. If not, one should double click on the Sophi icon. A green light (lower left-hand corner) is then on when the connection between the software and the camera is made. The light turns red otherwise.

Recording of spectra using either conditional analysis or ensemble averaging

As explained above, conditional analysis consists in recording one spectrum corresponding to one unique laser shot. This recording mode is retained when the probability to detect the element of interest is low, that is to say when the particle sampling rate is below 20%. The options to tick and values to key in are indicated in Table 8.

Table 8. Parameters of the detector.

Measure	The “continuous” option is to be ticked Clicking on the green button starts the acquisition
Type of measurement	Select “custom experiment” The “LIBS experiment” mode is not to be used
Dark correction	Tick the two options
General Settings	Averaging = 1 Shift speed corresponds to the readout speed of the CCD matrix. The higher

	the readout speed, the faster the recording of the spectra. Trigger = external
Shutter settings	Select “keep shutter open while measuring”
ICCD Settings	Gate mode : internal DDG Gain = 100 Gate delay and gate width: they depend on the analyzed atomic species (see Table 7)
Data Handling	Tick the “remove order crossover” option

“Averaging” in general settings is a key parameter to set either conditional analysis or ensemble averaging. “Averaging = 1” does correspond to conditional analysis whereas “averaging > 1 has to do with ensemble averaging. The Sophi window presents itself as shown in Figure 60.

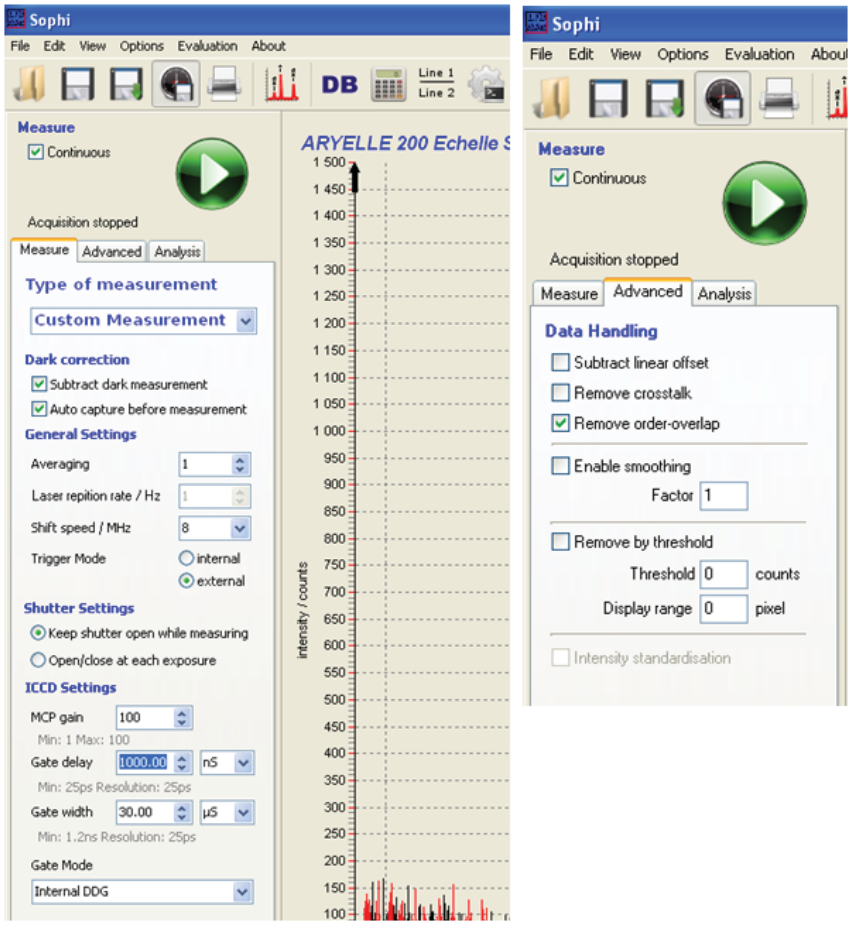


Figure 60. The Sophi software.

How to save the recorded files

The option menu is to be chosen to save files. Parameters are indicated in Table 9.. The options related to file saving are illustrated in Figure 61.

Table 9. Parameters for saving recorded files.

saving	Folder (D:\DATA\ ….) choose **.esf* (ASCII file like). The **.Ary* file format cannot be used with Excel. Select « *save every single spectrum* » to save all the spectra.
adv. CCD Settings	Binning : 1X1 ou 2X2. Allows resorting to megapixel thus increasing readout time when acquiring spectra

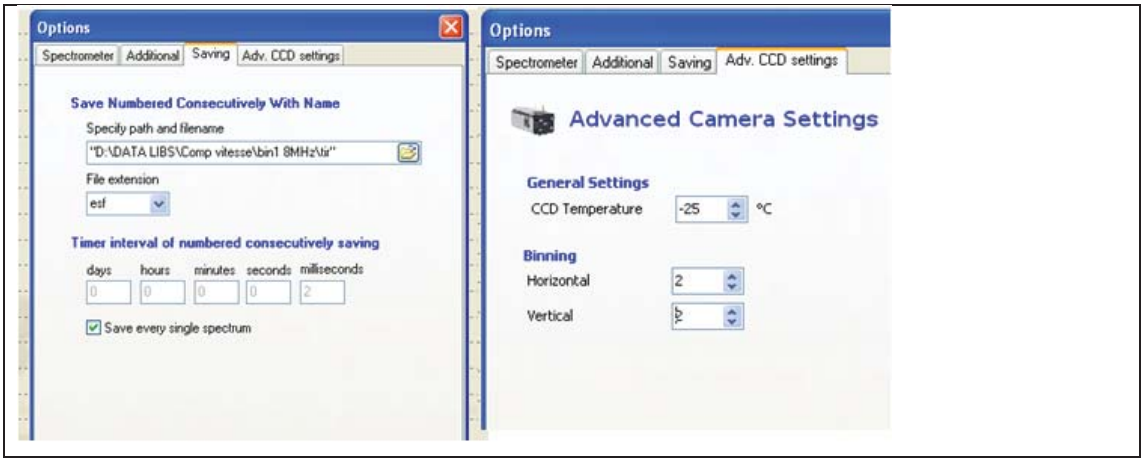


Figure 61. Illustration of the Sophi windows displaying parameters to key in to optimize file recording.

Data acquisition speed

Here are the steps when recording LIBS signal:

- The laser ignites a plasma and triggers the ICCD camera
- The ICCD camera captures plasma light emission with time delay and integration time keyed in by the user
- The CCD matrix is “emptied” and the obtained spectrum displayed on the computer screen
- The obtained spectrum is to be saved on the computer prior to the next laser shot

Data acquisition speed is a function of several parameters:

- The laser frequency
- The ICCD readout time
- The time needed to save a spectrum

Table 10. recapitulates the obtained acquisition frequency as a function of binning and CCD readout frequency.

Table 10. Acquisition frequency taking into account binning and CCD readout frequency.

CCD READOUT FREQUENCY (MHZ) / SHIFT SPEED (MHZ)	2		8		16	
ACQUISITION FREQUENCY	3	**9.8**	6	**15**	6	15
FILE SIZE *.ESF (KB)	1350	686	1350	686	1350	686

For conditional analysis to apply, that is to say recording of individual spectra corresponding to unique laser shots with a frequency of about 10 Hz, spectrum file size are to be limited resorting to a 2x2 binning.

Settings for field experiments

The settings to be used when carrying out field experiments are indicated in Table 11.

Table 11. Settings for field experiments.

ICCD PARAMETERS		
Readout frequency	2 MHz	
Acquisition frequency	9.8 Hz	
RECORDED FILES		
File size *.esf (KB)	686 KB	
1 minute	600 spectra	400 MB
10 minutes	6000 spectra	4 GB
16-17 minutes	10 000 spectra	6.86 GB
1 hour	36 000 spectra	24 GB

Annex 3: NANOBADGE sampler Standard Operating Procedure

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Aim(s) or Objective(s) of SOP
The scope of this standard operating procedure is the procedure for running sampling using the NB2013 aerosol sampler (NANOBADGE V3). This SOP includes the routine instrumentation maintenance.

Scope and required operating conditions

The operating principle of the NB2013 device is based on the sampling of the respirable particle size fraction using an impactor (d50 at 4 µm, as defined by the ISO7708 document) followed by the collection of the particles on a filter. The NB2013 sampler can be used for stationary measurements or personal sampling in the breathing zone, powered by an internal battery allowing up to 8 hours of continuous operation. The filters are analyzed offline using the state-of-the-art techniques listed in the nanoIndEx project Document of Work, namely SEM, ICP-MS or TXRF.

Materials and equipment needed

The main components of the NB2013 aerosol sampler are:

- NB2013 sampler.
- Filter units (single use) in individual zip bags. The unit is a sealed cassette containing a polycarbonate track-etched membrane to collect particles and equipped with a RFID chip to store data (sampling time, date, flow rate, errors, worker ID, sample ID, …).
- Impactor (the sampler can be operated with or without impactor).
- ID badge (personal use, one for each person operating the sampler).
- USB cable and power supply unit.

Setup of experiments

Set up the NB2013 sampler according to the following instructions.

- The NB2013 sampler is equipped with a rechargeable battery (lifetime up to 16 hours). It can be charged using the provided USB charger. Both lights will blink when the sampler is connected to a power source and the battery is charging. The battery is fully loaded when the lights go out. The sampling will not start unless the charge remaining in the battery is sufficient for 8 hours of operation.
- Before starting the sampler, remove the plugs on the filter unit and connect it to the sampler by pushing it down onto the white inlet barb located at the top of the device. The filter must be pushed all the way to ensure that it sits tight or the sampler will not start. Keep the plugs and the zip bag of the filter unit as you will need them to seal the exposed filter unit after sampling. Connect the impactor on top of the filter unit if necessary.
- To turn on the sampler, press the button on the front of the device while holding the ID badge against the marked area on the back of the sampler until both lights on the device turn green. A faint sound comes from the pump of the sampler when it is running properly.
- To stop the sampler, hold the button for a few seconds until the lights go out. The sampling time is automatically logged.

Zero check

Starting the sampler initiates a self-test routine. The device emits an alarm sound and shuts itself down if an issue is detected. The color of the LEDs indicates what kind of failure was diagnosed.

Left light (closest to the filter)	Right light (furthest from the filter)	Action required
Red	Red	Insufficient battery charge. Connect the sampler to a power source.
Green	Red	Sampling issue. Check that the filter unit sits tight on the sampler. If it does not solve the problem, try another filter unit.
Green	Green	The device is operating correctly.

Sampling

Once it has been turned on, the NB2013 device can be placed near a potential particle emission source, worn by a worker near the breathing zone in a chest pocket, or kept far from the release zone for background measurements. The sampler will go out when the battery is exhausted, if it is manually switched off, or in case of sampling failure (e.g. leak in the filter unit or clogging of the collection filter). The sampling time is automatically logged in all cases. At the end of the sampling, remove the impactor and filter unit. Seal the filter unit with the plugs and keep in a closed zip bag for offline analysis.

Measurement data

The filters are analyzed offline at CEA premises. The measurement parameters (user ID, filter ID and sampling time) are contained in the filter unit which is sent for analysis. It should not be opened by the user and kept sealed in a dry and clean environment prior to analysis.

Maintenance

There is no maintenance required on the user side, apart from loading the battery and keeping track of the filter units. If the outside of the instrument is contaminated with dust, it can be cleaned using wet wipes. If the interior is contaminated it has to be shipped in a sealed zip bag to the manufacturer for a proper decontamination.

Quality Control

Measures for quality assurance

- The NB2013 sampler runs a self-test at the beginning of the sampling and periodically during operation. The tests include the battery state and the pressure (detection of leaks or clogging of the filter unit).
- For each run, record the position of the sampler, the ID of the filter unit, the temperature and the relative humidity in a logbook. Keep a manual record of the sampling time to cross-check with the built-in time-logging system.
- Report anomalies during sampling and maintenance.
- Keep the filter units sealed in a dry and clean environment before and after sampling.

Annex 4: Procedure of airborne particle measurements with the Electrical Low Pressure Impactor (ELPI)

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Aim(s) or Objective(s) of SOP
The scope of this standard operation procedure (SOP) is the procedure for setting up and for making a measurement with the Electrical Low Pressure Impactor (Classical ELPI®, Dekati). In addition, this SOP describes the data treatment. It also encompasses the routine instrumentation maintenance. This SOP does not include the establishment of a sampling station that would include the Classical ELPI® instrument.

1. Scope and required operating conditions

The scope of this standard operation procedure (SOP) is the procedure for setting up and for making a measurement with the Electrical Low Pressure Impactor (ELPI®, Dekati). In addition, this SOP describes the data treatment. It also encompasses the routine instrumentation maintenance. This SOP does not include the establishment of a sampling station that would include the ELPI® instrument.

2. Notes

In an objective of harmonization and to help the user, the structure of this SOP follows the structures of the Standard Operation Procedures for assessing exposure to nanomaterials, following a tiered approach, that have been developed within the project “nanoGEM – Nanostructured Materials – Health, Exposure and Material Characteristics” [1].

This SOP relates to the Electrical Low Pressure Instrument designated as Classical ELPI® by the manufacturer. A new model of the Classic ELPI®, the ELPI®+ has been commercialized recently [2] and the Classic (original) ELPI® is no longer available. However, distributed since 1995 [3], the Classical ELPI® has become a widely used instrument by several research laboratories and companies around the world for many applications, including combustion aerosol and engine exhaust studies, pharmaceutical inhaler development, air quality measurements and general aerosol research. Its use for workplace measurements where nanomaterials are handled is less developed, probably due in part to its size, weight and cost.

The Classical ELPI® differs from the ELPI®+ by a certain number of points that make this SOP not applicable directly to the ELPI®+. Thus, it is envisaged in the near future to develop an adapted version of the SOP for the ELPI®+ and for its more recent development like the High Resolution ELPI®+ (HR-ELPI®+).

Finally, this document has been prepared on the basis of scientific literature, experience feedback from the various experimental studies performed at the Aerosol Metrology Laboratory from INRS, and the manuals listed in the "References" section [4, 5], including the notes and hints. It is strongly recommended to users of this SOP to refer to these manuals, ensuring that they have the very latest versions.

In any case, INRS would not be responsible for misuse of this SOP or that could result from any error that may remain in the document.

3. Basics

The Classical ELPI® belongs to the category of the so-called time-resolved size-resolved instruments [6]. As for all other instruments of this type (Scanning Mobility Particle Sizer, Fast Mobility Particle Sizer, Aerodynamic Particle Sizer etc.) a major drawback of the Classical ELPI® is its lack of differentiation of background airborne particles from nanomaterial related airborne particles. However, an advantage of the

Classical ELPI® compared to the other instruments belonging to the same category is that offline physical/chemical analysis can be further conducted on collected particles in several size fractions individually (see section 4.9).

3.1. Instrument description

The Classical ELPI® combines electrical detection with a conventional low pressure cascade impactor to measure airborne particle size distribution and concentration in real-time over the particle size range of 0.03 - 10 µm with 12 channels. With the filter stage configuration, the size range can be extended down to 7 nm.

As shown in Figure 62, the main components of the Classical ELPI® are a Corona charger, a low-pressure cascade impactor and a multichannel electrometer. Moreover, an external vacuum pump is used to ensure the inlet flowrate of 10 L/min. Finally, a personal computer or a laptop is needed to run the ELPIvi™ measurement software [5]. This software controls the Classical ELPI® instrument by sending commands to the unit internal computer via serial port; it also serves for data collection and logging.

In the Classical ELPI®, as shown in Figure 62, airborne particles are first sampled through an inlet at a flowrate of 10 L/min. Then, sampled airborne particles pass through a unipolar positive Corona charger where they are charged electrically by the small ions produced according to their mobility-equivalent diameter. After the charger, the electrically-charged airborne particles are classified (by inertia) according to their aerodynamic equivalent diameter in a low-pressure cascade impactor. This impactor is made of 12 insulated impaction stages. Each of them is connected via a contact needle to a multichannel sensitive electrometer (noise level ≈ 1 fA).

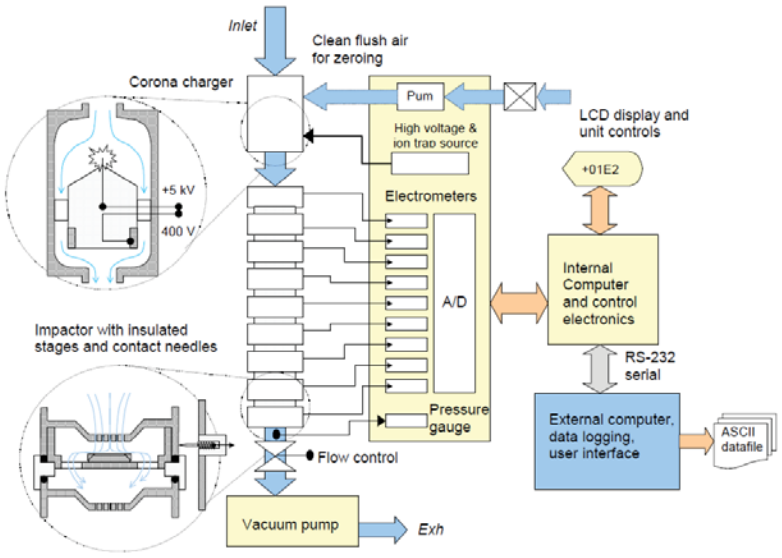

Figure 62. Schematic diagram of the operation of the Classical ELPI® [4].

When the particles are deposited onto the insulated impaction stages (based on their aerodynamic equivalent diameter), an electrical current is generated and measured. The number concentration in each channel can then be calculated by applying a charging law (or charger efficiency curve) dependent on mobility-equivalent diameter, itself related to the aerodynamic diameter as described in the section 5.3.3.

Because of its high time resolution (1-5 s), the Classical ELPI® is an ideal aerosol measurement instrument for the analysis of unstable concentrations and size distributions, or the evolution of airborne particle size distributions.

Figure 63. general view of the interior of the Classical ELPI® [4].

3.2. Specifications

Table 12 below provides the main specifications of the Classical ELPi® used to develop the present SOP.

Table 12: Main specifications of the Classical ELPI® [4].

Specification	Value
Nominal air flow	10 L/min
Particle size range	0.03 – 10 µm with filter stage 0.007 – 10 µm
Number of electrical channels	12
Time resolution	1 – 5 s
Operation temperature conditions	5 – 40 °C
Operation humidity conditions	0 – 90 % RH, non-condensing
Weight	35 kg
Dimensions	H560 x W400 x D250 (mm)
Inlet / outlet connections	R 3/8 and R 3/8 (NW16 flange)
Charger voltage / current	5 kV / 1 µA
Pump specification	minimum 21 m^3/h at 100 mbar
Pressure under the lower stage	100 mbar

Table 13 below presents/gathers the nominal specifications of impactor of the Classical ELPi®. For exact values, the user should refer to the signed calibration data sheet provided by the manufacturer.

Table 13: Cut-off and geometric mean nominal diameters of the Classical ELPi®.

Stage	Aerodynamic equivalent diameter	
	Cut-off $d_{a,50}$ (µm)	Geometric mean $d_{a,i}$ (µm)
13	9.970	
12	6.800	8.4
11	4.400	5.3
10	2.500	3.2
9	1.600	2.0
8	1.000	1.3
7	0.650	0.810
6	0.400	0.510
5	0.260	0.330
4	0.170	0.210
3	0.108	0.140
2	0.060	0.081
1	0.030	0.042

4. Making a measurement

This chapter describes the different steps in order to make a measurement with the Classical ELPI®.

4.1. Cleaning and assembling

Each airborne particle that is sampled by the Classical ELPI® during a measurement phase is deposited somewhere within the instrument according to different deposition mechanisms. While the primary deposition mechanism onto the collection substrates within the impactor column is inertia, there are secondary collection mechanisms (diffusion, image force and space charge) that are responsible for a diffuse particle deposition onto the inner surfaces of the Classical ELPI® (within the Corona charger, the impactor column). With time, these deposited particles may modify the charger efficiency. The inter-stage loss of particles may also modify the cutoff diameters of the impactor and alter the insulating properties of the insulator parts. Therefore, before any measurement phase a thorough cleaning of the charger and all parts of the impactor should be conducted.

The cleaning operations demands careful dismantling and assembly should be performed according to the section 6 of the Classical ELPI® manual [4]. A special attention should be focused on the cleanness of the impactor nozzles. Indeed, no particle should clog these nozzles and these should be kept open at all time. Also, a very tricky part is the cleaning of the charger; it requires some dexterity. If the corona needle is damaged during the cleaning, it should be replaced.

The cleaning protocol should use an ultrasonic bath and suitable solvents like distilled water, isopropanol, ethanol, and some dish washing detergents for few parts. The Teflon parts should be washed with isopropanol or distilled water only. It is recommended to use for wiping premium optical cleaning tissues (pure Cotton, non-Woven, low Lint). Optionally, drying can be achieved by means of a laboratory oven (max temperature of about 50 °C).

If cleaning is not satisfactory, pressurized air can also be used to clean or dry the impactor nozzles. However, this should only be performed under laboratory fume.

NB: After each measurement, inspection of various parts of the impactor must be performed. If clogging of the nozzles is noted, the measurement (or measurement series) should not be accepted.

4.2. Impaction plates and collection substrates

The Classical ELPI® can be used with two type of impaction plates: regular or sintered plates.

It is recommended to use collection substrates onto the regular impaction plates, even if samples are not collected for subsequent analysis. The collection substrates should be absolutely smooth and can be made from example of aluminium foil or polycarbonatemembrane. Due to the high air velocity jets within the impactor, particle bounce occur in the ELPI. Therefore, greasing of the collection substrates is absolutely necessary. Due to its purity and low evaporation properties, Apiezon-L grease is recommended. There are two options for greasing (brushing or spraying) but in any cases the layers should be thin (≈ 10 µm) and smooth on all substrates. See section 4.2 of the Classical ELPi® manual [4].

Sintered impaction plates are provided as an accessory for ELPI. The sintered collection plates have been specially designed to prevent impactor overloading and particle bounce. Instead of collecting the particles on smooth foil, the collection area is sintered and porous oiled metal. When the particles are collected on the plates the oils seeps up due to capillary forces and thus the impaction is always a particle to oil collision. The sintered collection plate allows 10–20 times more collected material on a plate compared to a regular plate depending on the type of particles. However, chemical analysis or gravimetric measurements cannot be made if sintered collection plates are used. It is highly recommended to use the sintered impaction plates when measuring high concentrations or for widely polydisperse aerosols or when the measurement time is important.

The greasing operation of the sintered collection plates is somewhat different of the one for the regular collection plates. The manufacturer recommend to use the vacuum oil that is used for the vacuum pump of the ELPI. An estimate of the amount of oil needed on each plate is 10–100 µl, which correspond to approximately 1-2 droplets.

The exact amount of oil or grease is a difficult point to assess. Too much oil or grease will cause fouling of the other impactor parts, and possible oil transfer in the impactor. Too few will cause bounces of particles on the lower impaction stages and it will result in changing the size distribution to smaller diameters. It is therefore strongly recommended to perform preliminary tests to optimize the greasing step. It is possible that the amount of oil is not uniform on all impaction stages (because of the increasingly high air velocity in the impactor towards the lower impaction stages).

4.3. Flowrate check

The flowrate check should be conducted after every time the impactor is assembled and before each measurement phase. A different value than the exact value indicated in the signed calibration data sheet given by the manufacturer indicates that the cleaning is not well done (impactor nozzles are clogged), or there is a leak within the instrument due to an improper assembly or the metering valve to get a reading of 100 mbar (±5 mbar range acceptable) is not well adjusted.

The flow rate can be checked using e.g. laminar flow element or mass flow meter. The result of the measurement should be compared to the flowrate given in the signed calibration data sheet (make sure that the comparison is performed with the correct inlet and outlet pressure of the impactor and temperature).

4.4. Leakage test

The leakage test should be conducted after every time the impactor is assembled and before each measurement phase. Because the lower stages of the ELPI operate at low pressure, it is important that the impactor does not leak. Any leakage will modify the cut-off diameter of the stage and then alter the determination of the size distribution. It is therefore recommended to follow precisely the protocol in the section 4.5 of the Classical ELPI® manual [4].

4.5. Warm up

For reasons of stabilization electrometers it is highly recommended to turn on the ELPI well in advance prior to any measurement. A minimum warm-up time of one hour is recommended.

4.6. Zeroing

In addition to cleaning and impactor leakage test, zeroing of the electrometers is an important step and should be conducted before each measurement. There are different protocols available in the Classical ELPI®

manual [4]. It is recommended to conduct first several “Main Reset” during the warm-up time, and then prior to any measurement a “Zero”. These zeroing protocols can be directly run with the ELPIvi™ software.

The manufacturer recommends conducting these protocols with the flush pump on. These protocols can also be conducted in a second phase by connecting a clean HEPA filter and flexible conductive tubing upstream of the Classical ELPI® which should ensure a particle-free air entering the instrument.

It is recommended to determine the Limit of Quantification (LOQ) in fA of each respective electrometer through a specific test carried out separately. This test consists of measuring a particle-free air through a HEPA filter with a previously cleaned ELPI. The HEPA filter is pre-tested with a CPC for leaks and cleanliness, so check that the number concentration downstream of the HEPA filter is zero. A recording of the currents measured by the ELPI through the HEPA filter is performed for about 5 min. These currents correspond to the noise signal of the electrometers. The limit of quantification is defined as 10 times the standard deviation of this noise. Figure 64 presents the LOQ obtained with the Classical ELPI® equipped with sintered plates for two measuring ranges. Once determined, these LOQ values can be used to check the zeroing conducted prior to each of the measurement.

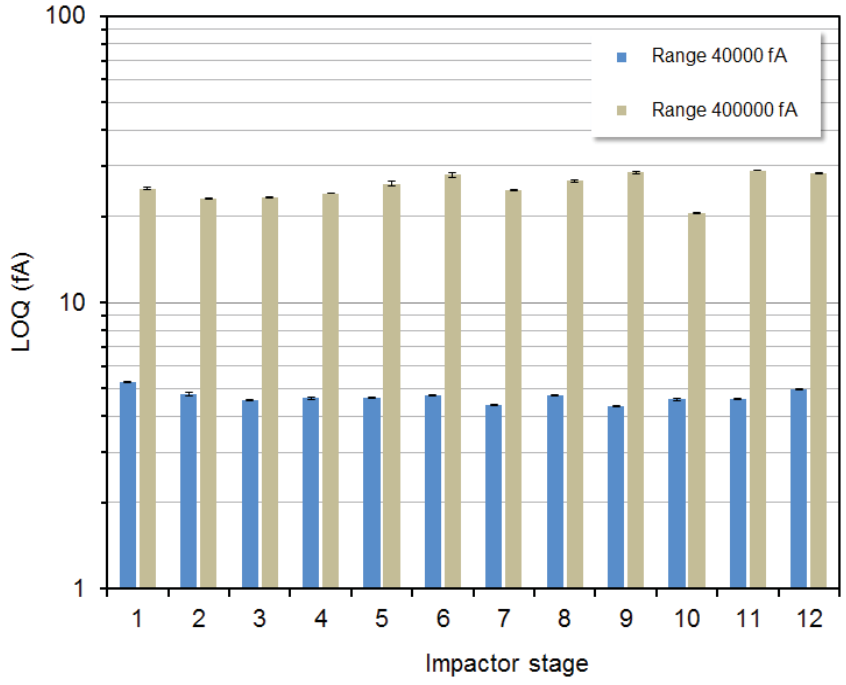

Figure 64: LOQ obtained with the Classical ELPI® equipped with sintered plates for two measuring ranges. Data presented with IC(95%).

4.7. Setting up the instrument

Setting up of the instrument consists of:

- Assembling the impactor the correct (greased or oiled) impaction plates.
- Assembling the charger, impactor, and connecting the external vacuum pump.
- Connecting the external computer and set-up the ELPIvi™ software.
- Opening the metering valve and switching the power on.
- Performing the leakage test and checking the inlet flowrate.
- Zeroing the unit.
- Performing and saving a 5-min measurement with a clean (and previously CPC checked) HEPA filter in order to compare the measured data with pre-determined LOQ (see section 4.6)

If the LOQ comparison is positive, the instrument is ready to be used for a measurement.

4.8. Setting up the ELPIvi™ software

The ELPIvi™ software controls the Classical ELPI® instrument by sending commands to the unit internal computer via serial port; it also serves for data collection and logging.

Before any measurement campaign, it is strongly recommended to read carefully the ELPIvi™ [5] measurement software 120-page document and make several preliminary tests. In particular, the sections corresponding to the charger set-up, the impactor set-up, the communication, the program, the defaults values (range, average time, save interval) and the data format for saving.

Concerning the default values, it is recommended to use a density default value of 1 g/cm^3 as well as for the dilution factor. The true values can be integrated later during the data treatment in the Excel sheet provided by the manufacturer.

In the same way, concerning the data format of the save values, it is recommend to save the corrected current values in the form dW (and not dW/dlogDp for example) and for the aerodynamic equivalent diameter (and not the Stokes diameter).

4.9. Off-line characterization of collected airborne particles

As airborne sampled particles are collected onto impaction plates during the measurement phase, subsequent physical/chemical analysis can be performed in several size fractions individually. These off-line characterization and elemental analyses can be conducted to obtain information concerning aggregate or agglomerate size or morphology, and chemical composition of the collected particles. Be aware that the collection substrate must be chosen in consideration of the analysis method.

The Classical ELPI® can eventually be used to as a conventional time-integrated low-pressure cascade impactor. For this, it is best to remove the charger part and use only the impactor column (together with the vacuum pump). Gravimetric analysis, though apparently simple, is subject to uncertainty arising from instability in the mass of the sampling medium and other elements which must be weighed. If gravimetric analysis is used, general method should be followed [7, 8, 9].

5. Data treatment

5.1. Using the Excel sheet

An Excel sheet is provided to the user by the manufacturer for the treatment of ELPI data. With this calculation tool, several information can be pointed out such as:

- Time series of currents,
- Time series of particle number, surface area, and mass concentrations,
- Number, surface area, mass size distributions (cumulated or not).

The user is asked to input inlet flow rate, particle density and dilution to potentially correct the data measured. An example of times series of number concentration (Figure 65) and of number size distribution (Figure 66) stemming from the calculation sheet are provided below.

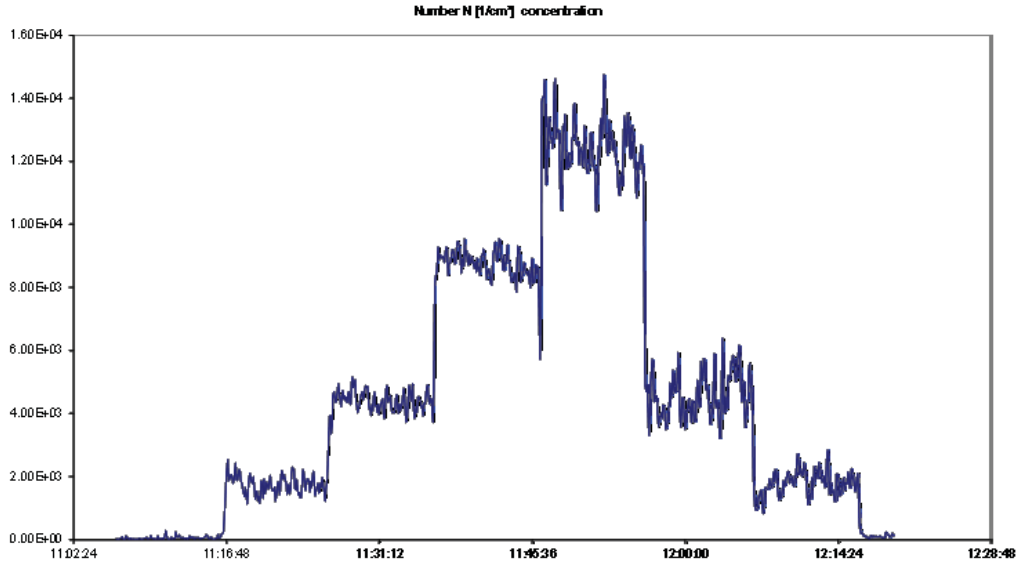

Figure 65. Example of time series of particle number concentration.

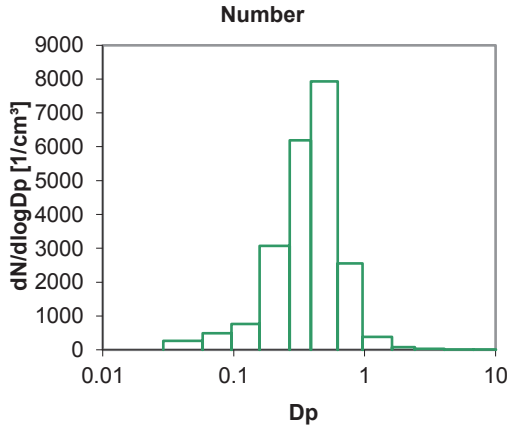

Figure 66. Example of particle number size distribution.

Using the Excel sheet makes it is also possible to check the absence of negative currents or current constant deviation with time, which is crucial to ensure reliable measurement data. Finally, the stages to be considered can be chosen by the user, i.e. 1 to 9 for the submicrometer range only, in case large parasite particles shall not be taken into consideration.

5.2. Data inversion

Presenting the cascade impactor data in a histogram ignores information on particle size distribution within each impactor stage and does not take into account the possible overlap between them, i.e. that the particles of a given diameter may deposit over several stages [10], be lost [11] or bounce [12]. Accounting for these different effects requires the use of complex inversion algorithms (e.g. [13]) to post-analyze data from ELPI measurements and provide a continuous curve, as shown in Figure 67.

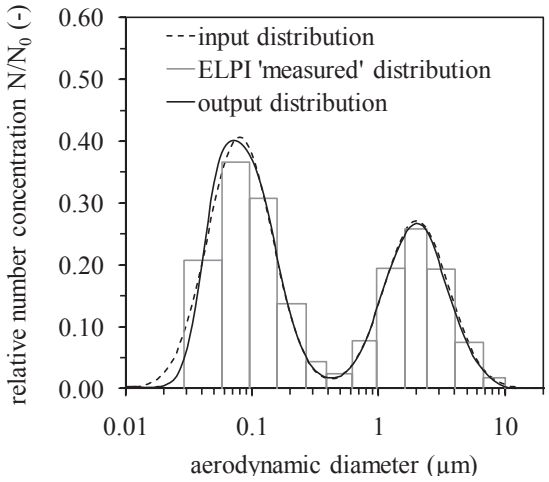

Figure 67. Example of data inversion (theoretical values).

According to several publications about inverse problems in cascade impactors, the software proposed by [13] realizes the data inversion using the Markowski method, and more particularly the Twomey iterative algorithm [14]. The algorithm consists of a deterministic method, its main advantage being that it does not assume a mathematical model for the aerosol size distribution resulting from the inversion procedure. The results provide a continuous curve defined by 100 to 1000 data points.

5.3. On the influence of particle density

As indicated in section 3.1, the number concentration in each channel is calculated from the measured current by applying the charger efficiency curve which is dependent on mobility-equivalent diameter, itself related to the aerodynamic diameter. The use of these two particle equivalent diameter concepts requires the knowledge of the effective density to convert one into the other [15].

5.3.1. Particle charging

In the ELPI, airborne particles are first drawn into a unipolar corona charger (+5 kV) by a vacuum pump at 10 L.min^{-1}. Here, they are positively charged to a defined level according to their mobility equivalent diameter [16, 17]:

$$E_C(d_m) = \frac{Q}{10} \cdot \begin{cases} 4.48 \cdot {d_m}^{1.9087} & \text{for } d_m < 0.095 \text{ µm} \\ 1.2930 \cdot {d_m}^{1.3805} & \text{for } 0.095 < d_m < 1.196 \text{ µm} \\ 1.3529 \cdot {d_m}^{1.1308} & \text{for } d_m > 1.196 \text{ µm} \end{cases}$$

where Q is expressed in L.min-1. The charging efficiency is thus defined with regards to the electrical mobility equivalent diameter d_m.

5.3.2. Cascade impaction

The charged particles are then size-classified from 30 nm to 10 µm in a 13-stage channel, multi-jet, low pressure impactor based on their aerodynamic equivalent diameter. A 12-channel electrometer is used to measure the charges carried by the particles impacted on each stage. All stages are electrically insulated from each other.

At this point in time, the behavior of airborne particles is mainly governed by their inertial properties; thus, their density is a key characteristic when calculating particle deposition within the cascade impactor. According to [17], the global collection efficiency of stage i in the impactor is given by:

$$E_i = 1 - (1 - E_i^P) \cdot (1 - E_i^D) \cdot (1 - E_i^{IM}) \cdot (1 - E_i^{SC})$$

where E_i^P corresponds to particle collection by impaction, E_i^D by diffusion, E_i^{IM} by image forces and E_i^{SC} by space-charge effects. For mathematical details, see [18]. The probability that a particle of aerodynamic diameter d_a will be collected on stage i is therefore calculated by the following equation:

$$k_i(d_a) = E_i(d_a) \cdot \prod_{j=i+1}^{j=13} \left[1 - E_j(d_a)\right] \qquad i = 1, \ldots, 12$$

The resulting current corresponds to the product of the deposition probability, the inlet number concentration and the charging efficiency:

$$I_i(d_a) = \sum_{d_m} k_i(d_a) \cdot C_N(d_a) \cdot E_C\big(d_m(d_a)\big)$$

5.3.3. Corresponding number size distribution

In other words, the current I_i measured in each stage is converted into number concentration $C_{N,i}$ according to:

$$C_{N,i}(d_a) = \frac{I_i(d_a)}{E_C\big(d_m(d_a)\big)}$$

Thus, the conversion of aerodynamic diameters into mobility diameters is required when using the measured currents I_i to determine airborne particle number size distribution. The density ρ of particles is related to the electrical mobility (d_m) and aerodynamic (d_a) equivalent diameters according to [14]:

$$\rho = \frac{Cu(d_a)\, {d_a}^2}{Cu(d_m)\, {d_m}^2}\, \rho_0$$

where Cu is the slip correction coefficient and ρ_0 the reference density ($\rho_0 = 1$ g.cm^{-3}). Therefore, an assumption must be made about particle density when seeking to determine the particle number size distribution based on the particle aerodynamic diameter.

A number of studies investigating how best to convert ELPI particle number distributions into mass distributions have been reviewed [20]. A constant density was assumed by some authors [21, 22]. Other authors [23] described how this assumption can result in extensive uncertainties in mass measurements and consequently recommended using the effective density when calculating the mass concentration from the number concentration. A limitation to the studies cited is that they only investigated how density affected the conversion of number into mass concentration, and not the conversion of ELPI current (raw data) into number concentration.

5.3.4. Influence of particle effective density on the calculated number size distribution

Figure 68 presents the case of particle with an aerodynamic diameter of 100 nm that deposit on the same impaction stage (similar inertia). The first particle considered has a density of 1, leading to a mobility equivalent of 100 nm, and a charging efficiency of 0.05 fa.cm^3 (solid line). The second particle has a density of 5.6 (case of an airborne salt-based CdI2 particle), corresponding to a mobility equivalent of 22 nm, and a charging efficiency of 0.003 fa.cm^3 (dashed line).

Consequently, for the same current measured, the number concentrations calculated will differ by a factor of 16. In other words, the concentration reported assuming a particle density of 1 (instead of 5.6) will be 16 times below the one obtained when the true density is input.

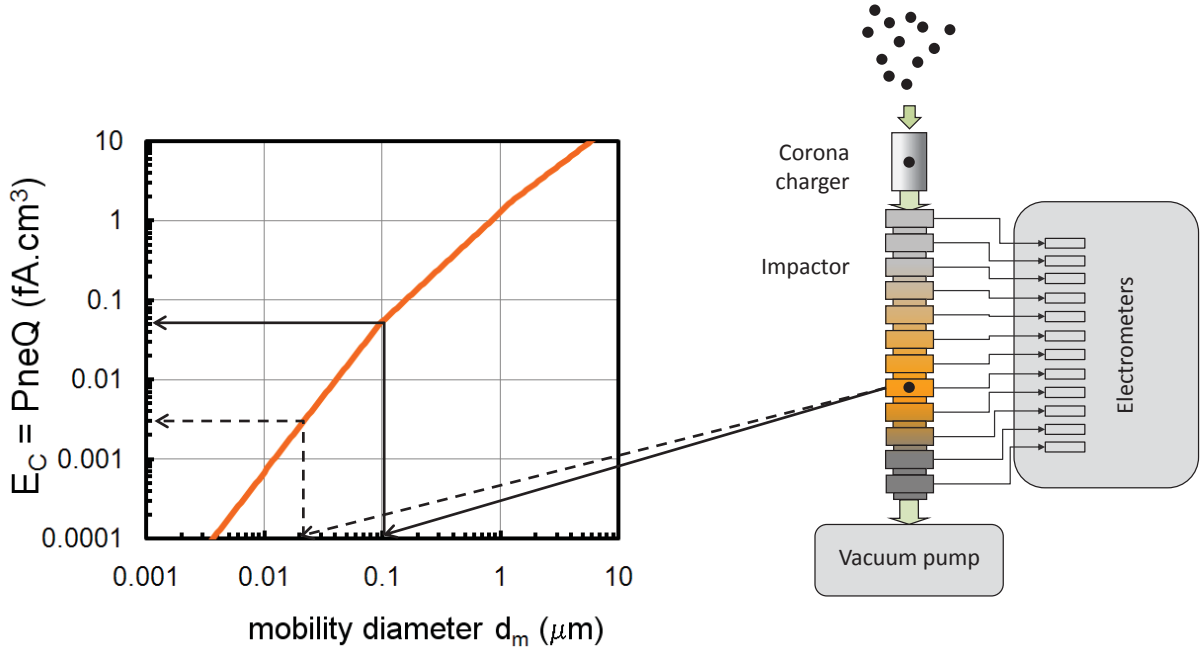

Figure 68: Schematic of the effect of particle density on charging efficiency

5.3.5. Case of size-dependent particle density (effective density)

The effective density ρ_e of airborne nanoparticles is defined as the ratio of aggregate mass and equivalent mobility volume:

$$\rho_e = \frac{m_a}{\frac{\pi}{6} \cdot d_m^3}$$

In addition to effective density, this combination of devices can be used to calculate the mass-mobility exponent of the aggregates [24]:

$$\rho_e = \frac{6 \cdot k_m}{\pi \cdot d_{pp}^{D_m}} \cdot d_m^{D_m - 3} \implies \rho_e \propto d_m^{D_m - 3}$$

where D_m is the mass-mobility exponent, k_m is a fractal prefactor, and d_{pp} is the diameter of primary particles making up the aggregate being analyzed. As suggested by the abovementioned equation, when D_m is less than 3, particle effective density decreases when particle size increases.

In his review of Diffusion Limited Cluster Aggregation (DLCA) aggregates in the slip flow regime ($0.1 < Kn < 10$), it suggests the mass-mobility exponent is close to 2 [25]. Thus, when the diameter of electrical mobility is multiplied by a factor of 10, the effective density of the corresponding aggregates is divided by a factor of 10.

In a recent study [26], the number (Figure 69) and mass (Figure 70) concentrations obtained by two reference devices (an SMPS and a TEOM) to those calculated from raw ELPI data, i.e. the current size distribution, are compared. ELPI data post-treatment was performed assuming different particle densities:

- the mobility-dependent effective density determined by the tandem DMA/APM;
- the density of the raw material ($\rho_{Zn/Al}$ = 5.72 g/cm^3);
- a standard density (ρ_0 = 1 g/cm^3);
- an average effective density (defined below and equal to 0.71 in this case) that takes into account the number size distribution (obtained from the SMPS) and effective density of airborne particles (obtained from the coupling of a DMA, an APM and a CPC):

$$\overline{\rho_e} = \frac{\sum_{d_m} C_N(d_m) \cdot \rho_e(d_m)}{\sum_{d_m} C_N(d_m)}$$

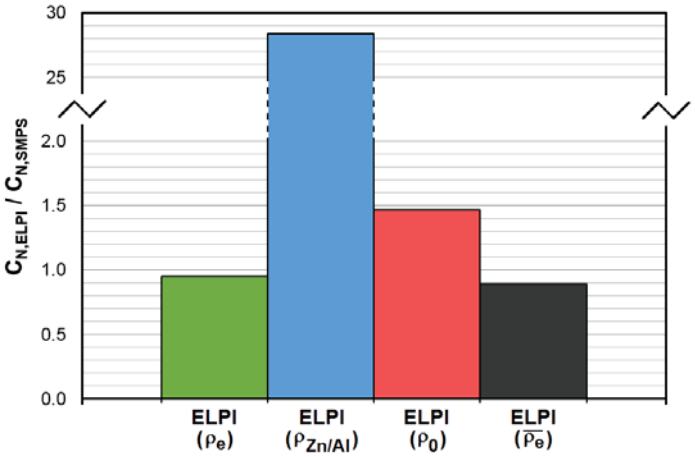

Figure 69. Ratio between ELPI and SMPS number concentrations as a function of density

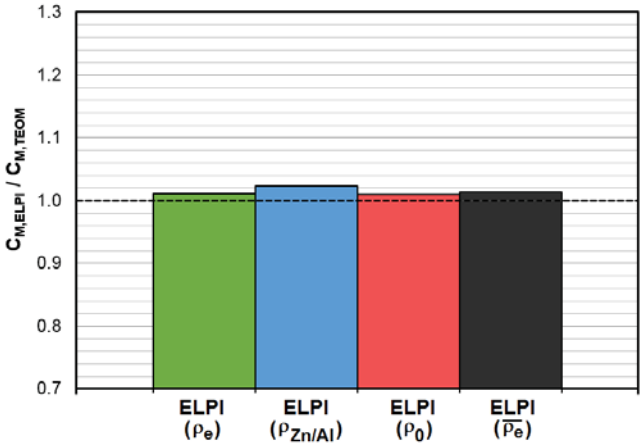

Figure 70. Ratio between ELPI and TEOM mass concentrations as a function of density

The results show that if aerosols are perfectly characterized, good measurements of mass and number concentrations can be obtained with an ELPI by using the raw material density for spherical particles and the effective density for agglomerates. In the latter case, additional raw data post-treatment is required.

If aerosols are not fully characterized (i.e., if the effective density is unknown), a constant density should be used, but the number concentrations provided by the ELPI may not be reliable. In particular, the standard density should not be considered as a universal choice. Indeed, depending on the particle size distribution, using the standard density could lead to incorrect or fortuitously correct results.

An approach consisting in using (even an erroneous) constant density twice (to convert currents into particle number concentration and then number concentration into mass concentration) provides an adequate estimation of the mass concentration, within roughly ± 20 %, whatever the particle size distribution and density. This approach is recommended rather than 1) using the standard density in ELPI software for conversion of currents into particle number concentration and 2) using the mobility-dependent effective density to convert the number concentration into a mass concentration.

5.4. General flowchart

Figure 71 is a generic flowchart describing how to deal with ELPI data.

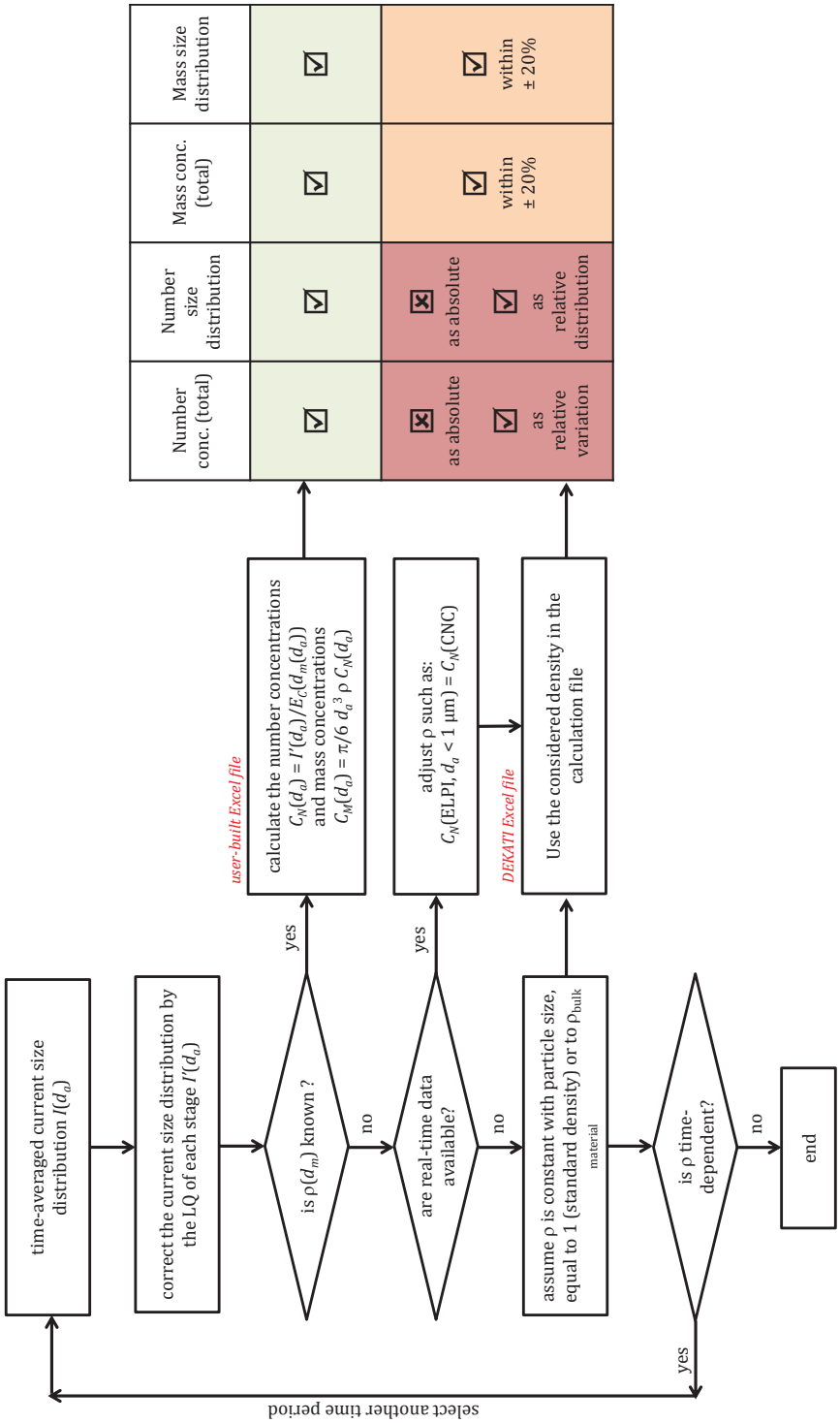

Figure 71. Flowchart for ELPI data treatment

6. Maintenance

In order to ensure proper operation of the Classical ELPI® a comprehensive service should be done every other year or three years. The need for the service depends on how frequently the Classical ELPI® is used. The service is done by the local distributor of the manufacturer Dekati Ltd.

7. References

[1] nanoGEM - Standard Operation Procedures for assessing exposure to nanomaterials, following a tiered approach. June 2012, 90 p. Available at http://www.nanogem.de/cms/nanogem.

[2] Järvinen, A., M. Aitomaa, A. Rostedt, J. Keskinen, and J. Yli-Ojanperä (2014) Calibration of the new electrical low pressure impactor (ELPI+). Journal of Aerosol Science 69 (0):150-159

[3] Keskinen, J., K. Pietarinen, and M. Lehtimäki (1992) Electrical low pressure impactor. Journal of Aerosol Science 23:353-360.

[4] ELPI™ User Manual ver. 4.12. Copyright © Dekati Ltd. 2014. 136 p.

[5] ELPI™ Softaware Manual ver. 4.12. Copyright © Dekati Ltd. 2014. 120 p.

[6] Kuhlbusch, T., C. Asbach, H. Fissan, D. Gohler, and M. Stintz. (2011) Nanoparticle exposure at nanotechnology workplaces: A review. Particle and Fibre Toxicology 8 (1):22.

[7] ISO 15767:2009 Workplace atmospheres — Controlling and characterizing uncertainty in weighing collected aerosols.

[8] HSE. MDHS14/4 General methods for sampling and gravimetric analysis of respirable, thoracic and inhalable aerosols. Available at http://www.hse.gov.uk/pubns/mdhs.

[9] Lodge JP, Chan TL eds.(1986) Cascade impactor sampling and data analysis. Akron OH: American Industrial Hygiene Association, p. 170.

[10] Dong Y, Hays MD, Smith ND and Kinsey JS (2004) Inverting cascade impactor data for size-resolved characterization of fine particulate source emissions. *Journal of Aerosol Science* **35**:1497-1512.

[11] Virtanen A., Marjamäki M., Ristimäki J. and Keskinen, J. (2001). Fine particle losses in electrical low pressure impactor. *Journal of Aerosol Science* **32**:389-401.

[12] Kuuluvainen H., Arffman A., Saukko E., Virtanen A. and Keskinen, J. (2013). A new method for characterizing the bounce and charge transfer properties of nanoparticles. *Journal of Aerosol Science* **55**:104-115.

[13] Bau S and Witschger O (2013) A modular tool for analyzing cascade impactors data to improve exposure assessment to airborne nanomaterials. *Journal of Physics: Conference Series* **429** 012002.

[14] Twomey S. (1975). Comparison of constrained linear inversion and an iterative nonlinear algorithm applied to the indirect estimation of particle size distributions. *Journal of Computational Physics* **18**: 188-200.

[15] DeCarlo P. F., Slowik J. G., Worsnop D. R., Davidovits P. and Jimenez J. L. (2004). Particle morphology and density characterization by combined mobility and aerodynamic diameter measurements. Part 1: Theory. *Aerosol Science and Technology* **38**: 1185-1205.

[16] Moisio M. (1999). *Real-time size distribution measurement of combustion aerosols*. PhD thesis, Univeristy of Tempere, Finland. ISBN 952-15-0328-9.

[17] Marjamäki M., Keskinen J., Chen D.-R. and Pui D. Y. H. (2000). Performance evaluation of the Electrical Low-Pressure Impactor (ELPI), *Journal of Aerosol Science* **31**: 249-261.

[18] Marjamäki M., Lemmetty M. and Keskinen J. (2005). ELPI response and data reduction. I: Response functions. *Aerosol Science and Technology* **39**: 575-582.

[20] Liang B., Ge Y., Tan J., Han X., Gao L., Hao L., Ye W. and Dai P. (2013). Comparison of PM emissions from a gasoline direct injected (GDI) vehicle and a port fuel injected (PFI) vehicle measured by electrical low pressure impactor (ELPI) with two fuels : Gasoline and M15 methanol gasoline. *Journal of Aerosol Science* **57**:22-31.

[21] Shi J.P., Harrison R.M. and Brear F. (1999). Particle size distribution from a modern heavy duty diesel engine. *Science of the Total Environment* **235**:305-307.

[22] Ushakov S., Valland H., Nielsen J.B. and Hennie E. (2013). Particle size distributions from heavy-duty diesel engine operated on low-sulfur marine fuel. *Fuel Processing Technology* **106**:350-358.

[23] Andrews G.E., Clark A.G., Rojas N.Y., Sale T. and Gregory D. (2001). Diesel particle size distribution: The conversion of particle number size distribution to mass distribution. SAE Technical Paper, 2001-01-1946.

[24] Shapiro M, Vainshtein P, Dutcher D, Emery M, Stolzenburg M, Kittelson DB and McMurry PH (2012) Characterization of agglomerates by simultaneous measurement of mobility, vacuum aerodynamic diameter and mass. *Journal of Aerosol Science* **44**:24-45

[25] Sorensen CM (2011). The Mobility of Fractal Aggregates: A Review. *Aerosol Science and Technology* **45**:765-779.

[26] Charvet A., Bau S., Bémer D. and Thomas D. (2015). On the importance of density in ELPI data post-treatment. *Aerosol Science and Technology* **49**: 1263-1270

Annex 5: Nasal paper flag Standard Operating Procedure

Method for the use of the nasal flag as an inhalation indicator to nanoparticles

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Aim(s) or Objective(s) of SOP
This SOP describes the development a method that allows the sampling and the analysis of nanoparticles deposited in the nose for the indication of a potential inhalation to nanoparticles.

Scope and required operating conditions

The assessment of external exposure thanks to air sampling is essential, but this type of measurement does not take into account the use of individual protective devices, and individual parameters such as absorption, distribution and elimination. Biomonitoring is the best approach to assess individual exposure to occupational and environmental toxicants. In general, biomonitoring is based on blood and urine analyses, but in the case of insoluble nanoparticles, toxicokinetics data would tend to prove that very few nanoparticles, or derivatives, would be found in those matrices following inhalation. This is why other methods have to be explored. A test able to verify the non-exposure to nanoaerosols seems to be of great interest, especially at work places where workers might be exposed to nanomaterials. Such a device might be also a useful tool in case of incidental situations. Therefore, nasal sampling is proposed in order to give an indication on the existence, or not, of an inhalation situation. The expected result from this procedure is not a quantitative level, since the sampling itself is not quantitative and standardized, and the chosen analytical procedure is semi-quantitative, but more an indication type yes/no.
The aim of this SOP is the development a method that allows the sampling and the analysis of nanoparticles deposited in the nose for the indication of a potential inhalation to nanoparticles.

Materials and equipment needed

Materials

The nasal flag is made of a rectangular piece of paper without impurities pasted on a wood stick and forming a sampling device in the shape of a paper flag.
Suprapure nitric acid is used for the digestion of the sampling tool and Inductively Coupled Plasma Mass Spectroscopy (ICPMS) analyses. ICP reference standards are used to calibrate the ICPMS analyses, and standard nanoparticles in suspension from Sigma Aldrich were used to develop the method.

Equipments

The first equipment is a freezer at -20°.

The second required equipment is a multiwave oven (MW3000, Anton Paar) to perform the digestion of the sampling tool.

The third equipment requested is an ICPMS apparatus for the elemental analysis (Nexion 300X from Perkin Elmer).

Setup of experiments

The experiment is divided in three parts, the sampling, the digestion of the sampling tool, and the analysis.

Sampling

Prior to sampling the paper of the flag is humidified with ultrapure water.

The paper flag is rolled in one nostril and put in a collection tube.

A second flag is rolled in the second nostril and put in a second collection tube.

Collection tubes are stored at -20°C prior to analysis.

Digestion of the nasal flag

Due to the diversity of nanoparticles and associated protocols of mineralization we have made the choice to develop only one protocol based on the mineralization of the sampling tool and not on the mineralization of the nanoparticles. Depending on their nature, nanoparticles are more or less digested by the procedure, and the result is therefore semi-quantitative. A calibration made of standard nanoparticles deposited on blank paper flags is prepared and digested as well.

For the acidic digestion, the paper is separated from the wood stick and placed in a Teflon liner and submerged in nitric acid and mineralized at 1400 W during 35 minutes.

Analysis of the samples

After digestion, digested flags are analyzed using ICPMS and an ionic calibration is prepared with the element constitutive of the searched nanoparticles.

An analytical internal standard is added to the digested solution prior to analysis in order to take into account the matrix effect of the digested flag.

Annex 6: Exhaled breath condensate Standard Operating Procedure

Approach for the search of biomarkers of exposure and effect to nanoparticles in exhaled breath condensate

Authors *(Authors who actively wrote the report)*	**Caroline Desvergne, caroline.desvergne@cea.fr** **Muriel Dubosson, muriel.dubosson@cea.fr** **Véronique Mossuz, veronique.mossuz@cea.fr**

Aim(s) or Objective(s) of SOP
This SOP describes an approach for the search of biomarkers of exposure and effect to nanoparticles in exhaled breath condensate.

Scope and required operating conditions

The assessment of external exposure thanks to air sampling is essential, but this type of measurement does not take into account the use of individual protective devices, and individual parameters such as absorption, distribution and elimination. Biomonitoring is the best approach to assess individual exposure to occupational and environmental toxicants. In general, biomonitoring is based on blood and urine analyses, but in the case of insoluble nanoparticles, toxicokinetics data would tend to prove that very few nanoparticles, or derivatives, would be found in those matrices following inhalation. This is why other methods have to be explored, and in this regard, exhaled breath condensate (EBC) seems promising. EBC is a totally non-invasive respiratory sampling that can be used in the field of occupational health. It contains small molecules emanating from the respiratory tract and potentially reflects pulmonary pathobiology. Both biomarkers of exposure and effect can be found in this matrix. The aim of this SOP is to describe an approach for the search of biomarkers of exposure and effect in exhaled breath condensate. For biomarkers of exposure, metal concentrations are quantified in EBC and nanoparticles are observed using electron microscopy. For biomarkers of effect, the approach focuses on the analysis of inflammatory cytokines.

Materials and equipment needed

Materials

Exhaled breath condensate is sampled using the RTube device (Respiratory research).

Suprapure nitric acid and ICP reference standards are used for ICPMS analyses.

Biorad kits are used for the analysis of cytokines.

Equipments

A deep-freezer at -80°C is required for the storage of EBC samples. A freeze dryer is used to concentrate EBC samples by freeze-drying. Cytokines measurement is performed thanks to a multiplex apparatus (Magpix technology, Biorad). Multi-elemental analyses are performed with an Inductively Coupled Plasma Mass Spectroscopy (ICPMS) apparatus (Nexion 300X from Perkin Elmer). SEM-EDS (Scanning Electron Microscopy- X-ray analysis system) and TEM (Transmission Electron Microscopy) observations are performed using Hitachi and Thermo systems.

Setup of experiments

EBC collection

EBC is collected with the RTube device according to the recommendations provided by the American Thoracic Society and the European Respiratory Society. Before using the device, an intensive flushing protocol with ultrapure water and a check for absence of background noise by dynamic light scattering are performed.

In brief, the sampling with nose blocked lasts for 15 minutes during which the subject breathes normally through the device. Water rinsing of the mouth is carried out and the subject is asked to abstain from drinking or eating for an hour before the sampling. A survey is also associated and allows these elements to be clarified. After collection, EBC is immediately stored at -80°C in low absorption tubes.

Multi-elemental analysis

EBC are diluted in nitric acid and analyzed with a multi-elemental analysis ICPMS technique allowing the simultaneous quantification of 17 elements (Zn, Al, Ti, Co, Cu, Zr, Ni, Cr, Ga, In, Pt, Mn, Fe, Se, Cd, Ge, Be).

Electron microscopy observations

EBC samples are diluted in glutaraldehyde and spotted on aluminum membranes or TEM grids to perform either SEM or TEM observations.

Cytokine analysis

Prior to cytokine analysis, EBC are concentrated by freeze-drying. They are then put in phosphate buffer saline and analyzed in duplicate for a panel of 29 markers according to the manufacturer recommendations. The inflammatory markers sought are classified by family: interleukines (Il-1b, Il-1ra, Il-2, Il-4, Il-5, Il-6, Il-7, Il-9, Il-10, Il-12, Il-13, Il-15, Il-17a), chemokines (Il-8, eotaxin, RANTES, IP-10, MCP-1, MIP-1a, MIP-1b) and others (TNF alpha, IFN-g, G-CSF, GM-CSF, PDGF, FGF, VEGF, ICAM-1, VCAM-1).

Annex 7: FFF-ICPMS Standard Operating Procedure

SOP for the detection/quantification of silver nanoparticles in an aqueous matrix by AF4-ICP-MS

Authors *(Authors who actively wrote the report)*	**Sylvie Motellier, sylvie.motellier@cea.fr**

Aim(s) or Objective(s) of SOP
This SOP describes an approach for the characterisation (size distribution, elemental composition …) of nanoparticles in suspension. This approach was evaluated using NM300 K material

Scope and applicability of the method

The procedure is applicable for the determination of electrostatically stabilized anionic silver nanoparticles with a particle size range 10-100 nm in ultrapure water. The method is based on on-line coupling of the particle size fractionation method asymmetric flow field flow fractionation with **I**nductively **C**oupled **P**lasma-**M**ass Spectrometry (AF4-ICP-MS).

Principle of method

The untreated sample, injected in the sample loop of an AF4 system is subjected to size fraction separation by means of AF4 with smaller particles eluting before larger ones (range 10-100nm). For detection and quantification of the size fractions, the AF4 is coupled online to an ICP-MS.

When analysing unknown silver colloidal samples the size of nanoparticles is determined by calibrating the system for size against elution time using near mono-dispersed silver standards. For quantification of silver mass, the ICP-MS instrument response must be calibrated using appropriate silver reference solutions or suspensions.

Materials and equipment needed

Reagents

Only chemicals of recognized high purity analytical grade should be used.

Water: Ultrapure (18 ohms resistivity)

Sodium hydroxide solution (NaOH): 0.1M in ultrapure water

Nitric acid, 67-69%: Ultrapure for trace analysis

Ionic silver standard solutions: Any commercially available certified ionic silver standard defined as being suitable for ICP-MS calibration

Internal standard for ICP-MS: Any commercially available certified ionic standard defined as being suitable for ICP-MS calibration may be used. Indium in nitric acid has been used here. It has been diluted in ultrapure water to a final concentration of 100 µg L^{-1}.

Eluent of the AF4 system: Ultrapure water adjusted to pH 10 with 0.1M NaOH solution. The eluent shall be prepared freshly and filtrated (0.1µm) every day. It was not acidified post A4F (pre ICP-MS).

ICP-MS carrier gas: Argon (99,9999%)

Stock solutions of (mono-modal) silver nanoparticles: Any commercially available suspension containing near mono-dispersed silver pseudo-standards of silver nanoparticles. 20 nm to 100 nm citrate stabilized silver nanoparticles dispersed in aqueous sodium citrate solution have been used here. The nominal sizes have been confirmed by TEM and DLS (hydrodynamic diameter).

Table 14. Monodispersed silver nanoparticle pseudo-standards.

Standard	Diameter TEM [nm]	Hydrodynamic Diameter(DLS) [nm]
AgNP20	20.6	26.9
AgNP30	32.3*	44.8*
AgNP40	40.6*	53.7*
AgNP50	52.4*	58.1*
AgNP60	59.0	62.4
AgNP70	68.5*	69.0*

* Manufacturer specification

Experimental set up required for SOP

General

The following apparatus should be available in the laboratory:

- Suitable calibrated pipettes for sample dilution
- Ultrasonic bath for homogenization of particle solution
- pH meter for preparation of eluent solution
- Filtration kit with regenerated cellulose filters (pore size 0.1 µm)
- Asymmetric flow field flow fractionation device (HPLC pump, solvent reservoir(s), injector, separation channel, UV/VIS, MALLS, RI detectors (optional) and data acquisition computer system).
- Inductively Coupled Plasma Mass Spectrometer (ICP-MS):

Figure 1 gives a schematic of the AF4 system coupled on line with the ICP-MS. The elution output from the AF4 separation channel (or detectors if present) is coupled directly (on-line) to the ICP-MS via a T-connector that allows the mixing of the eluent with the internal standard before entering the nebulizer of the ICP-MS. Both the eluent and the internal standard flows are continuously added on-line using the peristaltic pump of the ICP-MS nebulizer.

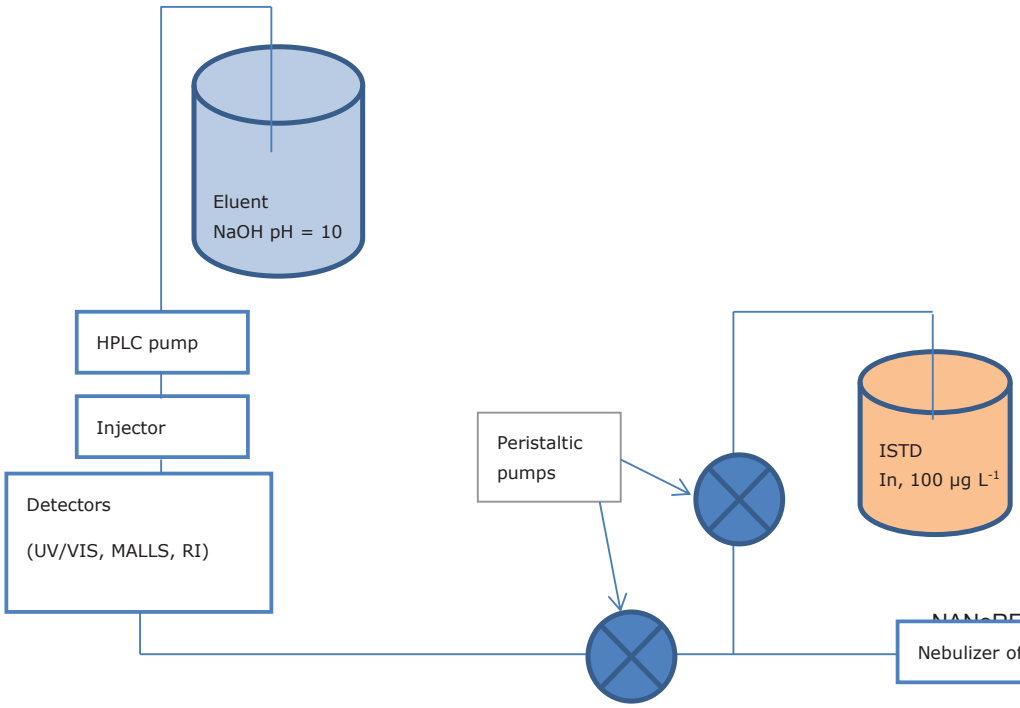

Figure 72. Schematic of the AF4-ICP-MS device.

Specific information of apparatus used by CEA

The following section provides specific information on the experimental conditions and parameters used by CEA. *This data is provided for information only.*

AF4 equipment used by CEA

The apparatus used by CEA was a Wyatt Eclipse 3+ composed of a flow monitoring system and a separation channel. The analytical conditions are listed in Table 15.

Table 15. Experimental conditions for AF4 separation.

Parameter	Characteristics / value
Channel type	Long
Channel length	240 mm
Channel width	21.5 mm
Channel spacer	350 µm
Channel membrane	Regenerated cellulose, 10 kDa cut-off
Injection volume	50 µL
Eluent	NaOH pH = 10, filtrated 0.1 µm
Detector flow	1 mL min^{-1}
Focus flow	0.5 mL min^{-1}
Inject flow	0.2 mL min^{-1}

The elution profile settings used for the separation of Ag NPs is given in Table 16. These conditions are only informative and the choice of starting conditions must be determined from operator experience or from manufacturer recommended values, according to the available equipment.

Table 16. Elution profile for AF4 separation.

	Duration (min)	From to	Vx start	Vx end	Gradient
Elution	2	0 – 2	1	1	
Focus	1	2 – 3			
Focus+inj	5	3 – 8			
Focus	1	8 – 9			
Elution	1	9 – 10	1	1	
Elution	20	10 – 30	1	0.1	exponential
Elution+inj	5	30 - 35		0	0

ICP-MS equipment used by CEA

The CEA ICP-MS device was an Agilent 7700x. It was used in Time Resolved Analysis (TRA) mode, with the conditions set as described in Table 17.

Table 17. Parameters used for ICP-MS instrument at CEA.

Parameter	Details
RF-Power	1550 w
Nebuliser type	Glass low flow, concentric
Nebuliser flow rate	0.3 L min^{-1}
Spray Chamber	Scott (quartz) double pass
Scan mode and Resolution	Time Resolved Analysis (TRA)
Integration Time	2.0 sec
Sampling Time	35 min
Monitored masses	Ag(107, 109) In(115)

Setup of experiments - Standard Operating Procedure

Sample preparation and sample storage

All samples must be stored at 4°C. Before using samples, they must be brought to room temperature and homogenized for 10s with a vortex mixer prior to injection. A short additional 10 s bath sonication can be performed to increase dispersion.

The standard samples of different AgNPs concentrations are obtained by dilution (weight) of the commercial near mono-dispersed silver pseudo-standards in the appropriate matrix.

The standard samples of different ionic Ag concentrations are obtained by dilution (weight) of the commercial ionic silver standards in the appropriate matrix.

Total Ag concentration

The total Ag concentration of the sample (AgNM300K) is determined after acidic digestion with HNO_3 65% (48h in a 3D-shaker) and proper dilution prior to analysis by ICP-MS as normally done in the laboratory using the ionic standards as described in section 6.1.

Particle size determination

Elution times will be used as a means to determine particle size. A calibration curve (elution time = f(diameter)) is obtained by injection of monomodal pseudo standards of different sizes into the AF4.

Particle size calibration

All single mono-dispersed standards are diluted to 1-10 mg L^{-1} (depending on the size of the AgNPs) and the resulting fractions are injected singularly. The resulting elution times (at the maximum of the peak) are plotted against the particle diameter (see example Figure 58). Where known, both the TEM and the hydrodynamic (DLS) diameters can be used. The latter returns a more proper value since FFF is based on hydrodynamic separation. However, DLS experiments must be performed with the exact same matrix as that of the FFF eluent to return an accurate evaluation of the hydrodynamic diameter distribution. For this reason, TEM diameter may be preferred.

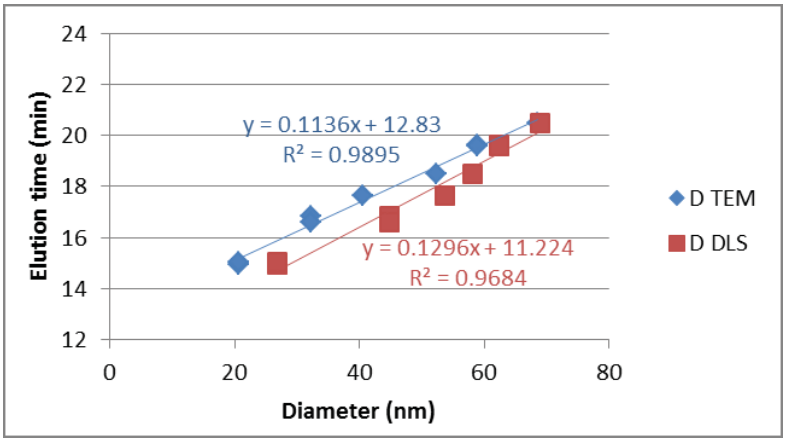

Figure 73. Example of dependency of elution time on particle size.

An interpolated data points fitting curve is plotted with most spreadsheet software packages (e.g. MS Excel, Origin).

Particle size calculation

The time scale of the sample fractogram is transposed into a size scale using the interpolated fitting curve of the size calibration obtained in section 6.3.1

Quantification of silver in identified particle size fractions

Silver concentration of size fractions (peaks) in the sample may be determined as usually done in the laboratory, i.e. after determination of the ICP-MS response (sensitivity in cps) to an ionic calibration curve. However, the following steps (sections 6.4.1 and 6.4.2) have to be done and the requirements fulfilled before validation of this simple quantification process.

In this approach, the AF4 outlet is disconnected from the ICP-MS and the peristaltic pump is used at the same speed to simulate the same flow as with the AF4. The ionic standards have to be diluted in freshly prepared AF4 eluent set at pH 10 at exact concentration using a range that is suitable for expected level of silver in the fractions eluting from the AF4. With the CEA instrument a 4 point calibration of 0, 1, 5, 10 µg L^{-1} in AF4 eluent was done.

The ICP-MS is calibrated by injecting either ionic silver or AgNPs through a capillary tubing whose end is placed in the vial of the calibration solution of known concentration. During the entire process, the internal standard has to be in place simulating the set-up of a normal AF4-ICP-MS run. Signals to be monitored are 107, 109, and 115 as described in Table 17.

For quantification, silver signal (107) is divided by ISTD signal (i.e. 115 for In) for calibrants. Ratio 107/115 is plotted *versus* silver concentration to create calibration curves. A simple linear regression function can be used, without forcing it through the origin.

Evaluation of ICP-MS detector response to particle vs ionic Ag

The calibration curves for ionic silver (from commercial ionic silver standard) and AgNPs (from single mono-dispersed standards) are established to check the recovery of the latter. Because of the high adsorption of the ionic Ag onto the tubings in NaOH pH = 10, a similar calibration curve can be done in any other suitable matrix. In the present case (see Figure 74), 1% HNO_3 was used to evaluate the analytical recovery of NPs versus ionic solutes.

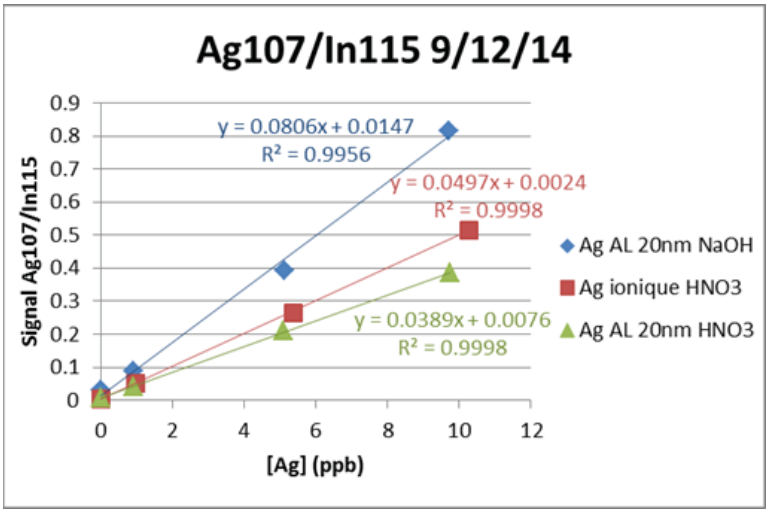

Figure 74. Example of dependency of ICP-MS sensitivity on the type of solute (ionic and NPs) in different matrices.

An interpolated data points fitting curve is plotted with most spreadsheet software packages (e.g. MS Excel, Origin).

The recovery rate may be lower than 100% (it was found to be ca. 78% between 20nm AgNPs and ionic Ag in 1% HNO_3). In this case, a correction factor is applied to the ionic calibration curve for quantitative purposes, or the detector response should be determined by injection of AgNPs for calibration curve establishment.

Evaluation of ICP-MS detector response to particle size

This section is intended to verify whether the ICP-MS response to the eluted Ag nanoparticles can be assumed to be independent of particle size.

Calibration curves of known concentrations of AgNPs of the different single mono-dispersed standards are established and the sensitivity of the response is deduced from the slope of each calibration curve (see example Figure 4).

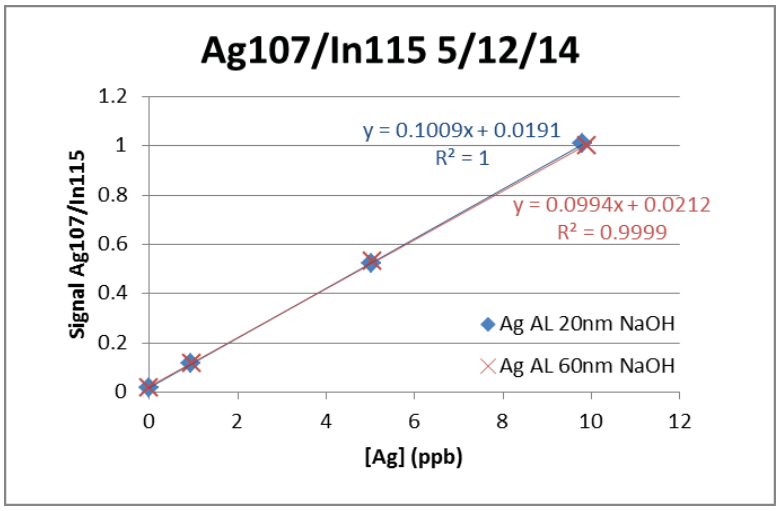

Figure 75. Example of dependency of ICP-MS sensitivity on particle size.

The calibration curves should be independent of the size of the particles for proper evaluation of the size distribution of the sample.

Particle size distribution

The signal scale of the sample fractogram is transposed into a concentration scale using the interpolated fitting curve of the calibration curve obtained in section 6.4.2.

Analytical requirements

ICP-MS tuning has been performed daily. Once usual quality requirements is fulfilled, both the size and quantification calibrations have to be run at least once a day. The latter should also be run every time the plasma is switched on/off and tuned.

Annex 8: Cryo-TEM Standard Operating Procedure

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Aim(s) or Objective(s) of SOP
This SOP describes an approach for the characterisation (size, morphology, elemental composition …) of nanoparticles in suspension. This approach was evaluated using NM300 K material

Scope and required operating conditions

Cryo-microscopy requires the freezing of the liquid sample. To do so, a machine allowing the repeatability of the preparation is used. The Vitrobot commercialized by FEI Company freezes the sample in controlled atmosphere and temperature (Figure 76).

Figure 76. Vitrobot machine allowing the freezing of hydrated samples (FEI).

Materials and equipment needed

The Vitrobot contains two separates parts:

- A command panel to control the parameters of the machine (temperature, humidity, Blot time, Blot force, Blot number, Wait time between blots, process steps).
- An operative part where the freezing of the sample occurs.

Setup of experiments

1. Command panel

The parameters controlled in this panel are mainly function of the sample viscosity. Thus, for a sample with a viscosity close to the water, the blot force, wait and time will be low while for sample with a higher viscosity

these parameters will be increased. It is important to notice that each change of viscosity requires a complete study of the best couple of parameters possible.

For water the best parameters found are the followings:

- Blot force: 5
- Blot time: 3 seconds
- Blot number: 1
- Wait time before blot: 1 second.
- Wait time between compression and freezing: 0 second.

2. Operative steps composing a freezing sequence.

2.1. Grid preparation

Commercial grids used for cryo-microscopy are coated with a hydrophobic film. The first step consists in removing this film. A Plasma Cleaner with the following parameters is used:

- Used gas: Oxygen 25 % / Argon 75 %.
- Power: 25 Watt.
- Time: 20 seconds.

The plasma cleaner allows a good wettability of the grid by the fluid (Figure 77 and Figure 78).

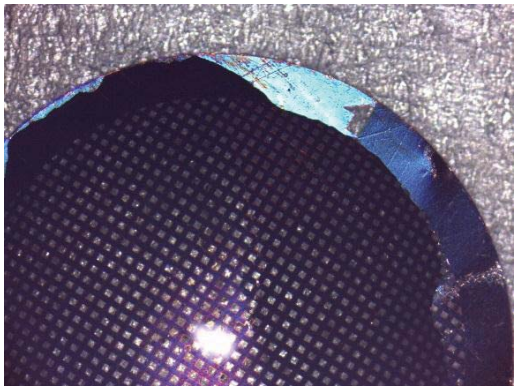

Figure 77. Optical image of a drop of water deposed on a non-treated grid for microscopy. The drop does not wet correctly the grid.

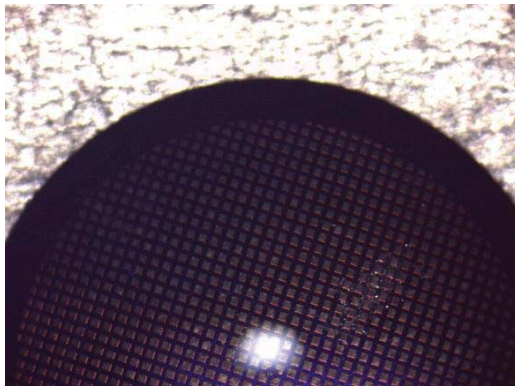

Figure 78. Optical image of a drop of water deposed on a treated grid for microscopy. The drop correctly wet the grid.

2.2. Freezing sequence of the sample

First, the Vitrobot atmosphere is set up (100% humidity, 22°C). The humidity content is very important to ensure a good blot process. The temperature setting ensures a good reliability of the process. Distilled water is evaporated using ultrasounds to set up the humidity content (Figure 79).

The blot of the excess of water is done using blot papers installed into the freezing chamber. These must be installed soon enough to remove the good amount of water (too dry papers would remove too many water while wet papers would have the opposite result, Figure 80).

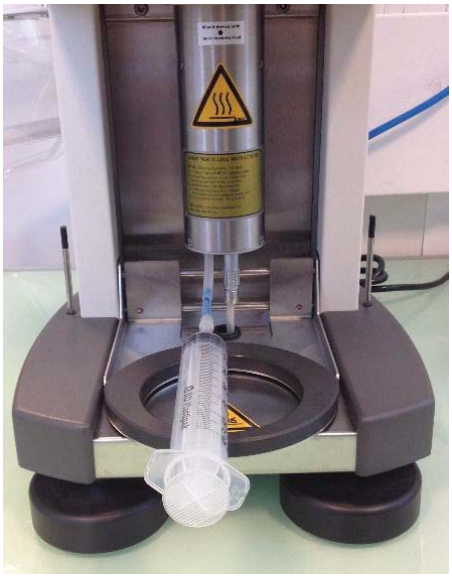

Figure 79. Ultrasound container to maintain 100% humidity into the freezing chamber.

Figure 80. Blot papers installed into the freezing chamber.

The freezing of the grid containing the sample is carried out into liquid Ethane. Indeed, the liquid temperature of ethane is under the amorphous point of the water. The ethane tank is frozen using liquid nitrogen. Once at the liquid nitrogen temperature, ethane gas is liquefied. To freeze the specimen at the lowest temperature and to obtain amorphous ice, the ethane is solidified and then liquefied again (Figure 81 and Figure 82).

Figure 81. Tank to freeze the samples. The central tank is for liquid ethane while the other tanks are for liquid nitrogen.

Figure 82. Frozen tank containing liquid ethane.

A small sample tank is also installed into liquid nitrogen for the further grid transfer.

Once the ethane liquefied, the polystyrene tank is installed on the machine. A carbon grid is blocked using a tweezer and blocked into the freezing chamber. The polystyrene tank is enclosed to the chamber to maintain a pure nitrogen atmosphere. Then, a drop of solution is deposed on the carbon grid into the chamber. The blot papers compressed the grid to remove the excess of water and the tweezer is quenched into liquid ethane.

The operator transfers the grid into the grid box which can be stocked into liquid nitrogen for TEM observations.

3. Microscope transfer

To transfer the frozen grid into the TEM holder and then into the TEM, a transfer station is used (Figure 83). This station is an isolated box working under liquid/gas nitrogen. The TEM holder is inserted at the opposite to install the frozen grid (Figure 84). The TEM holder contains a nitrogen Dewar to maintain liquid nitrogen temperature in the sample and keep the grid frozen.

Figure 83. Transfer station. The TEM holder is inserted on the right side. The blue grid box containing the frozen carbon grid is also installed for the transfer.

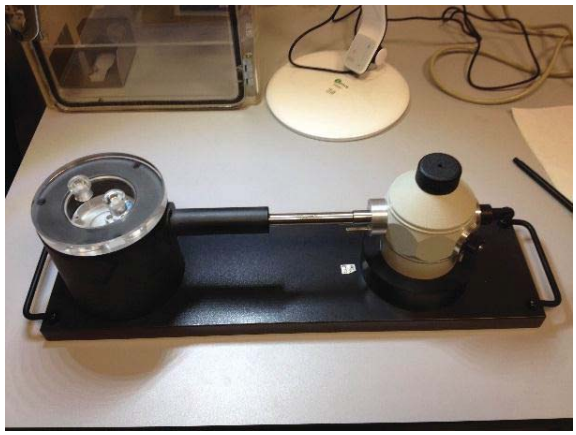

Figure 84. Transfer station with the TEM holder inserted.

Once the frozen sample installed on the TEM holder, this one is transferred into the microscope for observations.

4. Microscope set-up for cryo microscopy observations

Cryo microscopy requires a controlled dose of electron through the sample. This dose is controlled using a low dose mode installed on the microscope. Concretely, the sample is observed at low magnification and two separates areas are defined:

- A focus area where the sample will be destroyed to finely adjust the microscope focus.
- An acquire area where an image will be recorded.

After adjusting the microscope focus on the focus zone, the beam is blanked, the sample moved to the acquired area and an image taken. Immediately after the recording, the beam is blanked again and the sample moved in the focus area.

This mode allows to preserve the sample integrity and to avoid irradiation effect caused by the interactions between the sample and the electrons.

Annex 9: X-ray computed tomography Standard Operating Procedure

Methods for biological tissues or cells preparation for X-ray nanotomography measurement - CEREGE

Authors *(Authors who actively wrote the report)*	**Armand Masion, masion@cerege.fr**

Aim(s) or Objective(s) of SOP
This SOP describes an approach for the preparation of biological samples for X-ray nanotomography characterization.

Scope and required operating conditions

X-ray computed nanotomography (nano CT) measurements are based on X-Ray scans on different angles around the sample. To do so, the samples have to be perfectly dried and mounted on supports to avoid shifts during the scans. This method is describing the steps for dehydration, drying and scans of two kinds of samples *(i)* bacteria cells of *Pseudomonas brassicacearum* specie and *(ii)* roots of *Arabidopsis thaliana*.

Materials and equipment needed

Cells and tissues fixation

The first step consists in rinsing the cells/tissues.

The roots are immerged in 5ml sodium phosphate buffer 0.1M pH 7.2 (PBS)[1]. After 5 min PBS is removed and changed with a pipette. Repeating the operation, the tissues are rinsed 3 times. Then PBS is removed and changed by 5ml of 2.5% (v/v) glutaraldehyde[2] in PBS (2.5% G-PBS) and kept 12h at room temperature for tissues fixation in a sealed container under hood.

Bacteria cells (10^9cells/ml) are rinsed following these steps: *(i)* 5min centrifugation at 5000g, removing supernatant. *(ii)* Resuspension in 3ml PBS for 5min. These operations are repeated 3 times. After the last centrifugation and supernatant removal, cells are resuspended in 2.5% G-PBS and kept for 12h at room temperature for cells fixation in a sealed container under hood.

Cells and tissues dehydration

Before dehydration, 2.5% G-PBS is removed in a specific liquid container containing liquid glutaraldehyde wastes[2] and cells or tissues are rinsed 2 times in ultrapure water. This water may contain glutaraldehyde and have to be removed in the same specific container.

Several ethanol baths with increasing ethanol percentage are needed for dehydration steps. The ethanol series are (% v/v) 25, 50, 70, 90, 90, 100, 100. Roots are immerged for 20min in each bath and bacteria 5min.

Roots are kept in 5ml ethanol for 20min in each bath. Ethanol is removed with a pipette and replace as quick as possible by the next ethanol solution to avoid that tissue dry due to air contact.

Bacteria are kept for 5min in 3ml ethanol solution. After a 5min immersion, centrifugation at 5000g and supernatant removal, 3ml of the following ethanol solution is used for cells resuspension.

Cells and tissues drying

After dehydration root and bacteria are dried using a Leica CPD3000 Critical Point Dryer (CPD). This technic is based on the replacement of ethanol by liquid CO_2. Then, liquid CO_2 is rapidly transformed into gas by increasing the temperature (50°C) and decreasing the pressure. In this way cells are dried avoiding surface tension, samples morphologies and cellular architectures are preserved.

To do so, roots on 100% ethanol are transferred into cages immerged in 100% ethanol and closed with grills. Ethanol is exchanged with liquid CO_2 using 20 cycles of CO_2 flushing. CO_2 turned into gas after increasing the temperature to 50°C.

2ml of Bacteria suspended in 100% ethanol are placed into a dialysis bag (25kDa pore size) and immediately immerged into a large volume of 100% ethanol in the CPD. Ethanol is then exchanged with liquid CO_2 using 14 cycles of CO_2 flushing. CO_2 turned into gas after increasing the temperature to 50°C.

The resulting root and bacteria are perfectly dried.

Cells and tissues mounting and nano-CT measurement

Dried roots are slipped into a polymide capillary and mounted on a sample holder. For bacteria, a support made of polymide (MicroGripper, MiTeGen Society) is gently put in contact with the bacteria powder. In this way, some bacteria get attached to the support. You can found in fig. 1 (a) and (b) the examples of supports. Gold balls (≈900nm of diameter) are then deposited in the mounted sample for further calibration data treatment (using an Xradia microscope, picture in fig 1. (c)).

Nano-CT measurements are realized using the UltraXRM-L200 (Zeiss, Xradia) equipped with a copper anode (Cu, kα1, 8048 keV). Scans are realized using an acceleration voltage of 40kV. 901 projections are acquired (sample turning from -90° to 90° with a scan each 0.16°). The root scans are realized in large field of view with 60s exposure per scan for a total scans duration of 17h. Bacteria scans are realized in high-resolution mode with 250s exposure per scan for a total duration of 63h.

The sample shifts between each scan are corrected based on gold balls movements, using Autoalign 2.0.4 software. 3D reconstruction is realized using Avizo Fire 8.0 software.

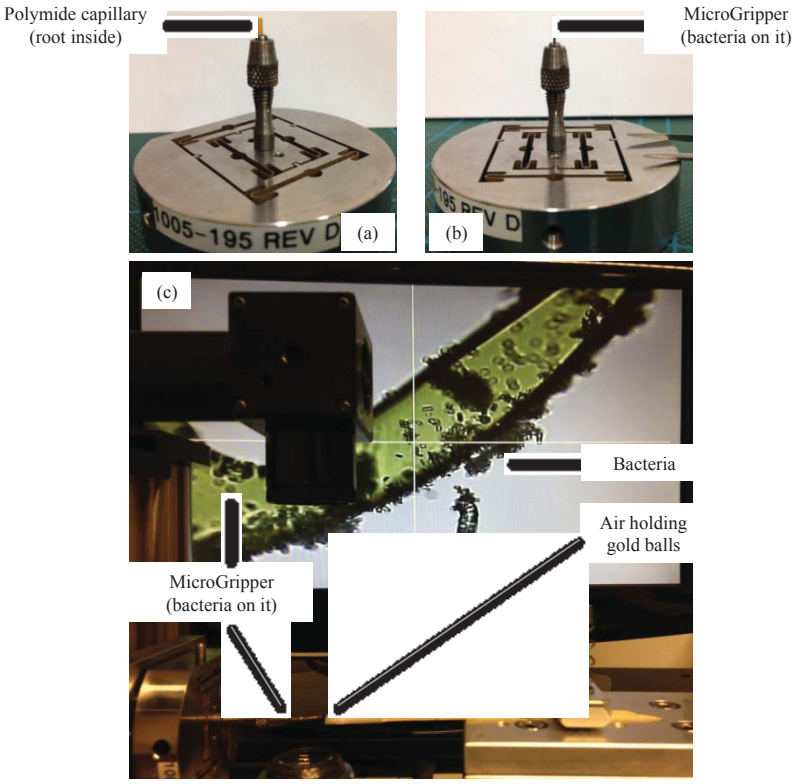

Figure 85: Sample mounting for X-ray computed nanotomography. (a) System for root and (b) for bacteria. (c) Microscopy for gold ball deposition (example of bacteria deposited in MicroGripper).

Annex 10: SAXS Standard Operating Procedure

Nanoparticles form and size characterization by Small Angle X-ray Scattering

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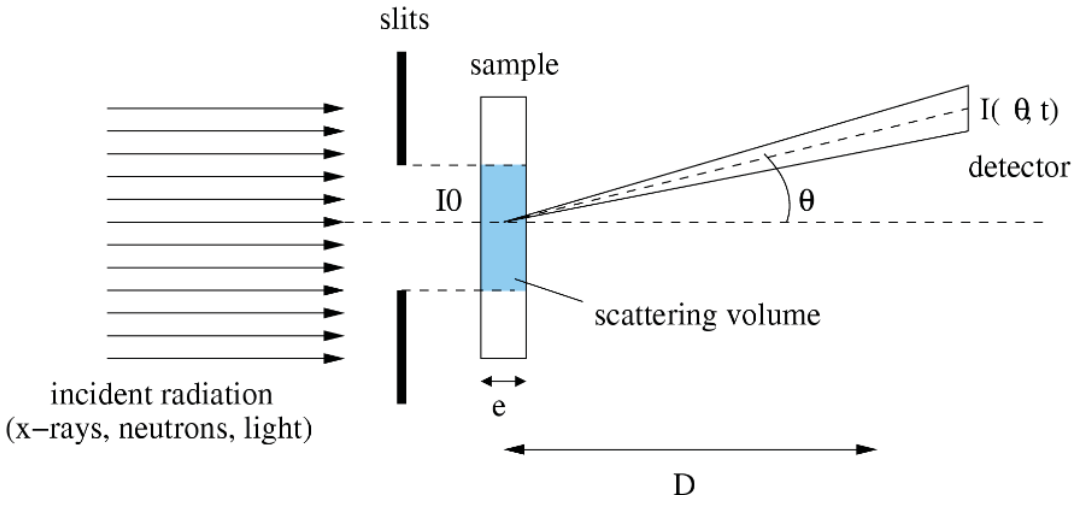
Aim(s) or Objective(s) of SOP
This SOP describes the characterization of nanoparticles size and morphology by Small Angle X-ray Scattering (SAXS)

Scope and required operating conditions

Scattering Experiments

X-ray scattering experiments are adapted to measure the size, shape, agglomeration state of nanoparticles (1-100 nm) in a sample. These properties are averaged over the whole scattering volume and thus over a very large number of nanoparticles. Indeed, typical concentrations are af the order of 10^{15} particles/cm^3., with a scattering volumes of the order of 10^{-3} cm^3, the measured properties are average over typically 10^{12} nanoparticles. The statistical relevance, the non-destructive observation and the possibility to measure *in situ* give SAXS experiments a significant advantages over more classically used TEM observations.

The operating principle of a typical scattering experiment is illustrated in figure 1. A monochromatic X-ray beam is incident on a sample. Part of the incident beam crosses the sample unaffected and some is scattered. A detector measures at an angle θ the scattered intensity I(θ,t). The volume illuminated by the incident beam and 'seen' by the detector is the scattering volume V.



The average value of I(q,t) as a function of q is used to obtain structural information such as size, shape, and agglomeration state. When proper precautions are taken, it is possible to calibrate the measured intensity. It has the advantage to yield, with almost no approximation or model, physical quantities (such as volume or specific surface) averaged over the whole sample.

When a flux N_0 (counts/s) of X-ray photons is incident on a sample (volume V and thickness e_s) then a part of the flux □N(**u**, □□□□□is scattered in a direction **u** with a solid angle DW:

□N(**u**, □□) = $N_0T(e_s/V)$ d□/d□(**u**) (1)

where dσ/dΩ(**u**) is the differential scattering cross section (m^2). This cross section is characteristic of the interaction between the material and the incident beam. The scattering intensity by a sample is the differential scattering cross section per unit volume and is expressed as the inverse of a length (m^{-1}).

I = 1/V d□/d□(**u**) = □N/(N0 T es □□□ (2)

This quantity is accessible experimentally, provided the thickness e_s is known and the transmission T is measured with the scattering properties □N/(N0□□□□□□f the exact composition of the sample is known e_s can be deduce from the transmission measurement according to the following relationship: T= □N(0)/N 0 = $e^{-\mu es}$. Where μ is the absorption coefficient. It only depends on the scattering volume composition.

The experimental set-up directly measures a differential scattering cross section per unit volume if the detector geometry is perfectly defined (□□) and if the same detector is used to measure the direct beam (N0) and the scattered beam (□□). Very special set-ups enter this definition as for example Bonse-Hart Ultra Small Angle X-ray Scattering apparatus because the same punctual detector is scanned from the direct beam to the scattered beam and because DW is precisely defined by the optics before the detector. However, generally, a standard must be used to calibrate the instrument. For X-ray scattering, a practical standard is pure water. Indeed, the scattering of pure water is related to its isothermal compressibility C_T and is well defined (I=0.016 cm^{-1}). The transmission of pure water also gives the thickness as the mass attenuation coefficient for this medium is known (μ/□water) = 9,91 cm^2/g at 8 keV). Using water as a standard requires however very sensitive instruments. Indeed, if the optimal thickness of e_s=1/μ is used then DN=(N_0 DW 0.016)/(μ e^1). With a typical flux of N_0=10^7 cps and a typical instrument having DW=2.510^{-9}, it is necessary to count 1.510^{-5} cps. For less demanding signal to noise ratio, other standard materials with known d□/d□□can be used. Lupolen (I_{max}=6cm^{-1}) or vitreous carbon are usable.

General expression of the scattering intensity

For an incident radiation of wavelength I, the incident beam as a wave vector $\mathbf{k_i}$ of amplitude $||\mathbf{k_i}| = k_i = 2\square\square/\square$. The scattered wave vector $\mathbf{k_d}$ making an angle q with $\mathbf{k_i}$ has the same amplitude for an elastic scattering process and defines a scattering wave vector $\mathbf{q} = \mathbf{k_d} - \mathbf{k_i}$. Thus the amplitude of the scattering wave vector is q= 4□□sin□□□□□□/ □□. A static scattering experiment yield structural information on the dispersed phase at a typical spatial scale of 1/q. For nanoparticles, the interesting range of scattering wave vectors is 10^{-2} to 1 $Å^{-1}$. This range is observable for angles between roughly to 0.1 to 10° for very small wavelengths that are typical of X-rays (λ~ 0.1 nm).

The scattering amplitude due to the scattering volume V is

$$A(\boldsymbol{q}) = \int_V \rho(\boldsymbol{r})\, e^{-i\,\boldsymbol{q}\,\boldsymbol{r}}\, d\boldsymbol{r} \quad (3)$$

where -**qr** gives the phase shift between two scatterers separated by the vector **r** and ρ(**r**) is the density of scattering length. The density of scattering length is ρ (***r***)=$\sum_i$ ρ $_i$***(r)***b_i ρ $_i$(**r**), r_i(**r**) being the local density of electrons and b_i is the scattering length. For x-ray, the scattering length is the Thomson scattering length of a single electron b_e=e^2/(4 □□□$_0$$mc^2$) = 0.282 10^{-14} m.

The scattering intensity per unit volume is given by

$$I(q) = \frac{A(q)A^{'}(q)}{V} \quad (4)$$

Sample preparation.

No particular limitations exist for the type of media that can be analyzed by SAXS. The sample can be a solid, a liquid or a gas. For liquid or gas, it has to be put in a container that has windows in the beam path. These windows should not interact too much with X-ray. Thin plastic sheets, thin mica sheets or Berilium windows

are some typical windows. The thickness of the sample is the most critical parameter. It has to be large enough such that X-ray can interact with the matter and thin enough such that both incident beam and scattered beam can cross the sample. For each sample type, there is an optimal thickness. Indeed, from equation (1), it is seen that the quantity of scattered photons DN is proportional to eT. Where e is the sample thickness and T is the transmission. As $T = e^{\mu e}$, the optimal transmission is obtained for the maximum value of $ee^{-\mu e}$ which is obtained for e = 1/μ. For example, with a 8 keV incident x-ray beam, in the case of water (μ/□=10 cm^2/g, □ = 1g/cm^3), the maximum scattered signal will be measured for a thickness of e = 1 / (10 * 1.) = 0.1 cm; for cerium oxides (μ/□=290 cm^2/g, □ = 6.5 g/cm^3), optimum thickness falls down to e = 1/(290*6.5) = 5 μm.

Typical values of μ/r at 8keV for nanoparticles materials:

SiO_2	35.16 cm^2/g
TiO_2	111.1 cm^2/g
CeO_2	281.5 cm^2/g
AlO_2	28.32 cm^2/g
FeO_2	150.27 cm^2/g
C	4.21 cm^2/g
CdS	188.7 cm^2/g
CdSe	158.9 cm^2/g

Scattering by a nanoparticle dispersion

A typical experiment is performed in a solvent of homogeneous scattering length densit ρ_{sol} containing N nanoparticles with a homogeneous scattering length density ρ_{np}. Then for one nanoparticle, we can rewrite equation (4) as:

$$A(q) = \int_{Vnp} \rho_{np} e^{(-iqr)} dr + \int_{Vsol} \rho_{sol} e^{(-iqr)} dr \qquad (5)$$

The scattering amplitude of this system can rewritten in terms of the scattering length density contrast between the nanoparticles and solvent □$\rho = \rho_{np} - \rho_{sol}$:

$$A(\boldsymbol{q}) = \int_{Vnp} \Delta\rho\, e^{(-i\,\boldsymbol{q}\,\boldsymbol{r})}\, d\,\boldsymbol{r} + \int_{V} \rho_{sol}\, e^{(-i\,\boldsymbol{q}\,\boldsymbol{r})}\, d\,\boldsymbol{r} = \int_{Vnp} \Delta\rho\, e^{(-i\,\boldsymbol{q}\,\boldsymbol{r})}\, d\,\boldsymbol{r} + \rho_{sol}\,\delta\, q \qquad (6)$$

The last term of equation (6) in only present at q=0 and can be removed for practical purposes. The scattering amplitude of a single nanoparticle is then only dependent on the electronic density contrast between the nanoparticles and solvent. The intensity can be written as:

$$I_{np}(\boldsymbol{q}) = A(\boldsymbol{q})\, A'(\boldsymbol{q}) = \int\int_{Vnp} \Delta\rho^2\, e^{(-i\,\boldsymbol{q}\,(r\,-\,v))}\, d\,\boldsymbol{r}\, d\,\boldsymbol{v} \qquad (7)$$

for a dilute suspensions of nanoparticles, the intensity per unit volume is obtained by summation of the scattering from each nanoparticles and is often written as :

$$I(\boldsymbol{q}) = \frac{N}{V} V_{np}^2 P(\boldsymbol{q}) = \phi V_{np} P(\boldsymbol{q}) \tag{8}$$

where P(**q**) is the normalized form factor which is defined as :

$$P(\boldsymbol{q}) = \frac{1}{V_{np}^2} \int \int_{Vnp} \Delta\rho^2 \, e^{(-i\,\boldsymbol{q}(\boldsymbol{r}-\boldsymbol{v}))} \, d\,\boldsymbol{r}\, d\,\boldsymbol{v} \tag{9}$$

The form factor P(**q**) is characteristic of the shape and scattering length density contrast of the nanoparticle. For example, the form factor of a spherical particle can be obtained as a function of the scattering wave vector amplitude by :

$$P(q) = \Delta\rho^2 \left(\frac{3[\sin(qr) - qr\cos(qr)]}{(qr)^3} \right)^2 \tag{10}$$

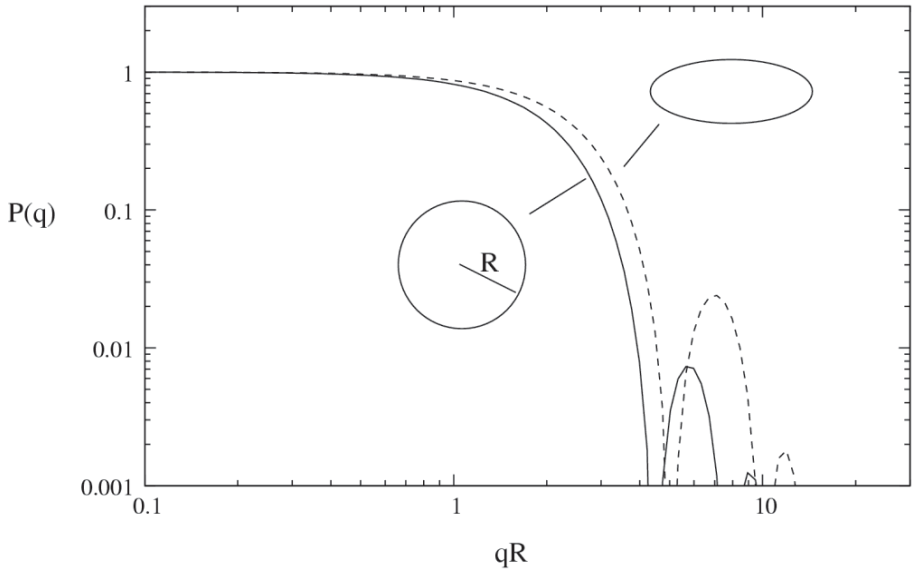


Figure 86. Shows the shape of the form factor for a sphere of 10 nm and an ellipse of the same volume.

When particles are interacting through long range forces in dilute suspensions or simply by collision in a concentrated suspension, the scattering of the different nanoparticles is no longer independent and interferences between nanoparticles must be accounted for. It can be shown that the scattering intensity of the suspension can be rewritten as:

$$I(\boldsymbol{q}) = \phi V_{np} P(\boldsymbol{q}) S(\boldsymbol{q}) \tag{11}$$

where S(q) is the structure factor accounting for the correlation between the NPs.

$$S(q) = 1 + \frac{1}{N}\left(\sum_i \sum_{j \neq i} e^{(i\,q(r_j - r_i))}\right) \quad (12)$$

A detailed quantitative analysis of the structure factor goes beyond the scope of this discussion. However, a qualitative description of the structure factor can give useful information on the state of dispersion of the nanoparticles. For a dilute suspension, S(q) = 1. For stable nanoparticle suspensions, the limit of S(q) when q approaches zero is less than 1. The value of S(0) is related to the colloidal liquid compressibility. For unstable nanoparticle suspensions, S(q) is larger than 1 when q approaches zero. The value of S(0) is proportional to the average mass of the nanoparticles aggregates. This is therefore particularly useful to immediately answer wheter or not a suspension of NPs contains aggregates or not.

Limiting behaviors of the form factor

In the limit of forward scattering and for dilute nanoparticles suspensions, the form factor can be approximated as an exponential:

$$I(q) \approx \phi V_{part} e^{\left(\frac{-(qR_G)^2}{3}\right)} \quad (13)$$

where R_G is the radius of gyration of the nanoparticle. This regime of forward scattering is called the Guinier regime and is valid for $qR_G < 1$.

In the limit of very large q, and provided that a sharp interface exists between the nanoparticles and the solution, then the following limit is observed:

$$\lim_{q=\infty} I(q) = \frac{2\pi(\Delta\rho)^2}{q^4}\frac{S}{V} \quad (14)$$

where □□ is the scattering length density contrast between the nanoparticles and the solvent, S is the total surface area of the nanoparticles and V is the scattering volume. This approximation allows the specific surface area of a nanoparticle to be measured. When the suspension is concentrated or if the solution is bicontinuous, the same formula holds. This large angle regime is called the Porod regime. The specific surface of the particles is conveniently extracted using a $I(q)q^4$ vs. q plot.

a SAXS experiment example:

Here follows a step by step description of a typical SAXS experiments aiming at characterizing the size and dispersion state of NPs in solution.

1) The stable solution of NPs is introduced in a special SAXS cell enabling thickness variation of the beam path. For a liquid sample, a volume of at least 4 ml is required. The concentration enabling a correct determination of the size and shape depends on the scattering length density of the NPs. Typically, several g/l are comfortable for oxides.
2) The sample cell is placed in the X-ray beam and the transmission is measured as a function of the beam path length. The thickness is optimized ie it is chosen such that $T=e^{-\mu e} = 0.368$.

3) An image of the scattered intensity is collected for typically 1000-4000s.
4) The thickness of the sample is then reduced by a known amount e.
5) An image of the scattered intensity with a reduced thickness is measured.
6) A special software is used to transform the bidimentionnal images into curves of the numbers of photon counts per unit time as a function of the scattering angle.
7) The scattered intensity of the NPs suspension is obtained by subtraction of the two curve.

From this curve, the size and shape of the NPs can be deduced. If the chemical formula of the NPs is known, the concentration of the suspension is also obtained.

Annex 11: Surface swab Standard Operating Procedure

Method for the evaluation of surface contamination in occupational settings where NOAA are handled

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Aim(s) or Objective(s) of SOP
This SOP describes a methodology for monitoring NOAA as surface contamination at workplaces during production and handling of nanomaterials and its potential for secondary inhalation and dermal exposures. This technique is used for sampling surfaces searching for any potential contaminant or cleaning residue present in irregular surfaces, hard-to-reach areas or heated and porous surfaces by sampling on the surface of workplaces with a dedicated material for posterior analysis.

Scope and required operating conditions

Surface swabs are used for sampling working surfaces. Any potential contaminant or cleaning residue present in irregular surfaces, hard-to-reach areas or heated and porous surfaces can be eluted from the swab and analysed down to trace levels using standard analytical techniques such as high performance liquid chromatography (HPLC), Inductively coupled plasma mass spectrometry (ICP-MS) or Total Organic Carbon (TOC) tests.

Swab sampling is applied once the handling of NOAA is finished in order to collect the NOAA deposited in different surfaces.

Recommended size of the surface area to be wiped is a 10 cm x 10 cm square to approximate the area of a worker's palm (~100 cm^2) in order to estimate the amount of contaminant transferred upon contact.

Materials and equipment needed

During the sampling, the operator must be equipped with disposable nitrile gloves which have to be changed in each sampling with the aim of avoiding any possible source of contamination.

The pad or patches can be of a great variety of sizes and shapes, depending on the area to cover or the time of exposure to monitor. To proceed with the assessment, an appropriate swab size and material regarding to the area to be tested must be selected.

The type of swab selected to perform the sampling must be one with a head made of laundered polyester knit fabric due this material provides the lowest levels of releasable particles, the highest recovery and the lowest background.

In order to preserve the integrity of clean surfaces. It is recommended to perform a swab analysis of surfaces weekly. For monitoring purposes, the frequency will depend on the regularity of the action.

Setup of experiments

The protocol followed to carry out the sampling consist on the following steps:

1. Delimit the selected area to carry out the sampling (10 cm x 10 cm) using sticky tape.
2. Unpack carefully the swab in order to avoid any contamination by contact with other surfaces.
3. Immerse the swab into a container of high-quality water and press both sides of the swab head against the side of the container in order to expel any air trapped in the fabric, and allowing the water to fully penetrate the fabric.
4. Remove the swab head of the water and drain the flat sides of the swab across the rim of the container to expel the excess of water and leaving the swab head moist. It has to be said that saturating the swab head with water may cause problems due the excess of liquid could spread the residue over the surface, avoiding the picking up of the residue.

5. Hold the swab handle to make a 30^0 angle with the surface and rub it slowly and thoroughly over the desired area 10 times to ensure an accurate and reproducible collection of residues, performing a concrete swabbing pattern that covers completely the test area (See Figure 87). The recommended pattern needs of two swabs and consists on the following steps:

   a) Swap horizontally 10 times the first side of the first swab.

   b) Flip swab and repeat ten times vertically over the same surface.

   c) Deposit the first swab in the vial.

   d) Repeat procedure with the second swab swiping the first side diagonally at 45° angles upwards ten times.

   e) Flip swab over and swap the second side diagonally in perpendicular direction downwards ten times.

   f) Deposit the second swab head in the vial for subsequent analysis.

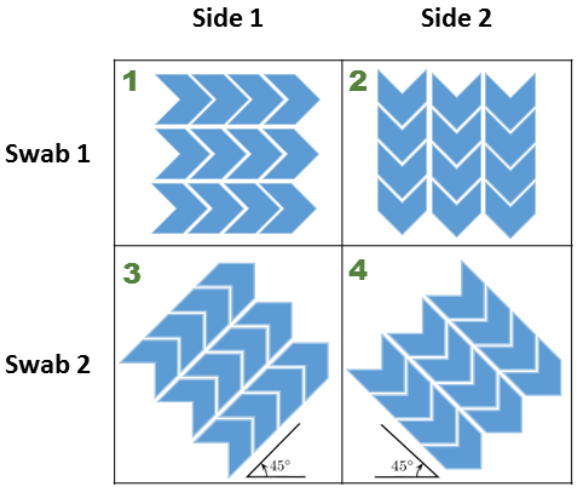

Figure 87: Schematic representation of the movements carried out in swab sampling of the surfaces

6. Collected nanoparticles are desorbed submerging the head swab in 2 mL of water under sonication conditions for 10 minutes.

7. A drop of the previous solution is placed onto a thin carbon grid and allowed to evaporate under vacuum.

8. The morphology of the NOAA picked up during the sampling was characterized by TEM.

9. This protocol has to be repeated with a second swab (except step 5) in order to obtain a blank sample.

Annex 12: Tape stripping technique Standard Operating Procedure

Method for the evaluation of surface contamination of nanoparticles in workplaces

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Aim(s) or Objective(s) of SOP
This SOP describes the implementation of a method for the evaluation of surface contamination of nanoparticles in workplaces.

Scope and required operating conditions

The scope of this protocol is to sample workplace surfaces to qualitatively determine the presence of contamination of nano-objects, their aggregates and agglomerates >100 nm (NOAA). It is also possible to semi-quantitatively assess the number concentration of the NOAA on a surface. With the tape sampling the release of NOAA from different work tasks can be evaluated. It is possible to assess if the NOAA exposure at workplaces is sufficiently controlled with safety and protection devices, if the cleaning routines are satisfactory and the potential for dermal exposure and secondary inhalation exposure.

Tape sampling is a complementary method to air measurements, and together these two methods provide a better view of the hygienic situation in workplaces where NOAA can be emitted into the work environment.

Materials and equipment needed

To collect a tape sample, ordinary clear transparent adhesive tape with a width of 15 mm (Staples Europe B.V., the Netherlands) is used (Figure 88). New sheets of plastic film, a permanent marker pen, new protective gloves of nitrile and new plastic covers are also required.

Figure 88. Tape used for collection of tape samples.

Selection of surfaces

Hard and smooth surfaces made of e.g. metal, plastic, laminate, glass, coated concrete, and coated wood, are suitable to be sampled. It is suitable to include both regular and seldom cleaned surface locations in the sampling.

Setup of experiments

Sampling procedure

Cut off a piece of tape with a length of ~15 cm and fold the ends (Figure 89). Press the sticky surface of the tape against the workplace surface to be sampled, and rub lightly to assure that the dust deposited on the surface adhere to the tape. Then, pull off the tape with a fluent and decisive movement, and place it with the sticky side down on a new sheet of plastic film and label it. Place the plastic film in a new plastic cover for storage until analysis. A new pair of nitrile gloves is used for each tape sample to be collected. Field blanks are also collected by placing the piece of new tape directly on the plastic sheet. The characteristics of the sampled surfaces is of importance and should be documented.

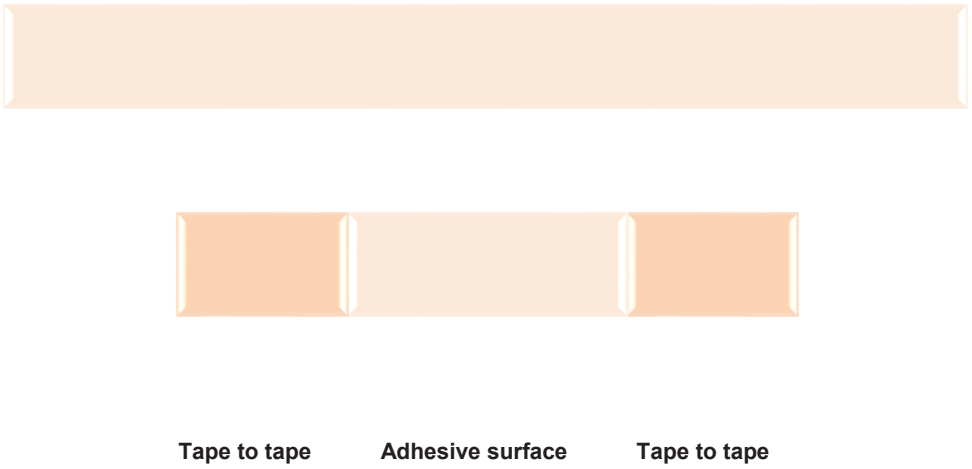


Figure 89. A piece of folded tape to be used for tape sampling.

Analysis procedure

Qualitative analysis

Prepare the tape sample for SEM analysis by removing it from the plastic film. Mount a piece of the tape (~1 cm in size) on a silicon wafer. Then coated the nonconductive tape with a thin layer of platinum on the glue side to avoid charging artefacts in the electron microscope image. The tape sample is qualitatively analyzed by SEM (FEI Nova Nanolab 600, FEI Company, USA) at a magnification of ×5000. If the SEM analysis show—based on size, shape, and elemental composition—particle characteristics of NOAA, a closer investigation is performed at a higher magnification to verify the detection. Example of SEM images of carbon nanotubes, carbon nanodiscs and GaAs nanowires can be seen in Figure 90. The elemental composition of the NOAA in the field samples can be analyzed using energy dispersive X-ray analysis (EDX; SDDXEDS, Oxford Instruments, Oxfordshire, UK). It is strongly recommended to perform SEM analysis of bulk nanomaterial before the tape sample analysis as this could be helpful when discriminating NOAA from background dust particles on the samples.

Semi-quantitative analysis

To evaluate the sampling recovery of the tape sampling method GaAs nanowires was been deposited on silicon wafers (N=5). These were then analyzed with SEM and the nanowires were manually counted. Tape sampling was performed on the silicon wafers. The tape samples were then coated with Pt and analyzed with SEM and the nanowires were manually counted. Based on these five samples the sampling recovery of the tape sampling method determined to be in average 71.5% (range 59.9-91.4%). Thus, by manually counting of NOAA collected with tape samples it is possible to semi-quantitatively estimate the number concentration of NOAA on surface areas.

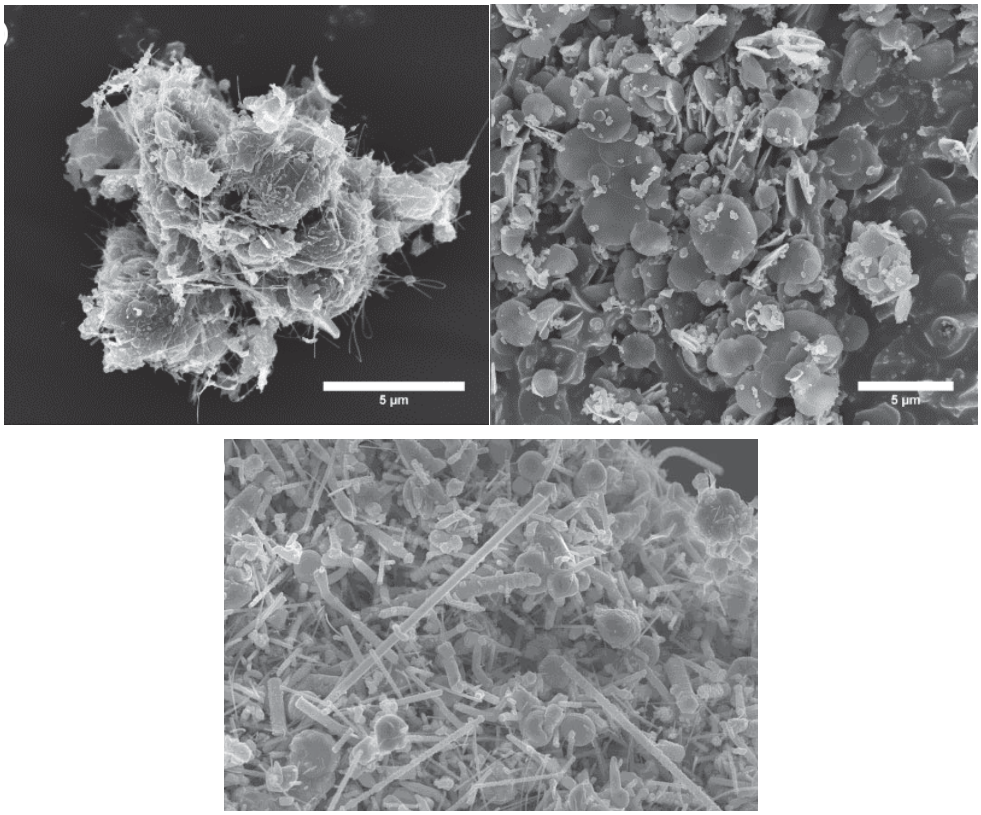
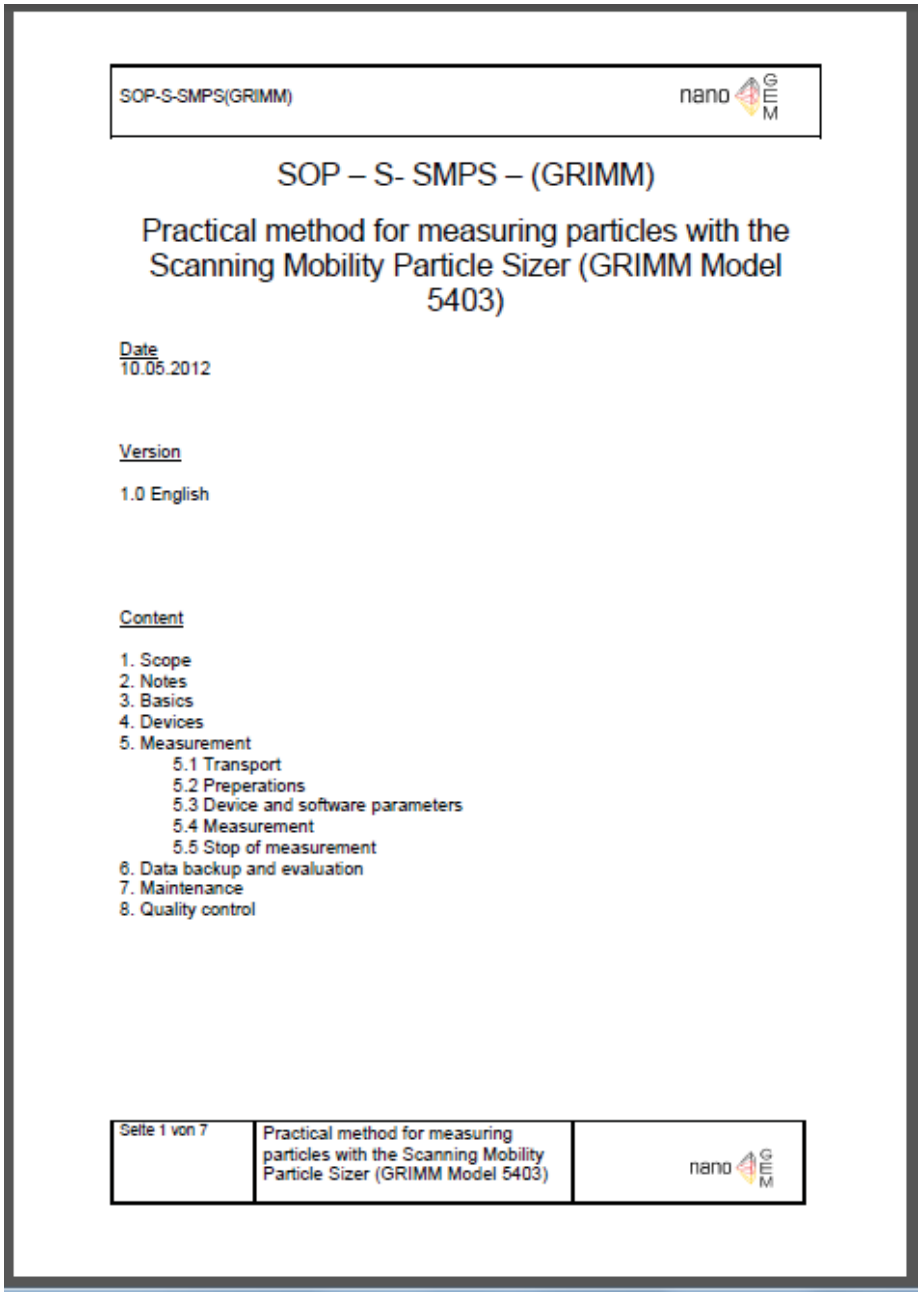


Figure 90. SEM images of surface contaminations of a) carbon nanotubes; b) carbon nanodiscs; c) GaAs nanowires.

Annex 13: SMPS (GRIMM 5403) Standard Operating Procedure



SOP-S-SMPS(GRIMM)

SOP – S- SMPS – (GRIMM)

Practical method for measuring particles with the Scanning Mobility Particle Sizer (GRIMM Model 5403)

Date
10.05.2012

Version

1.0 English

Content

1 Scope

Scope of this standard operation procedure (SOP) is the data acquisition and backup as well as the quality control of the measurement data obtained with the SMPS (GRIMM Model 5403) for the determination of the inhalative exposure to nanoscale product materials and ultra-fine aerosols in the workplace. The routinely maintenance of the devices is also covered.

2 Notes

This SOP – S- SMPS – (GRIMM) deepens the more general SOP-M-Expanded Measurement – "Measurements of inhalative exposure to nanoscale product materials and ultrafine aerosols at workplaces including the background concentration" and refers to ISO 15900 *Determination of Particle Size Distribution - Differential Electrical Mobility Analysis for Aerosol Particles".*

3 Basics

The Scanning Mobility Particle Sizer is a stationary, time-resolved measurement device for the determination of the size classified number concentration of nanoparticles. The system consists of a Differential Mobility Analyser (DMA) for the size classification of the particles, a condensation particle counter (CPC) for counting and the measurement software. The measurement data is saved by the software in a savefile on the computer (PC, Laptop) being used. It is possible to monitor the measurement online via RS232 interface. Additionally the measurement data can be saved on a data card.

During the measurement the particles are charged into a defined charge distribution and fractionized exploiting their different electrical mobility in an electrical field. Using a continuous change of the applied voltage of the electrical field a range of 5 nm to 1000 nm in a resolution of 44 (software version 1.35) / 89 (1.2.1) can be surveyed depending on the DMA type (M-DMA, L-DMA). Software 1.2.1 also makes it possible to measure monodispers aerosols with high resolution using up to 255 channels.

Due to the neutralizer installed in the DMA (alpha-radiator) the radiation protection directive as well as the handling authorization have to be followed during the handling or transport of the device. The device can only be used after a briefing by the radiation protection officer.

4 Geräte

The GRIMM-SMPS contains the following components:

- Electromobility classifier "Vienna U-Type" (Differential Mobility Analyser, DMA), alternatively L(ong) or M(iddle),
  Measurement range software version 1.35: 11.1 – 1083.3 nm or 5.5 – 350.4 nm without neutralizer AM-241 I
  Measurement range software version 1.2.1: 10.33 – 1093.3 nm or 5.15 – 351.04 nm
- Condensation particle counter (CPC), type 5.403 with butanol tank and condensate flask
- Laptop with Grimm-Software 5.477, control and data storage incl. data cable USB – serial
- Repair and spare part set with vacuum grease, sealing gasket, filter and tools, leakage test tool
- Manual, up-to-date radiation protection directive as well as the handling authorization

At this time two different version of GRIMM Software 5.477 (1.35 and 1.2.1) are being used. Comparative studies show that version 1.2.1 has a good comparability towards other devices of the same type (publication in preperation). Old measurement savefiles can be imported with the newer software,

5. Measurement

5.1. Transport

- For measurement outside the area of the facility the company where the measurement takes place and the nuclear legislative agency has to be informed of the measurement.
- The transport guidelines of radioactive sources have to be followed
- The Transport of the devices must be done horizontially without slope. If this is not possible, the saturator should be dryed beforehand (*Dryer Mode* for 12h at least). Switch power on (from *Charge Mode* press *Standby* and *Status*), empty condensate flask beforehand.
- The safety fuse has to be plugged out during the transport for the assurance of battery mode (old systems have the 5A type care fuse, new systems 6,3A safety fuse case)

5.2. Preperation

- Choose the DMA type (M/L) fitting the scope of the measurement campaign. The electrode has to be clean for DMA switch (clean with isopropanol)
- A test of leakage has to be conducted after DMA switch or cleanup (see manual)
- Check the impactor being used (choose impactor according to the DMA, check on cleaniness – malfunction can occur during the measruement due to clogging of the orifice, clean the deposition plate of the impactor and apply a thin layer of vacuum grease. See manual for impactor exchange)
- If high concentrations (~10^6 / cm^3) are being measured one might want to use a pre-impactor or dilute the atmosphere

- The SMPS system has to be acclimatized before measurement after storing the system in cold rooms or after transport in a cold environment (<10°C)
- Before measurement after transpport with a dry saturator the system has to be run without data storage (for the consistent humidification of the saturator)

5.3. Device and software parameters

- CPC and DMA must be arranged on the dedicated metal plate
- The safety fuse has to be plugged in the back side of the CPC. Without the fuse warning messages can be wrong
- Apply **empty** condensate flask at the *Condensate Outlet*
- Fill 1-butanol before measurement. Watch the green filling level indicator on the back site of the CPC
- Establish tube connection DMA – CPC

DMA		CPC
Sample Outlet (short connection with special tube)	➔	Sample Inlet
Sheath Air Inlet (yellow tube at the head)	➔	Sheath Air Outlet
Excess Air Outlet (black tube at the foot)	➔	Excess Air Inlet

- Connector DMA – CPC
- Boot up computer, log in, connect computer with CPC and look for the dedicated COM-port
- Start SMPS by switching the CPC on using the front planel. Wait for LCD-message "Warm UP?" and approve by pressing "+"
- Checking on flow rate
- Zero measurement with filter

5.4. Measurement

- Start GRIMM software 5.477 and follow instructions by software (set date/time, scheduling according to software)
- Start measurement using the software, initialization and calibration can last for up to 15 minutes
- All SMPS+C systems will conduct a selftest before every measurement. Such a selftest is composed by a leakage test, test of the DMA, check for stray current on the DMA and survey of the condensation process
- If possible, measure the aerosol directly without using a tube on the inlet. If one must use a tube, choose an anti-static sampling tube, connect it to the inlet und consider losses due to diffusion

- In small rooms in particular a carbon filter should be applied to condensate the butanol (Connector *Sample Outlet*, check the maximum periode of application, drying afterwards). Connecting a tube with access to ambient air is also possible.

Table: Error messages or failure of measurement routing and help guidlines

Error / Failure	Possible reason	Adjustment
No Connection to CPC, CPC does not react	• CPC not switched on • No USB cable attached before switching on CPC • Wrong COM port chosen when using a USB hub • Use of wrong connection cable	• Switch on CPC, restart software • Restart computer, choose right COM port • Choose right connection cable (Grimm 1.141A)
The zero test can not be finished in a reasonable time during the selftest	• System leakage • DMA contanimated • Laminator contanimated	• Test of leakage – if no leak is detected, move on, if not find leak, exchange tubes and gasket if necessary • Pull out and maintain impactor • Disassemble DMA and cleacn with isopropanol, blow out channels with clean compressed air • Disassemble neutralizer and clean carefully with compressed air (Pmax 0.5 bar) • Exchange filter • Clean laminator • If these steps are not successful, measurements with the DMA are impossible. Exchange DMA. After campaign send DMA for repair or maintenance to the manufacturer
Flow Error – termination of measurement	• Wrong impactor • Clogging of impactor • Tubes are applied wrongly • Saturation of absorption filter • Wrong outlet filter	• Exchange impactor • Detach impactor and clean • Look at tubes • Exchange / remove absorption filter • Use right filter (active carbon)

Error / Failure	Possible reason	Adjustment
Warm-Up will not finish	• Safety fuse is damaged or not applied the right way	• Exchange / Reapply fuse
Warm Up will not finish, Liquid Level Led stays red	• Butanol level in Liquid tank is low • Air bubble in tube between liquid tank and inlet	• Plug in butanol flask • Raise pressure
Warm Up will not finish, Temperature-LED stays yellow/red	• Ambient temp. >30 °C • Heating/colling is broken • Cooling liquid level is low • Broken cooling liquid membrane pump	• Measurement is not possible • Repair by manufacturer • Fill up cooling liquid • Repair by manufacturer
Measurement dows not run consistently	• Safety fuse is damaged or not applied the right way	• Exchange / Reapply fuse

5.5. Stop of measurement

- Stop measurement via software
- Save data on measurement computer (raw data, excel file of measurement data, excel file of statistical data)
- Clean device with alcohol if necessary
- Empty condensate flask

6. Evaluation

First results can be viewed using the Software (current Scan, particle number concentration, geometric mean of particle size). Software version 1.35 only shows the last and the current scan where 1.2.1 shows all scans after choosing.

Use SAA "Measurements of the inhalation exposure to nanoscale product materials and ultrafine aerosols at workplaces including the background concentration" for data evaluation

7. Maintenance

- Maintenance has to be conducted anually at the device manufacturer (GRIMM Aerosoltechnik)
- Test of "zero concentration" with zero filter periodically
- Cleaning of impaction plate and orifice of pre-impactor if necessary
- Cleaning of DMA's inner and outer electrode if necessary. If the system is used consistently this has to be done every six months, see manual.

8. Quality control

- Do and log the maintenance
- Log abnormalities during sampling and device maintenance. If possible, do frequent internal comparative measurements with suitable aerosol measurement devices.