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Revised OECD methods for determination of physicochemical NM properties

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1 Description of task

According to the NANoREG Description of Work, deliverables D2.3 and D2.9 cover the first paragraph of Task 2.3, which refers to studying the applicability of some OECD test guidelines to nanomaterials characterization: 'all procedures for establishment of the physicochemical information requested by REACH will be evaluated and revisions validated'. The assessment results for the various OECD test guidelines were presented in D2.3. In this report, D2.9, the methods mentioned in D2.3 are further evaluated and adapted to the specific properties of nanomaterials. The experimental evaluation of the following protocols and test guidelines are reported: TG 109 (relative density), TG 110 (granulometry), TG 105 (water solubility), and TG 112 (dissociation constant in water), which may be coupled with TG 108 (complex formation in water). In addition to the existing OECD guidelines, a method for the determination of dispersibility was elaborated.

2 Description of work & main achievements

2.1 Summary

The OECD Guidelines for the Testing of Chemicals are a collection of internationally accepted and relevant test methods for determining the safety of chemicals and chemical preparations, including pesticides and industrial chemicals. These guidelines cover the physicochemical properties of chemicals, environmental effects, degradation and accumulation in the environment, human health effects, as well as other areas. In D2.3 '*Experimental evaluation of OECD methods for analysis of physicochemical MNM properties*', important OECD guidelines relevant for particle characterisation were assessed for their applicability to nanomaterials (NM). In D2.9 the modification of the OECD guidelines proposed in D2.3 were experimentally developed and evaluated. Most of the modified technical guidelines are now ready for validation in inter-laboratory tests.

Table 1-1 shows the situation of the different technical guidelines after assessment and modification by members of WP2:

For **TG 109** additionally to the existing types of densities an effective density (density of the agglomerates or skeletal density) was added. This effective density is of high importance for the characterisation of the nanopowders in liquid dispersions especially for the prediction of dosage.

TG 110 is focussing on the determination of the hydrodynamic diameter of NM. The work in WP 2 shows that dynamic light scattering (DLS) is the most convenient method, but other methods based on the determination of the sedimentation rate are also useful. In all cases, it is of utmost importance that the NANoREG protocol for powder dispersion is applied when preparing the suspension.

TG 105 was the most difficult technical guideline to adapt. Depending on the elements to detect, existing analytical methods based on atomic spectrometry and electrochemistry are useful. It is important to note that a separation of the nanoparticles from the solution is necessary for some methods (ICP-MS) and not for others (conductivity measurement, voltammetry). It is evident that methods, which need a total separation of the undissolved nanoparticles, are more difficult to apply.

TG 115, surface tension of solution, can be applied directly to suspensions. Several publications exist which show in a convincing way that the well-established method described in the TG are also useful for suspensions.

TG 112 is not applicable to nanoparticles but the Zeta-potential (surface potential at the shear plane of particles in suspension) is as important as the dissociation constant of molecules in water. A protocol for the determination of the isoelectric point was therefore established. This parameter is of high importance to understand colloidal properties as well as adsorption of biomolecules.

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TG 108 also requires modifications in order to be applicable to nanoparticles, the most important of them being the replacement of the complex formation stability constant by the determination of the adsorption capacity and affinity of nanoparticles for dissolved heavy metal ions.

Table 1-1 List of OECD TGs assessed by WP2 and pre-assessment results from D2.3. The TG in bold are treated with high priority, the underlined TG's were treated with lower priority)

OECD Technical Guideline	Appropriate for NMs	Modified protocol established	Future work
TG 109 (relative density)	Partially	YES New protocol for effective density	Validation Validation
TG 110 (granulometry)	Partially	Yes, including dispersion protocol (CLS, DLS and SEM (image analysis))	Validation
TG 106 (sorption-desorption)	NO	From a thermodynamic point of view not possible	
TG 105 (water solubility)	Partially	YES Protocols for different analytical methods established	Validation
TG 115 (surface tension of aqueous solutions	YES	No modification necessary beside the replacement of solution by solution/suspension	
TG 107/117/123 (*n*-Octanol-water partition coefficient)	NO.	From a thermodynamic point of view not possible	
TG 112 (dissociation constant in water)	NO	Protocols were developed for other more relevant endpoint such as isoelectric point.	Validation
TG 108 (complex formation in water)	Partially.	Guideline modification for focusing on the adsorption of trace metals on NMs.	Validation
Dispersibility		New protocol developed	Validation

The discussion in WP 2 shows also that the **dispersibility** of nanoparticles is still not well defined, and no protocol exists for its determination. Based on existing protocols for dispersion of nanoparticles and size measurement, WP2 has developed a first draft protocol to determine in a reproducible manner the dispersibility of nanoparticles in water.

Finally as mentioned in Delivery 2.3 the OECD guidelines **TG 106** and **TG 107/117/123** are for thermodynamic reasons not applicable to nanomaterials.

In this report the new protocols developed for the mentioned technical guidelines are described and their application to some of the NanoReg standard powders reported. In a next step, the protocols will be applied to the other standard powders from the Nanoreg project, and finally the protocols have to be validated and integrated into the OECD guidelines. SOP's for the

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determination of Granulometry, Density (true and effective), water solubility, dispersibility and Zeta potential measurement were developed and up loaded to CIRCABC C-NANoREG results > NANoREG developed improved SOPs and Methods > WP2 developed improved SOPs and Methods .

2.2 Background of the task

The OECD Guidelines for the Testing of Chemicals are a collection of internationally accepted and relevant test methods for determining the safety of chemicals and chemical preparations, including pesticides and industrial chemicals. These guidelines cover the physicochemical properties of chemicals, environmental effects, degradation and accumulation in the environment, human health effects, as well as other areas. The OECD test guidelines are a unique tool for assessing the potential effects of chemicals on human health and the environment. In D2.3 'Experimental evaluation of OECD methods for analysis of physicochemical MNM properties', important OECD guidelines relevant for particle characterisation were assessed for their applicability to nanomaterials (NM).

The summary of the results elaborated in D2.3 are compiled in Table 2-1 in a slightly adapted form taking into account the discussions and modifications during the work in the last years. Most of the problems have their origin in the fact that nanomaterials form colloidal dispersions (not true solutions). The TG in bold are treated with high priority, the underlined TG's were treated with lower priority whereas TG 106 and TG 107/117/123 were not further treated, following the recommendations of Deliverable 2.3.

In addition to the mentioned test guidelines in Table 2-1, two drafts for additional guidelines were elaborated: i) measurement of effective density (an important parameter for the determination of the deposited dose) and ii) dispersibility.

2.3 Description of the work carried out

2.3.1 *TG 109 "Density of Liquids and Solids", (EPFL)*

OECD TG 109 is valid for 4 types of density:

a) Density (bulk material, true density)
b) Relative density (D20/4, density at 20 °C relative to that of water at 4 °C), which may be used to compare different chemicals and is not relevant for NM
c) Pour density (powders)
d) Tap density (powders)

D2.3 presented an assessment of the methods proposed in TG 109 for the determination of the densities of solids, especially nanosized powders. The only applicable method for true density determination was found to be the air comparison pycnometer. For nanosized powders, our own preliminary tests, presented in D2.3, as well as published results, showed two possible reasons for a deviation from the expected true density: i) increasing importance of hydroxyl layers on the surface of the particles with decreasing size and ii) influence of the gas displacement process. The protocol for true density determination was therefore slightly adapted.

For the determination of pour and tap densities, the assessment presented in D2.3 showed that all of the methods proposed in TG 109 are applicable. Therefore, no further work was carried out for the determination of these two types of densities.

Agglomerate density is a key parameter for several methods proposed to evaluate the toxicity of nanoparticles. As mentioned in D2.3, the determination of the density of agglomerates has to be

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included in the OECD guideline for density measurements. We propose the development of a method and SOP based on the recent work of DeLoid et al. (2014).

Table 2-2 Results of OECD TGs evaluation (adapted from deliverable 2.3).

OECD Technical Guideline	Appropriate for NMs	Proposal	Future work
TG 109 (relative density)	Verification necessary	Protocol evaluation.	Evaluation of the measurement of the density of agglomerates
TG 110 (granulometry)	OECD methods only partially applicable	Completely new protocols (CLS and DLS) are proposed	The proposed protocols will be evaluated and reported in D2.9
TG 106 (sorption-desorption)	Not applicable to nanomaterials	Observation of the literature	
TG 105 (water solubility)	Not directly applicable. Requires modifications.	-Kinetic study. -Control of variables such as pH or dissolved gases. -Protocol for dispersibility determination.	Modifications to solubility protocol will be evaluated and reported in D2.9. Relevant SOPs for *in vitro*, *in vivo* and environmental dissolution testing will also be presented in Task 2.4 (D2.6, D2.7 and D2.8) Dispersibility protocol will be evaluated and proposed as new protocol
TG 115 (surface tension)	Modifications necessary.	This protocol is considered relevant only for some NM.	Modifications will be evaluated and the relevance will be analyzed.
TG 107/117/123 (*n*-Octanol-water partition coefficient)	Not applicable.	It should be replaced by the determination of hydrophilicity/hydrophobicity.	Evaluation of new methods for testing hydrophilicity/hydrophobicity
TG 112 (dissociation constant in water)	Not considered adequate for its application to NMs in its current form	Develop protocols for other more relevant endpoints such as dissociation of water and hydration, surface acidity and isoelectric point.	Development of new protocols, especially for determination of isoelectric point
TG 108 (complex formation in water)	Not considered adequate for its application to NMs in its current form, but a similar protocol can be useful to determine NM as vector of toxic metals.	Guideline modification for focusing on the adsorption of trace metals on NMs.	Protocol evaluation with different metals in several conditions.

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2.3.2 *TG 110 "Granulometry"*

Partner Embrapa Instrumentação (Brasil) had modified the PROPESCT dispersion protocol for size measurement of the NMs from aqueous suspensions so that the Z-average values of the NMs were substantially improved. Typically, 10 mg of each NM was weighted and added to 20 mL of ultrapure water (Milli-Q trademark; resistivity of 18.2 MΩ cm at 25°C) in a beaker of 25 mL. The dispersion was sonicated using an ultrasonic digital sonifier Branson, model 450, Tapped Step Horn 1/2" Tip Diameter, operating at amplitude of 50%. The sonication time was varied in 1, 2 and 3 minutes. A new batch of NM suspension was used for each sonication time. The beaker was kept in an ice bath to avoid heating during the sonication treatment but even adopting this method, samples sonicated for 3 minutes suffered slight warming. After the sonication process, 1 mL of NMs suspension diluted to 50 mL of milli-Q water so that final 1 mg L^{-1} suspensions were produced. It is important to note that this dispersion protocol is different from the NanoReg dispersion protocol on calibration of the ultra sound equipment, much shorter sonication time). Samples for size distribution measurement by SEM were prepared in the following manner: Silicon wafers were firmly fixed on SEM sample holders using a conductive silver paint, and then maintained at 70°C in a laboratory incubator. Immediately after the sonication treatment, one drop of the NM dispersion was poured on the hot silicon wafer for fast drying, so that nanoparticle agglomeration was minimized. Drying took place at room temperature. More details are given in Annex 7.3. The comparison of hydrodynamic diameter and primary particle size determined by SEM shows that the powder is still strongly agglomerated after the dispersion process. The number of particles counted is still insufficient so that the result cannot be used for a comparison with the results presented by D2.10.

2.3.3 *TG 105 "Water Solubility", LEITAT, UdL, NRCWE*

According to D2.3, OECD TG 105 (water solubility) requires some revisions for application to NM. Water solubility is addressed as a key property, but there is some confusion in relation to terminology, as it is not always clear whether water solubility or dispersibility is addressed (Christensen 2012; Hankin et al. 2011).

NM water solubility is related to the NM hydrochemical reactivity and describes the dissolution of a NM into ions owing to the ionization of surface groups. Water dissolution kinetics is mentioned by several references as a key parameter, as well as dispersion stability, in RIP-oN2 and VCI (VCI 2008; Hankin et al. 2011). In contrast, NM dispersibility is related to the amount (or mass concentration) of a NM that can remain suspended in water as undissolved particulate material for a certain time and under fixed conditions, showing a certain particle size distribution in a defined liquid after the particles have undergone a dispersion process.

Water solubility is considered one of the most relevant parameters for a number of endpoints because it can determine the mobility of a test substance and its availability, exposure and fate within human and environmental compartments. Moreover, it is used to derive other environmental parameters (i.e. K_{ow}, K_{oc}, and Henry's law constant) or as input for some QSAR models. Solubility is also relevant for the regulatory classification of the nano-forms of a given material (*e.g.*: the paradigmatic case of ZnO in the cosmetic regulation [SCCS 2013])

It is our understanding that both water solubility and dispersibility are key properties for NM. Therefore, the current OECD TG 105 should be modified for application to NMs, and a new TG that considers NM water dispersibility should be developed.

2.3.3.1 Definitions

Water solubility

'The solubility of a substance in water is specified by the saturation mass concentration of the substance in water at a given temperature. The solubility in water is specified in units of mass per volume of solution. The SI unit is kg/m3 (grams per litre may also be used)' (see Regulation (EC) No 440/2008, A.6, section 1.2).

The original OECD TG 105 (water solubility) addresses the '*solubility in water of essentially pure substances which are stable in water and not volatile*'. As reported in the guideline, before determining the water solubility, it is useful to have some preliminary information about the

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substance, such as structural formula, vapour pressure, dissociation constant, and hydrolysis as a function of pH. Unfortunately, many of these parameters are not available or easy to determine for NM; moreover, these parameters can vary according to NM size, which can limit the application of existing data for similar chemical substances via a read-across approach. Only in those cases where NM is well defined in terms of chemical composition, crystallinity primary and polydispersity, there are thermodynamic relationships available to correlate solubility with parameters such as primary particle size (through the Ostwald-Freundlich equation), pH, temperature and salinity. However, these correlations have been tested in a limited number of NMs [Galceran 2012] Moreover, the above definition for solubility of a single substance in water is not applicable to substances that are multi-component or UVCB (unknown or variable composition, complex reaction products, or biological materials) substances. In these cases, instead of the water solubility, it is possible to determine 'the composition of the aqueous solution formed at equilibrium under a defined set of conditions'. However, equilibrium of all the components may not be achieved; therefore, it is recommended to establish and monitor some relevant experimental conditions, such as the amount of substance per unit volume of water, pH, temperature, time, type of agitation, presence of oxygen, and lighting conditions, which can play a crucial role in the measured water solubility.

2.3.3.2 Existing methods

As a result of the work carried out in Tasks 2.3 and 5.2, a critical review paper was released [Tantra 2016], addressing the different analytical methods currently available to measure solubility of nanomaterials and classifying them according to an array of criteria such as instrument and operator costs, sensitivity, selectivity, accuracy of measurement in complex matrices, etc. The general conclusion is that there is no generally accepted method for NM solubility testing able to meet all the analytical requirements as defined under *e.g.* the cosmetic regulation. One of the reasons is that the wide variety of combinations of solid-liquid separation methods and elemental analysis techniques lead to the measurement of different dissolved fractions (*e.g.*: total dissolved species below an arbitrary molecular weight cut-off measured by ultrafiltration plus atomic spectrometry; free dissolved ions measured by potentiometry or voltammetry without particle separation step, etc.). This work points out the urgent need for inter-laboratory multi-method comparisons with well-characterized NMs.

In TG 105, two methods are described for determination of water solubility: the column elution method and the flask method. Among existing methods, the OECD TG on transformation/dissolution of metals and sparingly soluble metal compounds (OECD 2001) could also be a suitable method, if followed by a proper filtration step to separate NM from their ionic fraction. In its current form, TG105 does not specify a protocol for the assessment of the efficiency in the solid/liquid separation step, nor the requirements of the elemental analysis technique and the relevance of the different chemical fractions of dissolved material (free metal ion, low molecular weight soluble complexes, metal bound to macromolecules, etc.). This poses a challenge for the application of solubility testing to complex matrices (in vitro culture media, in vivo compartments, ecotox media, etc.).

Column elution method

The column elution method 'is based on the elution of a test substance with water from a micro-column which is charged with an inert support material, previously coated with an excess of the test substance. The water solubility is given by the mass concentration of the eluate when this has reached a plateau as a function of time'. The application of this method to NM has many limitations:

- The loading of NM on an inert support is possible in principle, but the effect of NM aggregation during loading on the substrate should be evaluated and taken into account because strong size dependent effects are usually present when handling NM.
- The loading of NM on the substrate will expose less of the NM surface to the solvent, making it difficult to correlate the NM surface area and NM water solubility.
- The retention of NM on the column inert support may result in clogging related to the small size of the NM.
- Finally, proper filtration of the eluate is recommended to avoid the presence of any undissolved fraction of the NM before the determination of the substance concentration by an appropriate analytical technique.

It was proposed to disregard the column method due to practical considerations.

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Flask method

In the flask method, '*the substance (solids must be pulverized) is dissolved in water at a temperature somewhat above the test temperature. When saturation is achieved, the mixture is cooled and kept at the test temperature. Alternatively, and if it is assured by appropriate sampling that the saturation equilibrium is reached, the measurement can be performed directly at the test temperature*'. Subsequently, the mass concentration of the substance in the aqueous solution, which must not contain any undissolved particles, is determined by a suitable analytical method.

The application of the flask method to NM is possible with some modification/refinement. The term 'dissolved' should be changed to 'dispersed'. As the saturated condition cannot be reached according to the classical definition, preliminary tests should be performed to establish the substance concentration and equilibration time. In the case of ZnO NMs, the previous equilibration of dispersions at a larger temperature is discouraged, as this material shows a decrease in solubility with temperature (the dissolution of ZnO is an exothermic process). A strict control of pH is absolutely necessary for some NMs like ZnO and Ag, whose dissolution behaviour is strongly dependent of this variable, which strongly discourages the use of indicator strips recommended in the original TG. The presence of dissolved gases (CO_2, O_2) is also an important factor to be considered in the protocols due to its relevance for redox processes, precipitation of concomitant solid phases, and its influence on pH. For application of the flask methods to NM, a solid/liquid separation step is required to avoid the presence of any undissolved particles when assessing the mass concentration of the dissolved fraction of the substance in aqueous solution (except for some voltammetric techniques such as AGNES, where the solid/liquid separation step is not necessary). Different membrane filtration technologies are available to provide specific separation of NM from their dissolved fraction and solvent. These techniques include reverse osmosis, nanofiltration, and ultrafiltration, which differ in the membrane material, the membrane pore size cut-off, and the pressure required for filtration through the membrane. In the revised TG, we separated NM from their dissolved fraction using an ultrafiltration technique that employs a centrifugal filter device (Amicon® Ultra-15 Centrifugal Filter Device) and a filter membrane of low binding regenerated cellulose (Ultracel) with a molecular weight cut-off (MWCO) of 3 kDa, which corresponds roughly to a 1.5–3 nm size cut-off.

D2.9 presents the results of the experimental evaluation of the adapted flask method reported in OECD TG 105, followed by a filtration/centrifugation step, and ICP-MS analysis of the filtrate, which is useful for the determination of the water solubility of NM. A comparison with a voltammetric method (AGNES, without solid-liquid separation) is also provided, in the case of ZnO.

2.3.4 *TG 112 "Dissociation constant in water" and TG 108 "complex formation in water"*

According to D2.3, OECD TG 112 (dissociation constant in water) might be applicable under some circumstances or to some classes of manufactured NM (OECD 2009). This test guideline is currently referenced in the REACH Guidance (ECHA 2012); however, a number of alternative methods are also suggested. The original OECD TG protocol 'requires the knowledge of the stoichiometry of the dissociation reaction and a measure of the concentration of the associated and undissociated form of the chemical substance' under testing. This principle is hardly applicable to NM, as the surface of a NM, similar to that of a macromolecule, possesses a huge number of acidic/basic sites; therefore, as in the case of proteins, the net charge of the NM will result from the dissociation of many acidic/basic sites. For this reason, other specific endpoints, such as the surface charge/isoelectric point (IEP), surface acidity/basicity, and presence of redox active groups on the NM surface, could be considered meaningful.

The measurement of the zeta potential over a pH range to determine the NM IEP has been identified as a relevant endpoint for various regulatory needs, including environmental and human fate and exposure. In fact, the zeta potential of a NM is one of the factors that determine whether particles form a stable dispersion/suspension for a certain time or they agglomerate, aggregate, settle, or flocculate. For this reason, a new TG focused on IEP determination using a zeta potential versus pH titration is proposed.

A revision of TG 108 was recommended in D2.3. In this revision, the concept of stability constant of formation in the original TG (which only applies to complex species formed between metal ions and well-defined chemical substances) was replaced by an interpretation based on adsorption phenomena. Moreover, the classical polarographic method recommended in the TG does not take into account many recent developments in electrochemical techniques for the detection of trace metals, which are more accurate and require essentially the same equipment.

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2.3.4.1 Definitions

Adsorption isotherm is a graphic representation showing the amount of solute (the test metal ion) adsorbed by an adsorbent (the NM) as a function of the equilibrium concentration of the solute. This relationship is quantitatively defined by some type of partition function or adsorption isotherm equation that is statistically applied to the experimental adsorption data to allow interpolation/extrapolation and modelling of the adsorption process (e.g., effects of VSSA, amount of NM, etc.).

The experimental procedure to obtain an adsorption isotherm essentially consists of batch equilibrium experiments where a known amount of solute is equilibrated with a known amount of NM. The use of an elemental analysis technique able to probe the amount of "free" (i.e., non-adsorbed) solute at equilibrium plus the application of mass-balance relationships lead to the calculation of the dataset of values c_M vs. q, where c_M and q stands for the concentration of free metal and the loading (amount of adsorbed metal per mass/are unit of NM), respectively. The use of elemental analysis techniques such as atomic spectrometry will require a solid-liquid separation step (like in TG 105), whereas others (such as the voltammetric technique AGNES) can be performed directly in the stirred NM dispersion with negligible interference from the presence of solid particles [Galceran 2014].

Although many different mathematical equations can be used to fit the experimental adsorption isotherm, the simplest and most commonly used is the Langmuir equation:

$$\frac{q}{q_{\max}} = \frac{K_L c_M}{1 + K_L c_M}$$

Where q_{max} and K_L represent, respectively, the maximum loading and the Langmuir binding constant. This equation has the additional advantage of being formally equivalent to the use of a mass action law with a stability constant, as originally stated in the TG 108.

The procedure followed to obtain the adsorption isotherm of a given couple of metal ion and NM must take into account several considerations on the effects of equilibration time, mass/volume ratio, temperature, pH, and ionic strength. These considerations are conveniently addressed in *e.g.* the EPA guidelines for the testing of chemical adsorption on soil components [EPA 1992].

Zeta potential is the potential difference between the dispersion medium and the stationary layer of fluid attached to the dispersed particle. The zeta potential is caused by the net electrical charge contained within the region bounded by the slipping plane, and also depends on the location of that plane. The zeta potential or electrokinetic potential is positive if the potential increases from the bulk of the liquid phase towards the interface. When calculating the electrokinetic potential from electrokinetic phenomena, it is often assumed that a sharp shear plane separates the liquid adhering to the solid wall and the mobile liquid. However, if there is no reliable information on the values of the permittivity and the viscosity in the electrical double layer close to the interface, the calculation of the electrokinetic potential from electrokinetic experiments remains open to criticism. It is therefore essential, in all cases, to indicate which equations have been used in the calculation of the zeta potential (IUPAC 1968).

Zeta potential titration of the NM dispersed in a solution of KNO_3 10^{-2} [mol/L] over a pH range allows the determination of the NM **isoelectric point**, the pH value at which the NM net electric charge is zero and the NM possess a lower colloiadal stability.

2.3.5 Draft for a TG "Dispersibility", EPFL

The dispersibility of a nanopowder is of crucial relevance for other endpoint determinations and for standardized protocols, at the same level that solubility is determinant for traditional chemicals. For example, the dispersibility determines the particle size and size distribution, specific surface area, agglomerate density, dosimetry, and biodistribution. At the meeting in Rotterdam, Nov 2014, the members of Task 2.3 of WP 2 discussed the necessity of introducing guidelines for the determination of the dispersibility of nanoparticles and their colloidal stability.

2.3.5.1 Definitions:

In general, a **dispersion** is a two-phase system in which discontinuities of any kind (solid, liquid, or gas) are dispersed in a continuous phase of a different composition or

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state (Hackley 2001). More specifically, in the field of materials science and NM, the term dispersion is used to describe a *suspension of solid particles in a liquid medium*. In the following, we will discuss only suspensions, and the term suspension is used for all types and sizes of particles as dispersed phases. The term sol, in which the dispersed phase is <1 µm (colloid) and the dispersion medium is a liquid, is not used. The term sol is reserved for the case of a sol-gel process, where the sol is a liquid with dissolved molecules.

Stability of a Suspension (colloidal stability): Suspensions that do not aggregate at a significant rate are said to be colloidally stable. As colloidal stability is a form of kinetic stability, it is considered a metastable thermodynamic state. A suspension may exist for an appreciable length of time and therefore exhibit kinetic stability. A suspension that has sufficient kinetic stability prevents the occurrence of significant aggregation, as measured over a relevant time frame. Stability may be ascertained by various experimental parameters, such as particle size, turbidity, or sedimentation as a function of time and zeta potential.

The **dispersibility** of a powder can be defined by the particle size (mean and distribution) in a defined liquid after the particles have undergone a dispersion process. Moreover, the dispersion process has to be achieved using an ultrasound treatment. Although the term 'dispersibility' is not well defined, good dispersibility means that a stable dispersion is rapidly formed. The characteristics of a good dispersible powder are a high degree of dispersion (particle size in the dispersion is as near as possible to the primary particle size) and a high rate (low energy input) for dispersion of the powder.

2.3.5.2 Existing methods

Several methods have been developed for the determination of dispersibility, but these methods are mostly for particle sizes of micrometres and larger. In addition, the existing standards, such as 'Standard Test Method for Percent Dispersibility, Active Standard ASTM E1945', were developed for such powders. This test method is used to determine the percent dispersibility of dry pesticide formulations and is not applicable to NM. Similarly, ISO 8780-1 'Pigments and extenders—Methods of dispersion for assessment of dispersion characteristics', which describes the dispersibility of pigments larger than 1 µm, is not applicable to NM.

2.3.5.3 Determination of the "Dispersibility of Nanopowders"

The dispersibility of a NM could be expressed as the relative size D_{BET}/D as a function of the applied dispersion energy, where D is D_{50} measured by DLS and D_{BET} is the particle size calculated from the specific surface area and the known density of the primary particles.
To measure the dispersibility of a NM, the NANoREG procedure 'The ENPRA dispersion protocol for NANoREG', Version 1.0; Date: 11 July, 2014, including the calibration process described in the NANoREG SOP for probe-sonicator calibration of delivered acoustic power and de-agglomeration efficiency for in vitro and in vivo toxicological testing is a very useful base. The size and size distribution measurements have to be carried out following the 'NANoREG SOP for Measurement of Hydrodynamic Size-Distribution and Dispersion Stability by Dynamic Light Scattering (DLS)'. Combining these methods and carrying out dispersion of the powder with different amounts of energy input will allow determination of the powder behaviour during the dispersion process. In contrast to the NANoREG dispersion method, the aim of this procedure is not an optimal or reproducible dispersion, but rather the characterisation of the powder deagglomeration behaviour during the dispersion process. The use of D_{BET} is justified because the particle size calculated using the measured specific surface area is not or is only negligibly influenced by the degree of agglomeration, and this value therefore corresponds to the primary particle size. If D_{BET} is not available, the mean size measured by TEM, D_{TEM}, could be used.

The following results are expected:

- The dispersibility will be <1 if the mean particle size measured by DLS is much larger than D_{BET}. In such cases, the particle (agglomerate size) does not change significantly during ultrasound treatment.
- The dispersibility will be 1 if the mean particle size after ultrasound treatment is similar to D_{BET}.
- The slope of the change of the dispersibility with the energy applied (or specific energy if different volumes and concentrations are used) by ultrasound treatment (dD/dE) and the ultra sound energy input at the maximum of the dispersibility. As calibrated ultrasound equipment is used, the energy input could be replaced by the sonication time.

It is evident that parameters such as solvent type, pH (zeta potential), surfactant concentration, and ionic strength have to be known and must be mentioned in the measuring protocol.

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2.3.6 *TG 106 (sorption-desorption)*

The conclusion presented in Delivery 2.3 was confirmed by a publication from Nickel et al. [Nickel 2015] : "…The OECD test guideline 106 is designed for the testing of the adsorption behaviour of soluble chemicals to a soil matrix. Since the test is designed to determine adsorption, sedimentation of agglomerated ENM will be determined wrongly as adsorption. […] The test procedure "separation of the soil matrix from the supernatant by filtration or centrifugation", as described in the guideline, is not useful for the testing of ENM because the ENM suspended in the soil solution will be separated, too. Hence, no differentiation of adsorbed or agglomerated ENMs can be made using the OECD test guideline 106 and no valid information can be deducted for adsorption coefficients and isotherms. Therefore, it is concluded that the OECD test guideline 106 is not applicable for testing of engineered nanomaterial". This conclusion is also confirmed by Cornelis [Cornelis 2015].

2.3.7 *TG 107/117/123 „n-Octanol-water partition coefficient"*

The literature search has not shown any new and relevant paper regarding the n-Octanol-water partition coefficient of nanoparticles. The conclusion given in Deliverable 2.3 "not applicable" is therefore still valid.

2.3.8 *TG 115 „Surface tension of aqueous solutions""*

In deliverable 2.3, TG 115 was not investigated in detail. A literature search has been carried out and it turns out that OECD Guideline TG 115 can be also applied to suspensions with nanoparticles. It is shown that nanoparticles distributed in the solution could influence the surface tension. The proposed equipment and measuring method mentioned in TG 115 can be applied to suspensions as well [Tanvir 2012; Dong 2003].

2.4 Results

2.4.1 *TG 109 „Density"*

2.4.1.1 True density

To evaluate the influence of the adsorption of organic or water molecules on the surface of nanoparticles on the true density, the theoretical densities of spherical gold nanoparticles with 1, 2, and 3 nm thick adsorption layers were estimated. Figure 2-1 shows that the influence of the adsorbed molecule layer is very important, especially for small particles (diameter < 20 nm).

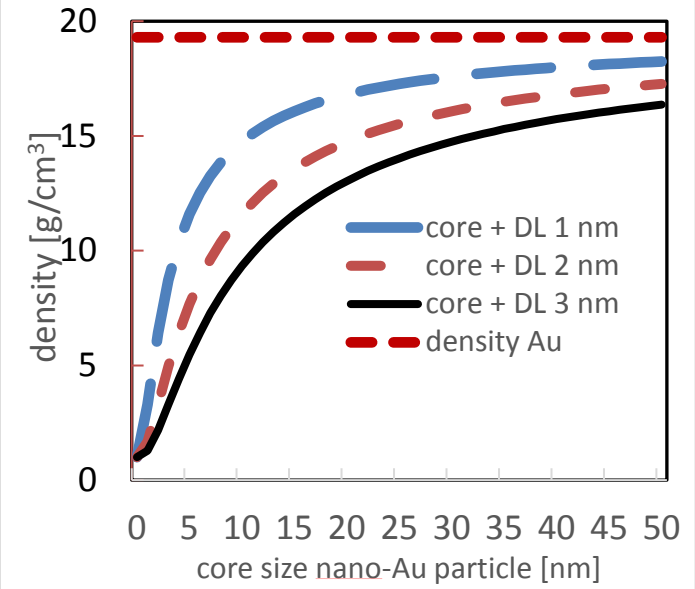

Figure 2-1 Influence of the thickness of a layer of adsorbed molecules (organic or water) on the measured density of gold nanoparticles.

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To test this assumption, the density of titania (TiO_2-P25 Lot PSI2818, Evonik) was measured with a Micromeritics AccuPyc 1330 pycnometer under two drying conditions: overnight at 60 °C or 120 °C in a laboratory oven. The measurements were carried out on 4–5 days with 8 repetitions per day.
Figure 2-2 and Figure 2-3 show the measured densities (4 samples dried at 60 °C and 5 samples dried at 120 °C, each with 8 measurements). In addition, the expected densities, calculated from the phase composition and the density of rutile and anatase, and the mean value of measurements 5–8 are also indicated (measurements 1–4 were not considered because they were too unstable). The figures show very clearly that during 8 consecutive measurements no stable values were obtained. Decreasing density values were observed during the measurement cycle. This decrease in density is especially pronounced during the first 5 measurement cycles. Moreover, the scatter of the results obtained over 1 week is large. Interestingly, the first 2 measured values are higher than the expected value. The samples dried at 120 °C show greater scatter for all 5 measurements and a continuous decrease of the mean values. Only one measurement gives a stable density.

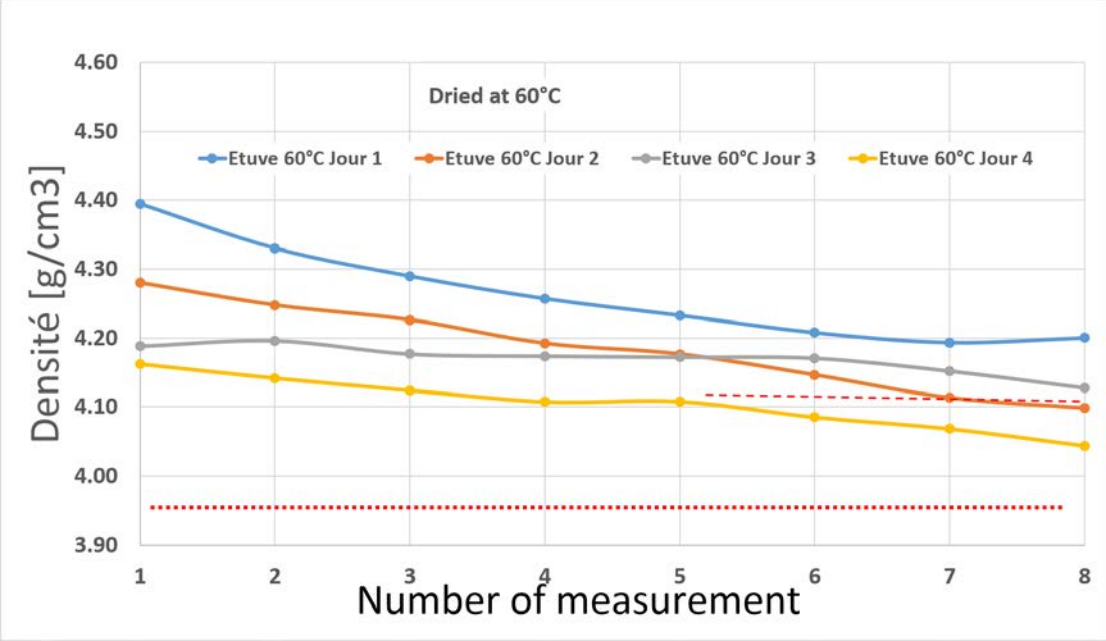

Figure 2-2 Density of TiO_2 (P25) powder dried overnight at 60 °C measured with a He-pycnometer. Dotted line: expected density (3.95, mixture of rutile and anatase), dashed line: mean.

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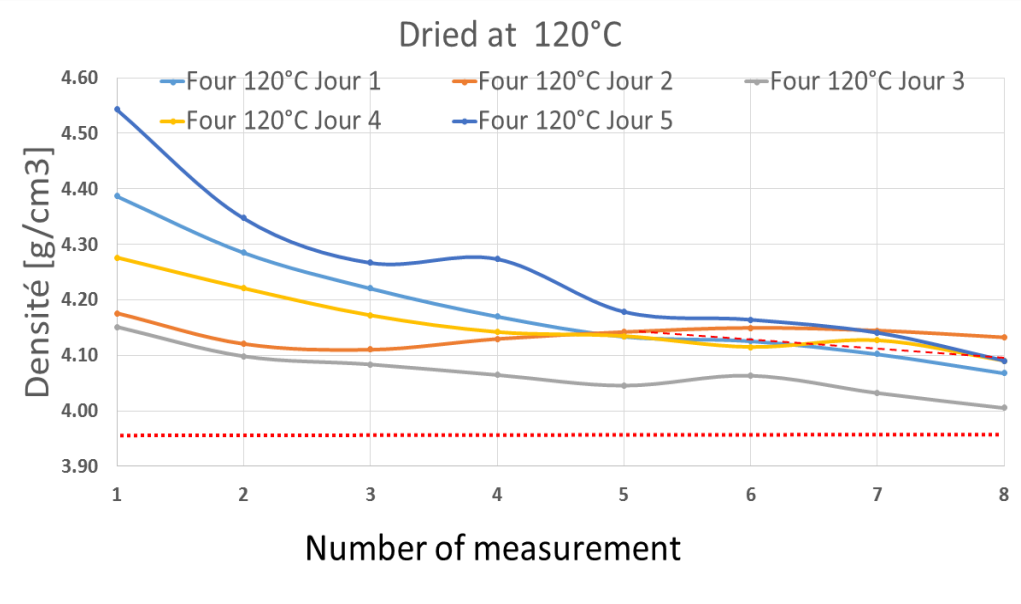

Figure 2-3 Density of TiO_2 (P25) measured with He-Pycnometer, powder dried at 120 °C over night. Dotted line: expected density, dashed line: mean value of measurements 5 to 8.

To validate these results, we checked other measurement protocols for nanosized alumina powder that were carried out some years ago with the same equipment, but another operator. This powder was also dried at 60 °C. Similar to the results for titania P25 powder, we observed a large variation over 4 measurements (3.6–3.39 g/cm^3). In addition, a tendency for decreasing density values with increasing measurement cycles was observed.

Based on these results, we concluded that the drying step is not the origin of the unstable density values. The equipment measures the volume of the sample using pressure differences. To calculate the density, the powder mass is taken as a constant value; therefore, a decrease in density could only be explained by an increase of the measured volume. One or two monolayers of water would be sufficient to obtain such a variation in density. However, the He-gas used in these experiments had a water content of <5 ppm, which is too low to cover the large surface of the powder with water. Additionally, this would not explain the values that are (much) higher than the estimated densities. To exclude other errors, the equipment was refurbished by the manufacturer. Subsequently, an investigation of the calibration procedure showed that the calibration of the equipment has to be adapted to NM because the measured sample volume of nanoparticles is relatively low compared with the volume of the sample chamber. Figure 2-4 shows the measured densities of titania powder P25 using the new calibration procedure. The measured values are constant over the measuring cycle, the standard deviation is lower and constant, and the values are somewhat lower than that estimated from the phase composition and the bulk densities of rutile and anatase. The final measurement protocol was applied to the standard powder NM-101-7926 + NM-101-7929, and the result reported to NANoREG (see Table 2-2). The final SOP for 'True density' is given in section 2.3.1.3.

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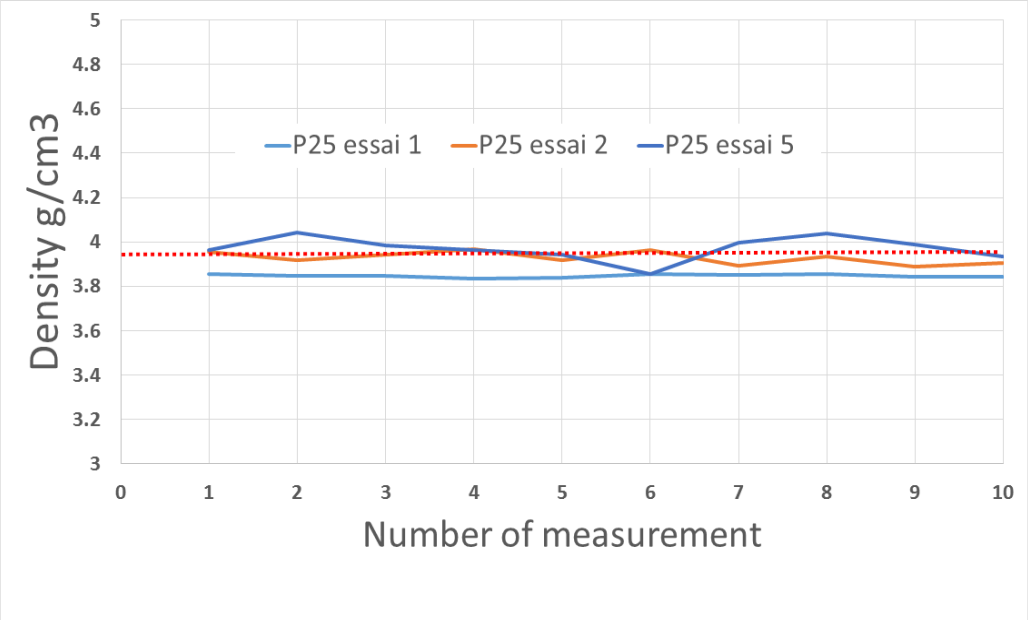

Figure 2-4 Density of TiO_2 (P25) measured using the new calibration method. Dotted line: expected density.

Replicate number	Vial number	Module (phys-chem, in vivo, in vitro)	Endpoint (e.g. OECD list, NANoREG relevant)	Assay / technique name (e.g. DLS, MTS)	Density (g/cm^3)	measurement uncertainty (SD)
1	NM-101-7926 + NM-101-7929	phys-chem	true density	He-pycnometer	3.045	0.0086
2	NM-103-6993 + NM-103-6995	phys-chem	true density	He-pycnometer	3.6079	0.0051
3	NM-303-6505 +NM-203-6509	phys-chem	true density	He-pycnometer	2.2421	0.0466

Table 2-3 Densities of NANoREG standard powders NM 101, NM 103, and NM 303.

2.4.1.2 Agglomerate density

As mentioned in D2.3, meaningful dose metrics are a basic requirement for in vitro screening and accurate characterization of agglomerate effective densities. DeLoid (2014) developed a simple, low-cost, and high-throughput method for estimating the effective density based on the volume of the pellet obtained by low speed, benchtop centrifugation of an engineered nanomaterial (NM) suspension in a packed cell volume (PCV) tube. We used this method and validated it for typical ENM. The only improvement necessary was the introduction of an additional step to fix the optimal quantity to generate a pellet volume that allows accurate determination of the pellet height in the tube.

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The effective agglomerate density is expressed as:

$$\rho_{ev} = \rho_{media} + \left[\left(\frac{M_{ENM} - M_{ENMsol}}{Vpellet \times SF}\right)\left(1 - \frac{\rho_{media}}{\rho_{ENM}}\right)\right]$$

The following protocol describes how to determine V_{pellet} for a given powder according to the volumetric centrifugation method (VCM). This method uses 1 mL PCV tubes that are graduated from 0.25 to 5 µL. A schematic representation of the PCV tube is presented in Figure 2-5.

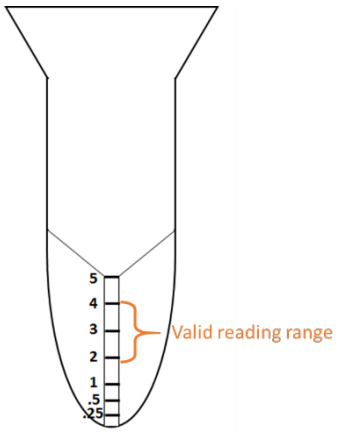

Figure 2-5 Schematic representation of a PCV tube.

After centrifugation of the tube, a pellet is observed in the capillary of the PCV tube. The volume of the pellet is measured with an easy-read measuring device. To facilitate determination of the pellet height, we decided that the pellet volume should be between 2 and 4 µL. Thus, the mass of an ENM powder required to fill the capillary to at least 2.5 µL when 1 mL of suspension is used was estimated. We considered the aggregate particles to be loosely packed in different ENM (corresponds to 50 vol-%) and thus the 'true density' of the ENM powder was divided by 2 to estimate the density of the powder. The mass (m_{ENM}) needed to fill the capillary to 2.5 µL was estimated as follows:

$$m_{ENM} = \frac{\rho_{ENM}}{2} \times 2.5 \text{ x } 10^{-3}$$

with ρ_{ENM}, the true density (g/cm^3) of the NM powder (determined with a He-pycnometer).

Then, an NM suspension at a concentration of m_{ENM}/mL was prepared (according to the dispersion protocol established by NANoREG) and three replicates were centrifuged in PCV tubes. If the pellet volume obtained after centrifugation was between 2 and 4 µL, the volume could be measured accurately with the easy-read measuring device and the effective density calculated. Otherwise, if the pellet volume was lower than 2 µL or higher than 4 µL, a new ENM suspension with increased or reduced concentration, respectively, was prepared and the centrifugation step repeated. The concentration of this second suspension can be estimated by multiplying/dividing the initial concentration by the same factor as required to increase the volume. (For example, if the initial volume obtained is 1 µL, the second concentration should be 2.5 × initial concentration).

2.4.1.3 Protocol for true density measurements with a He-Pycnometer

A. Method Version November 26th 2015 / Dr.Aurélie Walter

This method measures the volume of gas (helium) displaced by a known mass of powder, and gives the true density of the material. The sample must be completely dried.

B. Equipment:

Instrument: Micrometrics Acoupyc 1330
Analytical balance (accuracy 0.1 mg)

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Spatula for powder
Calibrated aluminium cylinder (volumes between 1–7 cm^3)

C. Protocol

Determination of the calibration volume:

- The sample should be dried at 60 °C overnight (around 15 h) before the measurement.
- Weigh the empty cell using the analytical balance and record the result (Wc).
- With the spatula, add a sufficient amount of powder into the cell (1–5 g) and record the result (Wt).
- The mass of the powder (Wp) is calculated using Wp = Wt − Wc.
- Put the cell with the powder in the instrument and run the measurement.
- Repeat the measurement ten times.
- From the measurement, determine the mean volume of the powder present in the measurement cell (Vp).

Instrument calibration

- Weigh the mass of the empty cell using the analytical balance and record the result (Wc).
- Instead of powder, place an aluminium cylinder with a volume close to Vp in the measurement cell.
- Run the calibration process.

Density measurement

- Once the calibration is complete, weigh the mass of the empty cell using the analytical balance and record the result (Wc).
- With the spatula, add a sufficient amount of powder into the cell (1–5 g) and record the result (Wt).
- The mass of the powder (Wp) is calculated from Wp = Wt − Wc.
- Put the cell with the powder in the instrument and run the measurement.
- Repeat the measurement ten times.
- Repeat the entire measurement procedure 2 more times to obtain 3 independent measurements of 10 runs.

2.4.1.4 Protocol for agglomerate density assay

A. Method Version February 26th 2016 / Dr. Aurélie Walter
This method determines the agglomerate density of powders suspensions.

B. Equipment

- All equipment required for the NANoREG SOP for probe-sonicator calibration of delivered acoustic power and de-agglomeration efficiency for in vitro and in vivo toxicological testing.
- Excel template for the probe-sonicator comparison ‘Template for Probe Sonicator Calibration’.
- All equipment required for the NANoREG ENPRA dispersion protocol.
- PCV tube, 1 mL.
- Easy-read measuring device for PCV tubes.
- Centrifuge (Eppendorf, 5702R), 4 × 85 mL swing bucket rotor with round buckets (rotor diameter: 14 cm).

C. Protocol

Probe-sonicator calibration

- Use the NANoREG SOP for probe-sonicator calibration of delivered acoustic power and de-agglomeration efficiency for in vitro and in vivo toxicological testing.
- Use the Excel template for probe-sonicator comparison ‘Template for Probe Sonicator Calibration’.
- Prepare the batch dispersion.

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- Prepare a stock suspension of X mg/mL in MilliQ water.
- Adjust the pH of the suspension to be within the electrostatic stabilization domain of the particles. Normally, a pH value 2–3 units below or above the pH of the IEP, but still far enough from the pH at which the solubility of the investigated powder increases, is used (see examples in Table 2-2).

Table 2-4 Examples of powder preparation for agglomerate density measurements

Sample	Bulk density	pH for measurement
NM-101	1.9 mg/mL	pH 9
NM-103	4.5 mg/mL	pH 4

Powder dispersion

- Use the stock suspension of particles in MilliQ water.
- Use the method described in the NANoREG ENPRA dispersion protocol, i.e. sonicate for 16 min, as described in the protocol.
- As soon as the sonication is finished, pour 1 mL of the suspension into the PCV tube. Prepare 3 replicates.

Centrifugation

- Set centrifuge at 20 °C.
- Centrifuge the tubes for 1 h at 4000 rpm with the in-house centrifuge or at 2504 *g*.
- Determine V_{pellet}.

Determination of the agglomerate density

- Use the excel template (see Annexe 7.1).

It is important to note that this method works only for particles that settle under the given experimental conditions. This was not the case for material NM-203, which means that the dispersion is very stable and/or the nanoparticles are too small and diffusion is the main transport mechanism. This is important information for application of this parameter (prediction of dosage), and the prediction could be carried out by taking diffusion into account. A template 'Agglomerate density' is in development.

2.4.2 *Granulometry*

2.4.2.1 DLS Measurment

The hydrodynamic size (Z-average size (d.nm)) of the dispersed powders were determined using the Nano-ZS (model ZEN 3600) equipment operating at 25ºC. Z-average values were acquired in triplicate with 120 s for equilibration at 25ºC and time of acquisition in the automatic mode. Because the dispersion protocol was different from the NanoReg dispersion protocol, the results cannot be compared with the results from other NanoReg partners. Interesting are the results in comparison with the investigation of the "Dispesibility" of NP (see chapter 2.3.7). The results show also that the chosen sonication time was too short.

2.4.2.2 SEM image analysis

SEM analyses were carried on a Jeol microscope, model JSM 6701, equipped with a field emission source (FEG – Field Emission Gun) and operating at an accelerating voltage of 10.00 kV and beam current of 10 µA. The working distance was kept at 3.5 mm. The sizes of the NM particles were obtained using the image processing software Image J. Approximately 120 single measurements of particle diameter were taken for each NM and the average values were summarized in this report. The results are presented in annex 7.3.

2.4.3 *TG 105 „Water Solubility" using a modified flask method, LEITAT, UdL*

The following SOP describes the experimental set-up and sample preparation for evaluation of the revised OECD TG 105 (water solubility). Measurements of the soluble fraction of NM-300K and NM-110 (JRCNM01100a) dispersed in purified water (or synthetic aqueous solutions) were

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performed by filtration/centrifugation experiments and ICP-MS analyses. For NM-300K, the results of one of the tested methods were compared with those obtained using Ag ion selective electrode (ISE) measurements. For NM-110, the results were compared with those obtained with AGNES measurements (without solid-liquid separation step).

2.4.3.1 Protocol for the measurement of water solubility

A. Materials to produce a stock dispersion for the NM water solubility experiment

- All equipment required for the NANoREG SOP for probe-sonicator calibration of delivered acoustic power and de-agglomeration efficiency for in vitro and in vivo toxicological testing.
- Excel template for the probe-sonicator comparison 'Template for Probe Sonicator Calibration'.
- All equipment required for the NANoREG ENPRA dispersion protocol.
- DI water (Milli-Q).
- pH meter.
- Closed bottles or vials.
- Magnetic stirrers and stir bars.
- Micropipettes and tips.
- Temperature controlled, orbital shaker incubator.

Materials to measure water solubility by ICP-MS

- ICP-MS
- Centrifuge
- Amicon® Ultra-15 Centrifugal Filter Device, cut off 3 kDa
- Microvials, 1 mL
- Micropipettes and tips

Materials to measure water solubility by Ag ISE

- Reference electrode and ISE for the ion under investigation
- $AgNO_3$ salts for the Ag ISE calibration curve
- Ionic strength adjuster (ISA) (5 M $NaNO_3$) for Ag ISE
- DI water
- Graduated flasks to prepare the standards for the ISE calibration curves
- Beakers, 30 mL
- Micropipettes and tips

Materials to measure water solubility by AGNES

- Potentiostat and polarographic stand
- Mercury capillary drop electrode
- Ag/AgCl reference and glassy carbon auxiliary electrodes
- Glass-jacketed thermostatic cell
- Water recirculating thermostatic bath
- pH meter
- water-saturated N_2/CO_2 (99.999% purity) gas mixtures for the removal of dissolved O_2
- Micropipettes and tips

B. Calibration

Probe-sonicator calibration

- Use the NANoREG SOP for probe-sonicator calibration of delivered acoustic power and de-agglomeration efficiency for in vitro and in vivo toxicological testing.
- Employ the Excel template for the probe-sonicator comparison 'Template for Probe Sonicator Calibration'.

ICP-MS calibration

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- Use the NANoREG SOP for ICP-MS measurements.
- Important: Metals may be leached from glass by nitric acid. Store all standard solutions in polypropylene or other inert containers. If glassware is used, it should be cleaned with nitric acid and dedicated for trace metals analyses. Standards prepared in glassware should be used immediately or transferred to suitable containers for storage.
- Starting from commercial 1000 ppm standard solutions, intermediate standards of 5 ppm are prepared. From these, a set of final calibration solutions is prepared for constructing a multipoint standard curve covering the range of analyte concentrations anticipated in samples.
- Measurements are performed for each sample at least in triplicate.
- Reagent blanks (generally, nitric acid diluted in MilliQ water) are employed, where the blank signal shows in all samples and standard solutions.
- Preparation blanks are also recommended, where the blank is from the sample preparation process only and not present in the calibration solution/standards. In this particular case, preparation blanks were necessary to check for analyte adsorption on the Amicon® Ultra-15 Centrifugal Filters.

Ag ISE calibration

- Maintain a constant temperature and stirring conditions during both the ISE calibration and the sample measurements.
- Prepare a concentrated stock solution of a completely soluble salt of the element of interest and add 5 M $NaNO_3$ as an ISA (2% ISA, v/v). Obtain different standard solutions by serial dilutions of the stock solution with distilled water and the appropriate amount of 5 M $NaNO_3$ to have 2% ISA (v/v) in all standards.
- Obtain a calibration curve in the expected concentration range. A typical curve for the selective electrode is composed of a liner area that ranges from 1 to 1000 ppm and a non-linear area that ranges from 0.01 to 1 ppm. In the linear range, the theoretical slope of the calibration curve for monovalent cations is 59.16 ± 5 mV/pAg at 25 ± 5 °C. In the non-linear range, the calibration curve should include measurements for at least five standards and polynomial interpolation is required.
- Before the first measurement, rinse both the reference and ISE electrodes with DI water and dry them carefully, taking care not to touch the ISE membrane
- Measure the standards in ascending order of concentration.
- Between measurements, rinse both the electrodes with DI water and dry them carefully, taking care not to touch the ISE membrane.

AGNES calibration

- Use the NANoREG SOP for AGNES measurements.

C. Solubility Measurement

Planning the experiment and practical considerations

- Establish the NM concentration that will be used and the time points to measure.
- Based on the MNM concentration selected, the time points considered, and the technique/techniques that will be used to analyse the samples, estimate the total volume of stock dispersion that should be prepared.
- If the NM is UV light sensitive, suitable amber coloured glassware should be employed to prepare and store the samples. If amber coloured glassware is not available, the vials, flasks, or beakers should be covered with aluminium foil to protect the sample against UV degradation.
- If the NM is known to be sensitive to oxygen and light, different aliquots of identical (twin) samples should be prepared for measurements at each time point. This procedure ensures that the NM concentration does not change during tests and the same conditions are maintained for all the samples.
- Temperature control must be ensured during incubation of the samples, and time needed for the handling of samples prior to analytical measurement must be minimized.

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- For the case of NMs that are very sensitive to pH changes (i.e., ZnO), a suitable control of eventual pH changes (e.g., due to variation in partial CO_2 pressure during experimental procedure) must be addressed (e.g., by use of a suitable buffer, continuous tracking of pH and/or use of correction factors, as described below).

Preparation of the stock dispersion

- Prepare a stock suspension of 2.56 mg particles/mL of MilliQ water, following the NANoREG ECOTOX dispersion protocol for producing reproducible dispersions of manufactured NM in environmental exposure media.
- Measure the size distribution of the stock dispersion using DLS according to the SOP developed in the NANoREG ECOTOX dispersion protocol.

Preparation of samples for NM water solubility testing

- Dilute the stock dispersion with water (or the desired test medium) to obtain the NM concentrations chosen for the water solubility tests.
- Transfer aliquots of the sample into closed (air-tight) bottles or vials, insert a stir bar, and leave the samples to stir, according to the time points selected. Alternatively, the sample vials can be stored in a temperature-controlled orbital shaker incubator. Check for pH changes before/after incubation.
- At the selected times, use the various aliquots of the sample to determine:
    - the total NM concentration and ionic fraction content by digestion plus ICP-MS and centrifugation/filtration plus ICP-MS of the filtrate, respectively.
    - the NM ionic fraction content by ISE measurement.
- For comparison (in the case of ZnO NMs), additional AGNES measurements can be performed with aliquots of the stock solution diluted directly in a suitable thermostatic glass vessel containing the test media under intermittent stirring. This procedure allows measurement of kinetic dissolution rates up to a few hours. No solid-liquid separation step is necessary.

Determination of total NM content by ICP-MS

- Place a 1 mL aliquot of the sample in a 1 mL microvial, store in the fridge until use, measure the total NM content by ICP-MS, and calculate the percent dissolution based on the total mass of NM employed.

NOTE: A proper digestion protocol is required before ICP-MS analysis for NM dispersions.

Determination of NM dissolved fraction by filtration/centrifugation followed by ICP-MS of the filtrate

- Pre-rinse the Amicon® Ultra-15 Centrifugal Filter Device with 10 mL of DI water, employing similar conditions to those used in the filtration/centrifugation experiment. This operation is useful to check if the filter works correctly or if it has been damaged or has any manufacturing defects.
- Dry the filtration device carefully before the filtration/centrifugation experiment.
- Place a 10 mL aliquot of the sample in the filtration device, cap the device, insert it into the centrifuge, and spin the device at 4000 RCF (G) for 10 min. These conditions allow approximately 5 mL of filtrate to be obtained.
- Place 1 mL of the filtrate in a 1 mL microvial, store in the fridge until use, and use for ICP-MS analysis.

NOTE: If the NM does not contain particles smaller than 1.5–3 nm (~3 kDa cut-off), the filtrate should contain only ions and other low-molecular weight dissolved species; therefore, a digestion protocol is not required.

Determination of NM dissolved fraction by ISE measurement (NM-300K, Ag NM)

- Check the operating conditions of the ISE electrode, including the pH range in which the electrode can work, and potential interference from other molecules or ions present in the sample. For Ag^+ determination, the pH of the sample should be between 1 and 9 to avoid reaction between the Ag^+ and OH^- ions. Moreover, S^{2-} and Hg^{2+} ions and protein should be absent.

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- Keep the temperature and stirring conditions constant during both the calibration and the sample measurements.
- Before the first measurement, rinse both the reference and SA electrodes with DI water and dry them carefully, taking care not to touch the ISE membrane.
- Place a 19 mL aliquot of the sample in a 30 mL beaker, and then add 1 mL of ISA while stirring.
- Immerse the reference and ISA electrodes in the sample, ensuring that no bubbles remain attached to the ISE membrane.
- Measure the Ag ion content in the sample three times, employing an already prepared calibration curve.
- Between measurements, rinse both electrodes with DI water and dry them carefully, taking care not to touch the ISE electrode membrane.

2.4.3.2 Experimental evaluation of modified OECD TG 105.

In this experiment, $AgNO_3$ is used to prepare the standards for the calibration curves. Four standards at 1, 5, 10, and 95 mg/L were employed for the linear range. For the non-linear range, five standards at 0.01, 0.05, 0.1, 0.5, and 1 mg/L were used. The calibration curves, equations, and the R values obtained are shown in Figure 2-6.

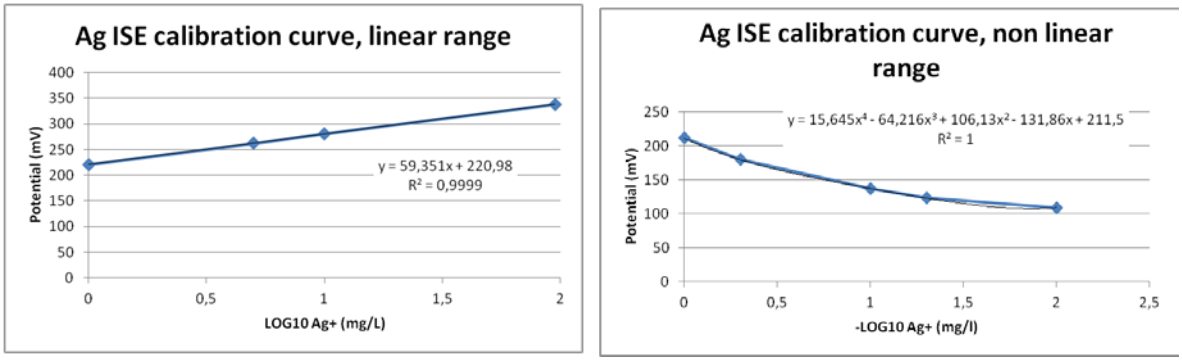

Figure 2-6 Calibration curves for Ag ISE in the linear and non-linear range.

According to the protocols described above, the following experimental parameters were used:

- The concentrations of NM-300K and NM-110 were 100 mg/L and the time points selected were t = 0, 4, 24, 48, and 200 h.
- As Ag NM-300K is sensitive to oxygen and UV light, different aliquots of identical (twin) samples were prepared for each time point. Moreover, all the glassware was covered with aluminium foil to protect the samples against UV degradation.
- Considering a NM concentration of 100 mg/L, five time points were selected and the volume of sample needed to perform the different measurements with the selected techniques were:
  - 10 mL of sample to digest before the ICP-MS analysis to check the total concentration of the element of interest in the starting sample.
  - 10 mL of sample for the separation through centrifugation/filtration, with 1 mL of the corresponding filtrate used to measure the dissolved fraction of the NM.
  - 20 mL of sample to measure the NM dissolved fraction by ISE.
  - A final volume of 50 mL of sample for each time point selected was required; therefore, a total volume of 50 mL × 5 time points = 250 mL of sample was required.
- To prepare the total volume of samples required for the measurements, 9.776 mL of stock dispersion was needed. Therefore, 10 mL of stock solution was obtained using the NANoREG ECOTOX dispersion protocol.

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- The appropriate amount of stock dispersion was transferred in a 250 mL graduated flask, and then distilled water was added to obtain 250 mL of sample with a concentration of 100 mg/L NM. This solution was divided into five aliquots of 50 mL.
- At each time point:
  - Place 10 mL of sample into a vial and store in the fridge. This aliquot is subjected to chemical digestion before ICP-MS to check the total concentration of the element of interest in the sample.
  - Place 10 mL of sample in the filtration device, cap the device, insert it into the centrifuge, and spin the device at 4000 RCF for 10 min. These conditions allow approximately 5 mL of filtrate to be obtained.
  - Place 1 mL of the obtained filtrate into a vial and store in the fridge. This aliquot is used to measure the dissolved fraction of the NM by ICP-MS.
  - Meanwhile, add 1 mL of ISA to 19 mL of sample to measure the NM dissolved fraction by ISE.

The Excel file employed to set up the experiment is shown in Figure 2-7.

NM-300K ecotox prot	Stock NM Conc. [mg/ml]	Vol. Available [ml]	N° of final samples to prepare	Tot. Vol. Stock NM will be used [ml]	Tot. Vol. all Final samples [ml]		
	2,56	10	5				
Final vol. of each sample to measure [ml]		**50**			250		
Final conc. of each sample to measure [mg/ml]		**0,1**					
Vol. of stock NM sample to prepare each sample to measure [ml]		**1,953**		9,766			
Vol. of H_2O to pick up for each sample to measure [ml]		**48,047**					
							
Time [h]	**0**	**4**	**24**	**48**	**200**		
Volume [ml]	50	50	50	50	50	**250**	**Tot. Vol. [ml]**
Concentration [mg/ml]	0,1	0,1	0,1	0,1	0,1		
NM [mg]	5	5	5	5	5	**25**	**Tot. NM [mg]**

Figure 2-7 Experimental set-up for water solubility measurement.

Figure 2-8 and Figure 2-9 show the results obtained for NM-300K and NM-110 respectively.

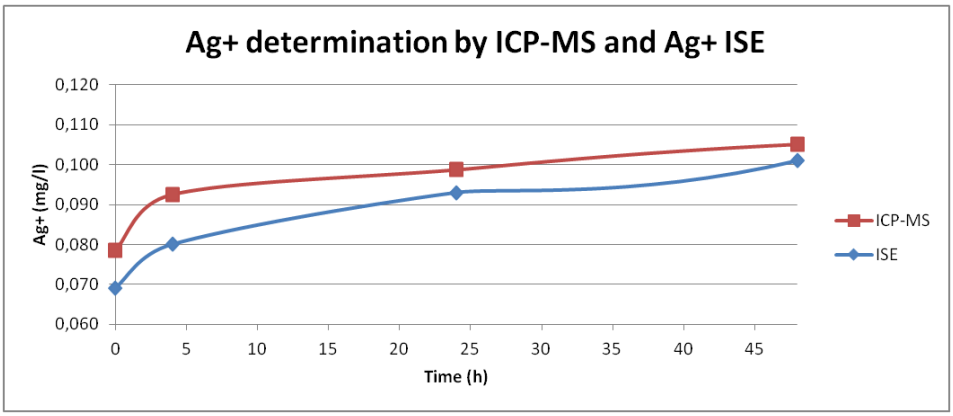

Figure 2-8 Ag^+ determination performed by ICP-MS and Ag ISE, Powder NM 300K.

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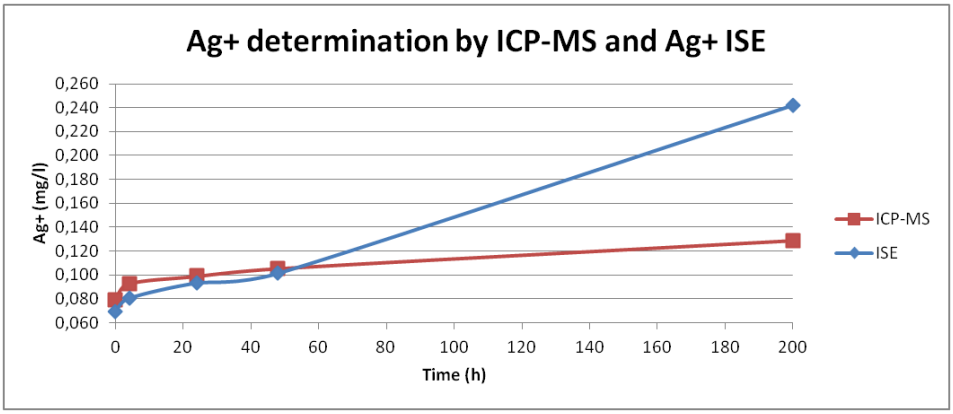

Figure 2-9 Ag^+ determination performed by ICP-MS and Ag ISE, Powder NM-110.

The obtained results show that the adapted flask method, followed by a filtration/centrifugation experiment and ICP-MS analysis of the filtrate, provides reproducible results. Further studies are required to assess the dependence of NM dissolution on the initial NM concentration, NM size, and type of solvent (e.g. pH and composition of the media).

An aliquot of the same sample was measured using Ag ISE as an alternative method for evaluating the presence of dissolved Ag ions. As shown in Figures 2-8 and 2-9, the ICP-MS data is comparable with those obtained using Ag ISE measurements. The significant deviation observed at the 200 h time point for the ISE measurement is probably due to the long interval between ISE calibration and measurement or interference during the ISE measurement.

2.4.3.3 Experimental evaluation of modified OECD TG 105 in a synthetic aqueous solution (UdL).

This section describes the results of the revised OECD TG 105 (water solubility) described above, applied to the dissolution testing of NM-110 (JRC01101a) dispersed in synthetic saliva (in absence of organic matter), selected as a model of in vivo/in vitro test media (NM111 was not tested because it was not possible to prepare a suitable stock dispersion following the ECOTOX protocol due to strong adsorption of the solid on the air-liquid interface)

The composition of the test medium is described in Table 2-3. The medium was adapted from D5.2 and [Walczak 2013]. The original recipe includes 200 mg/L urea, 290 mg/L amylase, 15 mg/L uric acid and 25 mg/L mucin, which were not included in this test to avoid interferences of Zn complexes with organic matter. The results were compared with thermodynamic calculations using Visual Minteq speciation software [Gustafsson 2013].

Table 2-5 Composition of solubility test medium

Synthetic Saliva
• 896 mg/l KCl
• 200 mg/l KSCN
• 1021 mg/l $NaH_2PO_4 \cdot H_2O$
• 570 mg/l Na_2SO_4
• 298 mg/l NaCl
• 1694 mg/l $NaHCO_3$
pH = 6.8 ± 0.1, I = 0.0587 mol/L

For these experiments, 6x2 glass vials were partially filled with 20mL of synthetic saliva, pre-equilibrated at pH 6.8 under a N_2/CO_2 atmosphere, and a suitable aliquot of the 2.56g/L NM110 sonicated stock solution

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(prepared according to the NANoREG ECOTOX dispersion protocol; Z-average size of NM110 measured by DLS: 226.8nm (PDI 0.125)) was added to each of them to obtain a range of total concentrations from 0.035 to 62 mgZnO/L (0.029 to 50 mgZn/L). A control sample was prepared with $Zn(NO_3)_2$ (0.32 mgZn/L) at the same conditions, in order to check for Zn adsorption on the Amicon filters. After 24 or 48h, two subsamples were taken from the vials to analyze the total Zn content (acid digestion, no filtration) and the dissolved Zn (after centrifugal ultrafiltration). All experiments were carried out in duplicate. The results (corrected for pH differences and Zn adsorption on the filters) are shown in Figure 2-10.

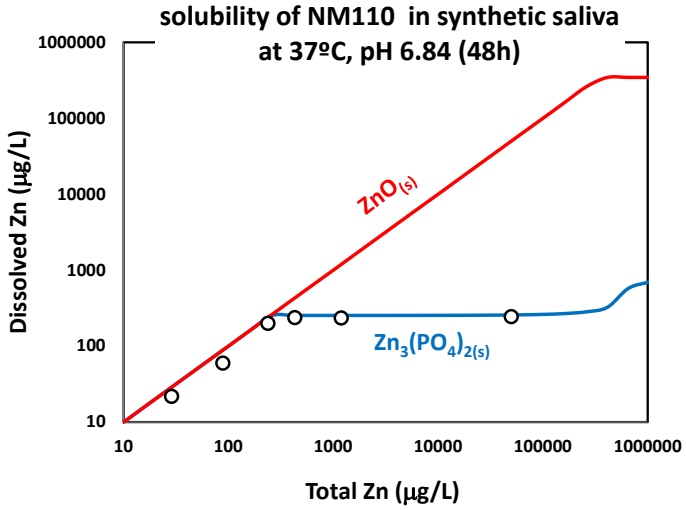

Figure 2-10 Symbols: Solubility of ZnO NM110 (JRC01101a) in synthetic saliva (Table 2-4) at pH 6.84, incubated at 37ºC for 48h, measured by ICP-MS after centrifugal ultrafiltration (3kDa). Lines: theoretical concentration of Zn dissolved from of bulk ZnO (zincite) according to Visual Minteq (using Davies model for activity coefficients) at the same conditions assuming: (a) no precipitation of other solid phases (red); and (b) precipitation of solid zinc phosphates (blue).

Control samples indicate a loss of 2.5% of dissolved Zn due to adsorption on the Amicom 3kDa filter membranes. Fluctuations of dissolved Zn due to changes in pH during incubation and handling prior to ICP-MS measurements were significant (between 6.8 and 7.3) and, therefore, the results were corrected according to the following factor:

$$\text{solubility}_{(pH\,6.84)} = \text{solubility}_{(test\ pH)} \times \frac{c^{theor}_{pH\,6.84}}{c^{theor}_{test\ pH}}$$

Where "*test pH*" refers to actual pH conditions (measured at the end of incubation), and c^{theor} refers to the theoretical dissolved Zn concentration calculated with Visual Minteq at each pH.

Regarding the thermodynamic calculations, the dissolution of the solid NM is a result of the hydrolysis / ionization equilibrium of ZnO:

$$ZnO_{(s)} + H_2O \overset{K_{sp}}{\rightleftharpoons} Zn^{2+} + 2OH^-$$

which is described by the solubility product K_{sp}. The value of K_{sp} for ZnO NMs is larger than for bulk material (as described by the Ostwald-Freundlich equation), but the differences have been found to be negligible above a primary particle size of 70 nm [David 2012]. Therefore, the thermodynamic value of the bulk material (zincite) is considered accurate enough for NM-110. The same inorganic composition of the digestion systems as listed in Table 2-4 was used for the calculations (with the value of the ZnO dissolution enthalpy included in the standard database). In these conditions, the overall solubility is conditioned mainly by T, pH and the concentration of phosphate.

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The results indicate that NM110 is **completely dissolved (at equilibrium) below 0.24 mgZn/L** (0.30 mgZnO/L). **Above this concentration, NM110 seems to be transformed into solid $Zn_3(PO_4)_2$,** due to the presence of phosphates in the test medium, leading to a maximum concentration of dissolved Zn of 0.25 mgZn/L. The formation of solid zinc phosphate is consistent with Visual Minteq calculations and the complete disappearance of the characteristic ZnO absorption band in UV-vis measurements performed on the test media after incubation.

Regarding the kinetic dissolution rates, the solubility results at 24 and 48h were statistically identical, so the results are assumed to be at equilibrium, and **dissolution kinetics is expected to be faster than 24h** in the experimental hydrodynamic conditions of the orbital shaker incubator. Further experiments at a smaller timescale were carried out using AGNES in a stirred batch reactor, as described below.

2.4.4 *TG 105 „Water Solubility" using a stirred batch reactor method and AGNES technique, UdL*

The above mentioned SOP for solubility testing (based on centrifugal ultrafiltration with 3kDa filters+ ICP-MS) has two main disadvantages. First, it does not assess the presence of residual particles in the filtrate, which would then contribute to the total element concentration measured by ICP-MS thus leading to an overestimation of the solubility. Second, the procedure does not allow a time resolution in the scale of minutes, which would be needed to characterize dissolution processes faster than a few hours. For this reason, the method was compared for NM-110 using an alternative procedure involving a continuously stirred batch reactor where AGNES measurements are performed *in situ* (*i.e.*, without solid-liquid separation step). This is possible due to the ability of AGNES of measuring free Zn^{2+} concentrations without interference from the particles present in the dispersion.

AGNES measurements were carried out in a glass vessel thermostatted at 37ºC, containing 50mL of synthetic saliva (see Table 2-4) pressurized under an atmosphere of water-saturated mixture of N_2 and CO_2 (10%) to remove traces of dissolved O_2 and keep pH constant at 6.8. At t=0, a suitable aliquot of the stock solution of NM-110 (prepared according the ECOTOX dispersion protocol) is added, and AGNES measurement of Zn^{2+} concentration is carried out as a function of time (for a maximum of 4h). The results are shown in Figure 2-11.

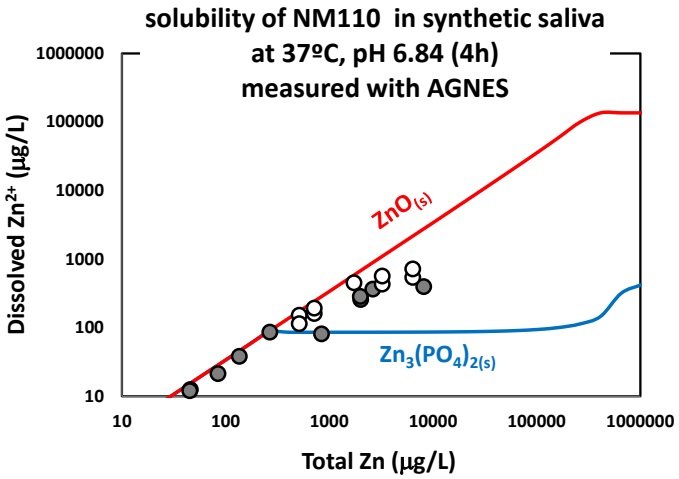

Figure 2-11 Solubility of ZnO NM110 (JRC01101a) dispersed in synthetic saliva (Table 2-4) at pH 6.84 and 37ºC after 4h, measured by AGNES (without solid-liquid separation). Grey symbols: samples incubated under 10% CO_2 atmosphere. White symbols: samples incubated without CO_2, pH adjusted with HCl. Lines: theoretical concentration of $Zn2^+$ ions dissolved from of bulk ZnO (zincite) according to Visual Minteq (using Davies model for activity coefficients) at the same conditions assuming: (a) no precipitation of other solid phases (red); and (b) precipitation of solid zinc phosphates (blue).

The possible influence of the formation of zinc carbonates was disregarded by comparison of datasets obtained with $CO_{2(g)}$ buffering with those obtained in absence of $CO_{2(g)}$ and pH control using HCl.

The results indicate that NM110 is **completely dissolved (after 4h) below 0.27 mgZn/L** (0.33 mgZnO/L), in good agreement with the UF-ICP-MS procedure. Above this concentration, the measured free Zn^{2+} concentrations were below the theoretical value predicted by Visual

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Minteq for the equilibrium values obtained from complete dissolution of solid ZnO, but still higher than predicted concentrations in equilibrium with solid zinc phosphate. In this situation, the rate of dissolution was negligible up to 4h (i.e., AGNES measurements in the first 10 min and after 4h were statistically identical). All this suggests that formation of soluble Zn complexes with phosphates may be fast, whereas the subsequent precipitation of $Zn_3(PO_4)_2$, may take place in an intermediate timescale between 4 and 24h.

2.4.5 *TG 105 „Water Solubility“ using a stirred batch reactor (SBR) method (NRCWE)*

This SOP describes the experimental set-up and sample preparation for evaluation of an alternative test procedure to the revised OECD TG 105 (water solubility) described above. The method is demonstrated by dissolution testing of NM-110, JRC01101a (new sub-sample of NM-111) and NM-300K dispersed in purified water. In this case, quantification was performed by ICP-MS after filtration/centrifugation through a 3KDa filter as described above. This approach is a modification from the previous stirred batch reactor procedure at NRCWE due to an agreement on best practice between the NANoREG WP2 and WP4 partners performing dissolution testing.

This method was originally developed to investigate the short-term biodurability and hydrochemical reactivity (acidity and redox activity and particle dissolution/precipitation dynamics) of NM and dissolved chemicals when incubated with synthetic lung fluids under highly controlled pH-levels and atmosphere composition (e.g., Jensen et al., 2011). In the current setup for determination of water-solubility, the system is only controlled in regards to water-temperature and atmosphere by supplying purified air by bubbling into the stirred batch reactor. To follow and document the conditions during the dissolution testing, pH and redox potential (E_h) is measured online using a pH- and redox potential electrode, respectively. The redox potential is used as an integral measure for oxidative/reductive capacity of the particle.

In the examples shown in this report, the dissolved NM is measured 6 times during 48 hoours to obtain the “0”, “1”, “2”, “4”, “24”, and “48” hour solubility. The batch dissolution rate can then be estimated from the amount dissolved at the different time-points.

The system is established as follows. The flow-through test reactor is placed in the TIM856 titrator and connected to a thermostatic bath. A Teflon tube is connected to a flow-controller to administer HEPA-filtered air humidified by bubbling through Nanopure Diamond UV water prior to leading the test atmosphere into the flow-through reactor. A pH-meter is fixed in position in a suitable hole in the reactor cap and connected to the computer used to control the TIM856 titrator. The redox electrode is fixed in position in a suitable hole in the reactor cap and connected to a multimeter (PHM240). The printer port is connected to a computer with a RS232 data cable to record data using the terminal program. A temperature electrode is coupled each of the pH and Eh electrodes to enable correction for temperature fluctuations. Before starting the tests, the flow-through reactor is carefully wrapped in aluminum foil to inhibit possible artefacts caused by photocatalytic reactions.

2.4.5.1 *Protocol for the measurement of water solubility using the stirred batch reactor method*

A. <u>Chemicals and equipment for dissolution testing</u>

- Purified water (Nanopure Diamond UV water)
- pH 7.4 calibration pH-buffer (e.g, IUPAC pH 7.413)
- pH 4.0 calibration pH-buffer (e.g, IUPAC pH 4.005)
- Redox buffers (e.g, Hamilton (DURACELL) +271 and +574 mV)
- Thermostatic water-bath (Polyscience AD07R-40-A12E) for controlling reactor temperature at: 25°C
- Atmosphere: HEPA-filtered cleaned air
- Gas-mixing and humidifier control unit
- pH electrode: PHC2401 (Radiometer Inc.)
- E_h electrode: MC3051 PT-9 (Radiometer Inc.)
- Two temperature electrodes: T201 (Radiometer Inc.)
- pH-logging: Titrator: TIM856 (Radiometer Inc.) installed with TIM85 vs. 1.6 or higher
- Multimeter for E_h monitoring: PHM240 (Radiometer Inc.)

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- Reactor: 100 ml water-flow Pyrex glass reactor
- Aluminum foil for light-impermeable wrapping of the water-flow glass reactor
- Magnets for stirring
- One computer to control the titramaster and log results from pH-measurements and titrant dosing
- One computer to log redox data sampled by the PHM240 and operate the Malvern DLS

Specifications of Nanopure Diamond UV water:

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

Resistivity: ≤ 18.2 MΩ-cm at 25ºC
Pyrogens: < 0.001 EU/ml
Total Organic Carbon: < 3.0 ppb
Other: nuclease-free (RNase andDNase).
Filter: 0.2 μm filter (γ-irradiated Barnstead D3750 Hollow fibre filter)

B. Chemicals and materials for water-sampling and filtration

- Needle syringe for water-sampling
- Amicon® Ultra-4 Centrifugal Filters, cut off 3 kDa (Z740186)
- Micropipettes and tips
- Centrifuge (SORVALL RC 6+ with SH-3000 Swinging Bucket Rotor head with insert)
- Weigh with an accuracy of up to ±0.1 mg (weighing mas of liquid filtered into Amicon® tubes)
- 2% ultra-pure HNO_3 in nanopure water to prevent reprecipitation growth in filtered water-sample

C. Chemicals and equipment for making a batch pre-dispersed nanomaterial

- Purified water (Nanopure™ water)
- Test material
- Probe-sonicator (Branson 400 watt; 20 kHz, 13 mm tip probe-sonicator)
- Vials: 20 mL Scint-Burk glass pp-lock+Alu-foil (WHEA986581; Wheaton Industries Inc.) for weighing out MN and sonication
- Steel and glass spatula
- Pipette and pipette tips
- Weighing boat/weighing paper
- Electrostatic neutralizer to prevent powder loss and weighing problems due to electrostatics
- Weigh with an accuracy of up to ±0.1 mg
- Control or reference weights
- Ice (Ice-water)
- Insulating styrofoam box for packing ice-water around the material vials during sonication

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

The probe-sonicator must be placed in a fume-hood or under strict local exhaust ventilation to avoid spread and inhalation exposure to aerosolized droplets (nanomaterial dispersions).

D. <u>Probe-sonicator calibration</u>

In case the sonicator performance has not been documented, the sonicator is calibrated using the the NANoREG SOP for probe-sonicator calibration of delivered acoustic power and de-agglomeration efficiency for in vitro and in vivo toxicological testing.

E. <u>Elemental analysis of dissolved test material</u>

Different procedures may applied depending on the elements or compounds to be quantified. Easy sample analysis with a minimum time and work for pre-treatment can be made by Wave-Dispersive X-ray Fluorescence (WD-XRF) analysis and Inductively-Coupled Mass Spectrometry (ICP-MS). In this case, the elements were quantified by ICP-MS as a commercial service by Eurofins Denmark.

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F. Cleaning

All re-used glass-ware is thoroughly cleaned and washed in acids for high-purity chemical analysis.

G. Consideration of nanomaterial concentrations and dispersing agents applied in the test

The concentrations to be tested should normally be higher than the expected solubility limit, S_o, but also still feasible for preparation of a batch dispersion. In the current set-up, a concentration of 0.25 mg/mL was used, which can be prepared by dispersion of 25 mg in 10 mL water (2.5 mg/mL) and add this total volume ad 100 mL to the stirred batch reactor. This allows sampling of 6 times 4 mL through the reactor cap during the test using a needle-syringe.

Protocol step-by-step

1. Sample and verify NANOPURE water
2. Prepare materials and vials; weigh in 3 KDa centrifuge vials without the filter mounted (m_1).
3. Turn on the water-bath and adjust the temperature to 20°C (or a different selected temperature)
4. Turn on computers, PHM240, and gas-flow controllers.
5. In Titramaster, choose and export the suitable "application" for online monitoring of pH, temperature to the TIM856
6. Choose method for logging redox potential and temperature at 5 min interval on the PHM240
7. Add 90 mL Nanopure water to the reaction vessel.
8. Take scintillation vial with pre-weighed NM to achieve the target batch dispersion concentration (e.g., 2.5 mg/mL).
9. Add the required volume (ca. 10ml) of Nanopure water to the scintillation vial to reach the target concentration (e.g., 2.5 mg/mL)
10. Disperse the NM by shaking, ultrasound or ultrasonic probe depending on protocol.
    Here we followed the dispersion conditions established in the NANOGENOTOX, which is 16 min at 10% amplitude and 400 W in a ice-water bath using the 400 Watt Branson Sonifier S-450D (Branson Ultrasonics Corp., Danbury, CT, USA) equipped with a standard 13 mm disruptor horn (Model number: 101-147-037).
11. Start the terminal program on computer 2, to log the redox potential and associated temperature
12. Calibrate the redox electrode using one or two redox reference solutions (e.g., Hamilton (DURACELL +271 and +574 mV). Use 5 min test logging each 30 seconds to verify stability.
13. Calibrate the pH electrode using at least two standard such IUPAC pH 4.005 and 7.413 following the calibration SOP in the TIM85 leading to sensitivity above 98.5%.
14. Install the batch reactor with the 90 mL nanopure water in the TIM856 and mount all electrodes, temperature sensors and add the magnetic stirrer.
15. Start magnetic stirring at 400 rpm using the interface of the TIM856 (adjustment of speed may be needed)
16. Place the Teflon tube leading test atmosphere into the reactor into the Nanopure water and position it 1 cm above the magnetic stirrer and initiate bubbling with 150 ml/min humidified and HEPA-filtered air.
17. Start the titrator application in TIM85 for pH- and temperature monitoring and the PHM240 for monitoring of the redox potential each 5 minutes.
18. After 1 min, slowly add the test material dispersed into the 90 ml Nanopure water into the reactor using a 10 mL pipette.
19. Sample immediately (t = 0 hours), and at 1, 2, 4 , 24, and 48 hours duration of the experiment, 3 mL of the dissolution medium for immediate 3 KDa centrifuge* and subsequent ICP-MS analysis.
20. At the end of the experiment (48-hour reaction)
21. End TIM85 application and the E_h recording and save printouts and data
22. Immediately withdraw the last 3 ml supernatant for ultracentrifugation* and subsequent ICP-MS analysis

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23. Transfer the data to a common data folder
24. Switch of and clean all equipment for next experiment.

* 3 KDa centrifuge filtration is conducted immediately after each sampling of supernatant. The centrifugation is completed for 30 minutes at 4.000G (RCF) and fast acceleration (9) and deceleration (9). Immediately after filtration, the filter is removed from the vial and the vial is weighed with the screw-cap on. The weight is recorded (m_2) and $m_2 - m_1 = m_{sample}$. The m_{sample} is added 1 mL 2% ultrapure HNO_3 for stabilization of the supernatant and to prevent growth during storage.

Data treatment

All data data are collected into one excel sheet and time-matched and plotted for demonstration of the test conditions.

The following data are collected:

- Time 1
- pH
- Temperature 1
- Time 2
- E_h
- Temperature 2
- Total concentration of leached/dissolved constituent elements

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

E_h results may be used as monitored or re-calculated to determine the theoretical electron concentration in the water according to the equation

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

The pH data can inform about the caustic, neutral or acidic effect of the test materials

Together, the pH and Eh data can be plotted to evaluate the environmental stability conditions of the simple system.

The elemental concentrations are calculated by taking the 1 mL 2% HNO_3 water dilution into account. The concentration data can be used to display and document the temporal evolution of the dissolution process directly. The element composition data can also be used to estimate the dissolution kinetics and the type of reaction by rate laws by using the differential approach for batch reactor tests. A simple way of analyzing the kinetics is to plot and determine the relationship between $\Delta C_t = C_t - C_o$ versus time (t), where C_t is the concentration at a given time point; C_o is the initial concentration.

All together the data can contribute to predictive modelling of the NM dissolution and phase transformation in a given environment using reaction chemical modeling.

2.4.5.2 Experimental evaluation of the stirred batch reactor (SBR) method (NRCWE).

Based on the SOP, the tests were conducted with water sampling at 0, 1, 2, 4, 24, and 48 hours after adding the dispersions into the batch reactor. Centrifugation was completed within ca. 40 minutes after sample collection. Tests have shown that most liquid has passed the 3KDa filter within the first 15 minutes. Therefore, we assume that a fully effective filtration has been completed 30 minutes after the sampling from the reactor. This time is added to the data-plot belows with an error bar pointing backwards by 30 min (Figure 2-12 A).

The results from the chemical analysis from dissolution-testing of NM-300K (AgNP) shows a reverse pattern, where the concentration drops from 162 µg/L as the initial concentration (30 min into the test), which decreases to ca. 77 µg/L at termination of the test at 48 hours. The observed Ag concentration values are in the same range as those measured in the modified flask method mentioned above. The reverse pattern observed in the SBR method needs confirmation. However, it is not unlikely that probe-sonication in pure water could have caused greater fraction of dissolved Ag at the start of the test (free Ag^+ has been detected in the original NM-

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300K dispersion sample). Negligible dissolution and in fact possible precipitation also appears to be the result after subtraction of the initial Ag concentrations resulting in a horizontal or slightly negative natural logarithmic regression function in Figure 2-12 B.

The results from the dissolution testing of NM-110 and JRC01101a (NM-111) show that both of the ZnO nanomaterials dissolve gradually during the test. It is evident that equilibrium was not reached within 48 hours of dissolution testing of any the two ZnO samples. The initial concentrations are very comparable (712 and 754 µg/L), but the dissolution of NM-110 appears to be considerably faster than for JRCNM01101a, resulting in approximately 2 times higher concentrations of dissolved Zn in tests with NM-110 as compared to the test with JRCNM01101a. This is also evident from the slopes on the regression curves in Figure 2-12 B.

The different dissolution rates and levels of these two ZnO NM is noteworthy, as the NM-110 is uncoated and JRCNM01101a is chemically surface modified by a hydrophobic silane compound. Since ethanol prewetting was not applied in this test, the differences may partially be ascribed to poorer dispersibility in the water medium, which may have resulted in different accessibility to the ZnO surface than for NM-110.

Considering the test conditions during the experiment, the temporal evolution in pH and Eh, shows that all NMs modify the conditions as compared to that of Nanopure water (Figure 2-12 C). All NMs increase the pH and reduce the Eh as compared to Nanopure water. On the long-term the two ZnO samples cause the highest increase in pH, but the initial acute effects of the NM-110 as well as NM-300K dispersion is most evident (increase in ca. 2 pH units over pure water). NM-110 is the material causing the greatest effect on the redox potential, where a drop in ca. 200 mV can be seen as compared to only ca. 100 mV in the test with JRCM01101a and NM-300K.

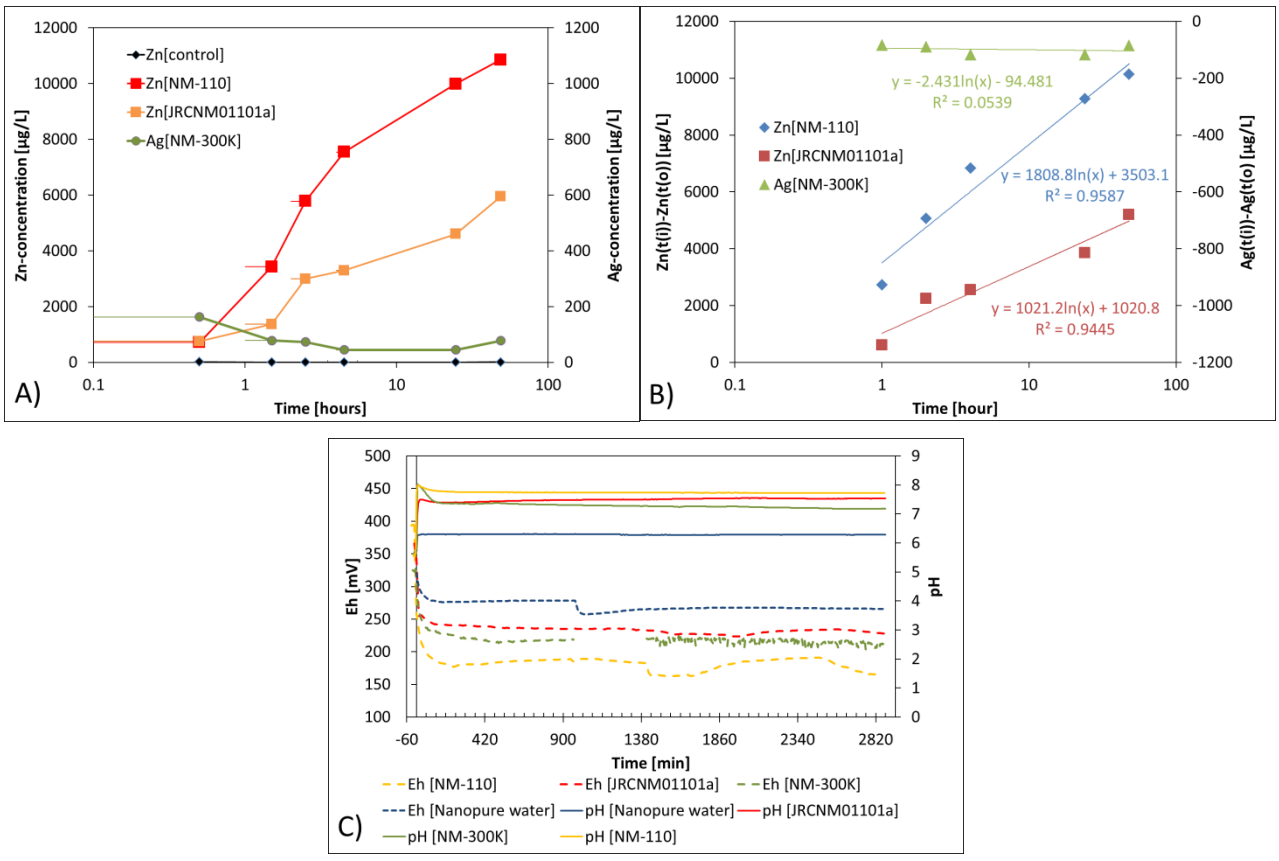

Figure 2-12 A) Concentration of dissolved Zn and Ag as function of time during dissolution of ZnO and Ag nanoparticles in Nanopure water™ equilibrated with ambient air using the 20°C temperature-controlled stirred batch reactor (note temperature could ot be controlled in the test of NM-300K due to failure of thermostat and hot weather). B) Temporal increase in elemental concentration corrected for

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initial elemental concentration C(t(o)) = C(t_o) in the tests. Preliminary correlation assessment in excel suggests to different natural logarithmic decreases in dissolution rate as function of time for the two ZnO samples. NM-300K does not appear to dissolve, but rather re-precipitate Ag dissolved during sonication, after dosing into the batch reactor. C) Temporal evolution in Eh and pH as function of time illustrating the effect of particle reactivity and dissolution on the test conditions.

The results provided by the established stirred batch reactor method provides detailed information about the pH and Eh test conditions, which can either be controlled or not controlled as needed. Contradictory results were observed for NM-300K, which may not be further dissolved after sample preparation in this study. The method may need further refinement for assessment of the dissolution of NM with very low dissolution rates. A harmonized procedure for determination of the kinetic dissolution rates must be established. Further studies are required to assess the dependence of NM dissolution on the initial NM concentration. Considerations should also be made on whether the water composition and pH values should be controlled by buffering or titration rather than made free as applied in the current set up. Data obtained on well-defined conditions may be of greater use for technical purposes as well as fate modelling and risk assessment.

2.4.6 *TG 112 (dissociation constant in water) and TG 108 (complex formation in water), EFPL, UdL*

The following SOP describes the experimental set-up and sample preparation for the experimental evaluation of a new OECD TG on IEP determination using a zeta potential versus pH titration. The following protocol was applied to NANoREG NM-212 and NM-101.

2.4.6.1 Protocol for the determination of TG 112 dissociation constant in water) and TG 108 (complex formation in water)

This method can be employed to identify the IEP and the complex formation (adsorption) with a trace metal of NM powders dispersed in water by ultrasonic dispersion.

A. Equipment

Materials to produce a stock dispersion for the zeta potential versus pH titration

- All equipment required for the NANoREG SOP for probe-sonicator calibration of delivered acoustic power and de-agglomeration efficiency for in vitro and in vivo toxicological testing
- Excel template for the probe-sonicator comparison 'Template for Probe Sonicator Calibration'
- All equipment required for the NANoREG ENPRA dispersion protocol
- DI water
- NaCl
- HCl, 0.1 M
- NaOH, 0.01 M
- pH meter
- Beakers, 30 mL
- Magnetic stirrers and stir bars
- Micropipettes and tips
- Syringe (1 mL) to fill the folded capillary cell
- Folded capillary cell plus caps
- Zeta potential transfer standard

Materials for the measurement of complex formation in water (metal adsorption)

- Potentiostat and polarographic stand
- Mercury capillary drop electrode
- Ag/AgCl reference and glassy carbon auxiliary electrodes
- Glass-jacketed thermostatic cell
- Water recirculating thermostatic bath
- pH meter
- Water-saturated N_2/CO_2 (99.999% purity) gas mixtures for the removal of dissolved O_2

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- Micropipettes and tips

B. Calibration

Probe-sonicator calibration

- Use the NANoREG SOP for probe-sonicator calibration of delivered acoustic power and de-agglomeration efficiency for in vitro and in vivo toxicological testing
- Employ the Excel template for probe-sonicator comparison 'Template for Probe Sonicator Calibration'

AGNES calibration

- Use the NANoREG SOP for AGNES measurements.

C. Measurement of the zeta potential

Planning of the experiment and practical considerations

- Establish the MNM concentration that will be used and the pH points to measure.
- Based on the MNM concentration selected, the pH points considered, and the technique/techniques that will be used to analyse the samples, estimate the total volume of stock dispersion that should be prepared.

Build-up of SOP for the size measurement that will be run before and after the zeta potential measurement

- Include the NM refractive index.
- Select water as the solvent.
- Set the temperature to 25 °C and the equilibration time to 60 s.
- Select the folded capillary cell.
- Select automatic for all the measurement conditions.
- Set up one size measurement.

Build-up of SOP for the zeta potential measurement

- Include the NM refractive index.
- Select water as the solvent.
- Select the Smoluchowski approximation.
- Set the temperature to 25 °C with no equilibration time before the measurement.
- Select the folded capillary cell.
- Select automatic for all the measurement conditions.
- Set up three zeta potential measurements with 60 s of equilibration time between each measurement.
- Set up a combination of three SOPs (SOP play list): SOP size, SOP zeta potential, and SOP size.

Preparation of the stock dispersion

- Prepare a stock suspension of 2.56 mg particles/mL in MilliQ water, following the NANoREG ECOTOX dispersion protocol for producing reproducible dispersion of manufactured NM in environmental exposure media.
- Measure the size distribution of the stock dispersion using DLS according to the SOP developed in the NANoREG ECOTOX dispersion protocol.

Preparation of samples for zeta potential versus pH titration measurements

- Rinse the folded capillary cell by flushing with water, then rinse the cell with 50% EtOH in water (v/v), and finally rinse again with water. Dry the cell under a nitrogen flow. Visually check the electrodes and cell for the presence of defects.
- Dilute the stock dispersion with an appropriate amount of NaCl solution to obtain a final concentration of 10 mM NaCl in the samples for titration.
- According to the starting pH and the pH points to measure, divide the sample into two aliquots which allows the pH changes in acid and basic directions. Store the aliquot that will be measured later in a closed bottle or vial and perform the titration in the shortest time possible.
- Start the first titration leg versus acidic or basic pH.

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- Under magnetic stirring, add the proper volume of HCl/NaOH to reach the selected pH point. Let the sample stir until the pH stabilizes.
- Use the syringe to remove a 700 µL aliquot of the sample and insert it into the capillary cell, taking care that no bubbles remain in the cell or attached to the electrodes.
- Run the SOP playlist.
- After the measurement, discard the sample and clean the cell by flushing with water several times.
- Dry the cuvette under a nitrogen flow and check visually that no residue is left in the cell.
- Repeat point 5 and follow the procedure described from that point to reach the following pH point of the titration.

Determination of the NM IEP

- Plot the zeta potential measured at each pH point of the titration and determine the NM IEP as the pH point at which the zeta potential is equal to zero.

2.4.6.2 Experimental evaluation of the proposed OECD TG XX (isoelectric point).

Following the above-presented protocol, the zeta potentials of NM-1001a and NM-212 were determined as a function of pH. The pH of the IEP was 6.4 for NM-1001a and 8.8 for NM-212, as shown in Figure 2-13.

The measured zeta potentials are very reproducible, and the pH values of the IEP fit well with published values for these types of materials (6–7 for anatase and 8–9 for ceria).

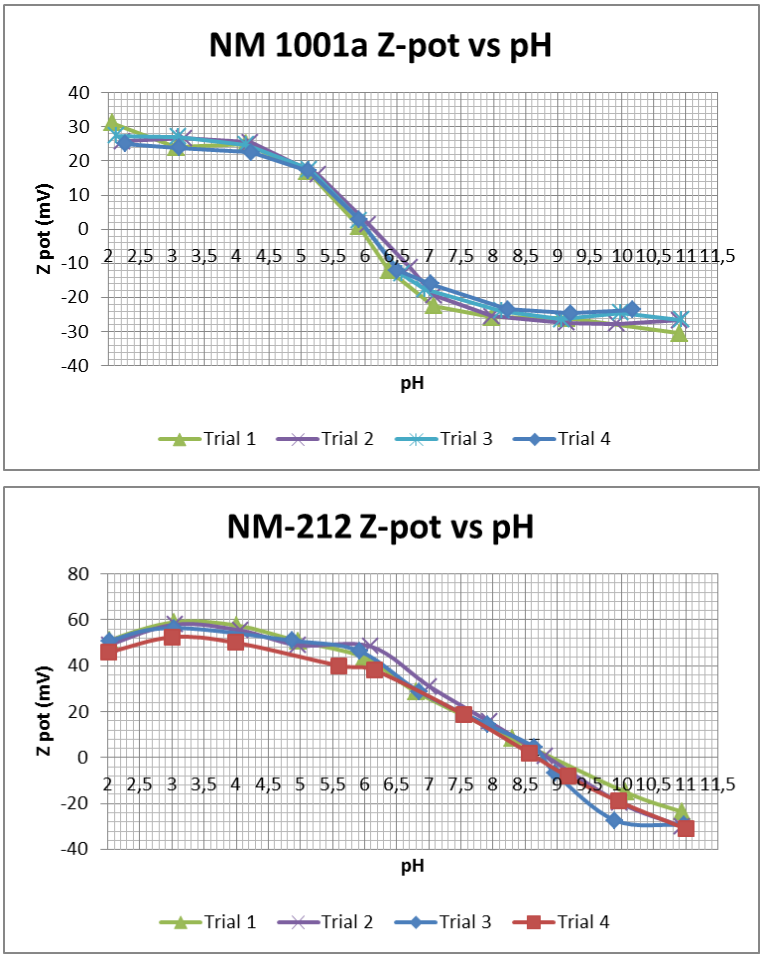

Figure 2-13 Zeta potential as a function of pH for NM-1001a and NM-212 powders.

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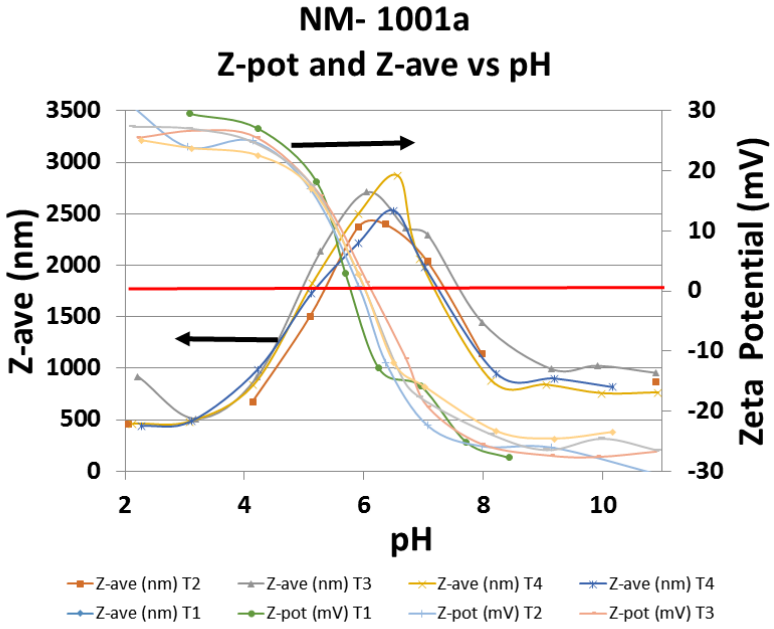
The SOP playlist set up, in which the NM hydrodynamic diameter is measured both before and after each zeta-potential measurement, can give information on the impact of the zeta-potential measure on the samples by the comparison of the sizes recorded before and after the zeta-potentialmeasurement, in fact if the NM is ruined from the measurement, the size value recorded after the zeta-potential will give a bigger hydrodynamic diameter and a wider PdI (data not reported).

Furthermore, using the SOPs playlist, the size recorded before the zeta-potential measurement could be plotted in the graph to underline the relationship between NM size, zeta-potential and colloidal stability. As expected from the DLVO theory, the colloidal stability is decreasing leading to an increased agglomeration expressed by a larger hydrodynamic size (Z-average) and particle size distribution (not shown), Figure 2-14.



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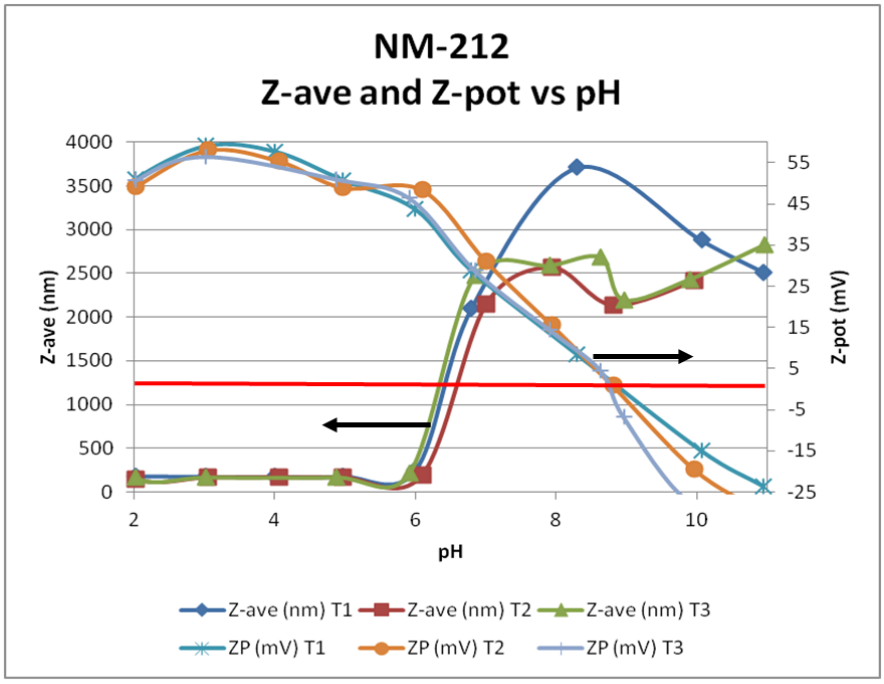

Figure 2-14 Zeta average and zeta potential as a function of pH for NM-1001a and NM-212 powders.

2.4.6.3 Experimental evaluation of the proposed OECD TG 108 (complex formation in water).

The adsorption of Pb^{2+} ions on NM212 CeO_2 was studied in experiments at 25.0±0.1ºC with AGNES. Calibrations used free Pb concentrations in the micromolar range with a gain Y=20 and resulted in a proportionality factor η =0.0025 A M^{-1}. The dispersions of CeO_2 nanoparticles where buffered to pH 6.2 with 2-(N-morpholino)ethanesulfonic acid (MES) 0.005 M in KNO3 0.01 M background electrolyte. Measurements of free Pb in the dispersions, once equilibration was reached after each addition of nanoparticles, were obtained with a gain of 50 and the 2-pulse strategy with deposition times t_{1a} = 50 s and t_{1b} = 150 s. The free Pb concentrations ranged between 1.25×10^{-7} M and 6.25 ×10^{-7} M.

Results were described by a linearized Langmuir isotherm:

$$\frac{1}{q} = \frac{1}{q_{\max}} + \frac{1}{q_{\max} K_L} \frac{1}{c_{Pb}}$$

where q stands for the loading (moles of Pb adsorbed per gram of nanoparticles, as calculated from the mass balance before and after each addition) and c_{Pb} is the measured concentration of free Pb^{2+} ions. Figure 2-15 lends support to the assumed isotherm model.

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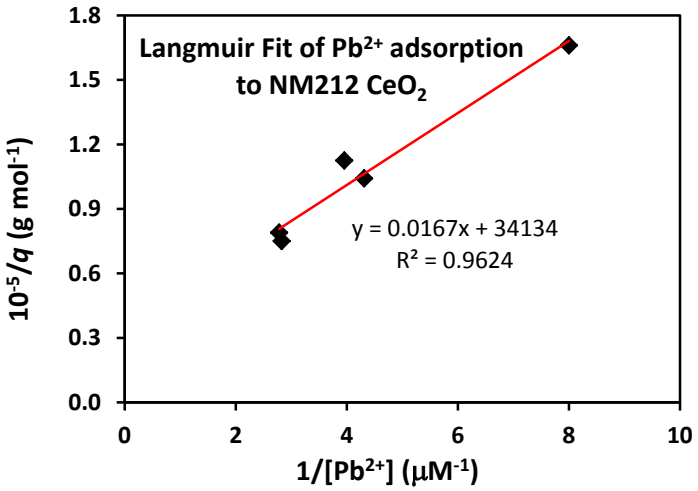

Figure 2-15 Symbols: experimental data (free Pb^{2+} concentration in equilibrium with the loading *q*) obtained with AGNES in 20 to 80 mg/L dispersions of NM212 (CeO_2) prepared in 0.01M KNO_3 background solution buffered at pH 6.2 and 25.0 ± 0.1°C. Line: linear fit according to a Langmuir isotherm, with an adsorption capacity of q_{max}=2.93×10^{-5} mol/g and an adsorption constant of K_L=2.04×10^6 M^{-1}.

2.4.7 *New proposed TG for determination of " Dispersibility"*

The following protocol for the measurement of dispersibility was developed and tested with several NANoREG powders.

2.4.7.1 Protocol for the determination of the Dispersibility

A. Equipment

- All equipment required for the NANoREG SOP for probe-sonicator calibration of delivered acoustic power and de-agglomeration efficiency for in vitro and in vivo toxicological testing
- Excel template for the probe-sonicator comparison 'Template for Probe Sonicator Calibration'
- All equipment required for the NANoREG ENPRA dispersion protocol
- Excel template for the dispersibility determination (HH template)

B. Probe-sonicator calibration

- Use the NANoREG SOP for probe-sonicator calibration of delivered acoustic power and de-agglomeration efficiency for in vitro and in vivo toxicological testing
- Use the Excel template for the probe-sonicator comparison 'Template for Probe Sonicator Calibration'

C. Preparation of the batch dispersion

- Prepare a stock suspension of 2.56 mg particles/mL in MilliQ water.
- Adjust the pH of the suspension to be within the electrostatic stabilization domain of the particles.
- Carry out DLS measurements, as described in the NANoREG SOP for probe-sonicator calibration of delivered acoustic power and de-agglomeration efficiency for in vitro and in vivo toxicological testing (Note: the medium of the suspension is water and not serum, as described in the NANoREG protocol).

D. Determination of the dispersion curve

- Use the stock suspension of particles in MilliQ water.
- Use the method described in the NANoREG ENPRA dispersion protocol.

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- Instead of sonicating for 16 min, as described in the protocol, sonicate the suspension for 1 min.
- Carry out DLS measurements, as described in the NANoREG SOP for probe-sonicator calibration of delivered acoustic power and de-agglomeration efficiency for in vitro and in vivo toxicological testing.
- Then, replace the suspension in the 10 mL beaker with fresh suspension (from the batch suspension) and sonicate the suspension for 2 min.
- Carry out DLS measurements.
- Repeat steps 5 and 6 with 3, 4, 5, 10, 15, 20, and 30 min of sonication.

E. Determination of the dispersibility of the powder

- Use the Excel template for dispersibility determination to calculate the dispersibility/agglomeration factor of the powder (Annexe 7.2).
- **Calculation of the 'Dispersibility' using 'Excel template for the dispersibility determination'.**
- **Definitions:**
- Dispersibility is a measure of the strength of the agglomerates of the powder. It is expressed as D_{BET}/D_{DLS}, where D_{BET} is the primary particle diameter calculated from the BET value and D_{DLS} is hydrodynamic diameter (taken as the z-average) of the agglomerate after ultrasound treatment at a fixed power and time. In the case of a very good dispersibility and weak agglomerates, the dispersibility value can reach 1 (particle and agglomerate sizes are the same). The following definitions could be useful:
- **Dispersibility value equal to 1** corresponds to an ideal powder with no agglomerates. In principal, this value cannot be reached because D_{DLS} is larger than D_{BET}.
- **Dispersibility value of 0.5–1** corresponds to a powder with very good dispersibility, with a maximum of 4–5 primary particles forming an agglomerate (loose packed configuration) and an agglomerate diameter corresponding to 2 particles.
- **Dispersibility value of 0.3–0.5** corresponds to a powder with good dispersibility, with around 5–15 primary particles forming an agglomerate and an agglomerate diameter corresponding to 3 particles.
- **Dispersibility value of 0.25–0.3** corresponds to a powder with medium dispersibility, with an agglomerate diameter that is 3–4 times larger than the primary particle (15–32 primary particles in an agglomerate).
- **Dispersibility value of 0.1–0.25** corresponds to a powder with bad dispersibility, with an agglomerate diameter that is 4–10 times larger than the primary particle (32–500 primary particles in an agglomerate).
- **Dispersibility value of <0.1** corresponds to a powder with very bad dispersibility, with an agglomerate diameter that is more than 10 times larger than the primary particle (more than 500 primary particles in an agglomerate with a loose-packed configuration).
- **Note**: The agglomerate factor, which is the inverse of dispersibility, is often used in ceramics.

2.5 Evaluation and conclusions

2.5.1 *TG 109 Density*

TG 109 (density) is applicable to NM. For measurements of true density, only a small adaption of the calibration method is necessary. Tap and pour densities are measurable if the flowability of the nanopowder is sufficient. The method proposed in the literature for measurement of the agglomerate density, which could be important for dosage estimation, was tested and a SOP elaborated. The method is not applicable to powders with very low densities that are agglomerated because under the chosen centrifugation conditions the agglomerates do not sediment. However, this has no influence on the method because such powders will also not sediment during in vitro tests.

2.5.2 *Granulometry*

In summary, DLS measurements carried out according to the modified PROSPECT dispersion protocol were more correctly performed. As shown from data in Table 7-3, the Z-average values of most NMs reduced after simply increasing the sonication time from 20 s to at least 1 min. However, the polydispersity index (PDI) of most NMs were still high. New studies on improving the dispersion method will be conducted in order to

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improve the PDI data. Other NMs, mainly the NFC fine coarse and NFC medium coarse, are not suitable to be sized by DLS. This limitation is expected to be solved by FEG-SEM. The complete characterization of all NMs by DLS and FEG-SEM, also including BET surface area determinations, will be reported in the next deliverable.

2.5.3 *TG 105 - Water Solubility*

TG 105 was not directly applicable for testing the solubility of NM in water. The revised versions; using either the modified flask method or the atmosphere-temperature-pH-controlled stirred batch reactor with 3 KDa filtration of supernatant appears to provide suitable results for determination of water-solubility – or rather dissolution rates in water. For NMs with low solubilities, electrochemical methods may be advantageous, but one should be very careful about artefacts and electrode drift using these methods.
The results provided by the established stirred batch reactor method provide detailed information about the pH and Eh test conditions, which can either be controlled or not controlled as needed.
A harmonized procedure for determination of the kinetic dissolution rates must be established. Further studies are required to assess the dependence of NM dissolution rates on the initial NM concentration. Considerations should also be made on whether the water composition and pH values should be controlled by buffering or titration rather than left free as applied in the current set up. Data obtained on well-defined conditions may be of greater use for technical purposes as well as fate modelling and risk assessment.
Further studies are required to assess the dependence of NM dissolution on the initial NM concentration, NM size, and type of solvent (e.g. pH and composition of the media).

2.5.4 TG 112 (dissociation constant in water) and TG 108 (complex formation in water)

The proposed replacement of “dissociation constant in water” and “complex formation in water” by the isoelectric point of dispersions and the adsorption capacity of NM for dissolved heavy metal cations seems to be possible. The developed protocols are reproducible and give important additional information regarding the physico-chemical properties of the material. The isoelectric point is important to estimate the colloidal stability of nanoparticle suspensions at biological pH or for the determination of the dispersibility. The adsorption capability for heavy metals is important to understand possible secondary effects regarding toxicity measurement (reduced content of soluble heavy metals). Both methods need a validation by interlaboratory tests.

2.5.5 *TG 115 Surface Tension of aqueous solutions*

TG 115 is applicable to dispersion with nanoparticles

2.5.6 *New TG for determination of “Dispersibility”*

The developed SOP for the determination of dispersibility was tested with several NANoREG powders. The SOP works well and gave reproducible results. Unfortunately, several partners were not able to test the method because the necessary equipment was not available.

2.5.7 *Other methods (TG 106, TG 107/117/123)*

In D2.3, it was mentioned that TG 106 (sorption-desorption) and TG 107/117/123 (*n*-octanol-water partition coefficient) are not applicable or only partially applicable to nanoparticles. A detailed literature search shows that this conclusion is still valid for these two properties. Praetorius et al. [Praetorius et al. 2014] reinforced this conclusion, stating that the ‘use of ‘partition coefficients’ instead of attachment efficiencies in predictive environmental fate or bio-concentration models for ENPs will very likely lead to erroneous results, thereby making risk assessment based on these results meaningless’. Cornelis [Cornelis 2015] has also investigated in detail the partition coefficient and the interaction of nanoparticles with soil by comparing three possible fate descriptors for NMs in soils:

- batch partition coefficients (K_d values)
- batch retention coefficients (K_r values)
- column attachment efficiency

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As mentioned in D2.3 K_d values are not appropriate fate descriptors for ENP because the equilibrium assumption is not valid. The kinetic interpretation of batch studies offered by K_r values may be confounded by high shear conditions during batch tests, making it difficult to use this parameter in transport or bioavailability calculations. Moreover, column experiments need a number of operationally defined parameters and do not lead to a widely applicable physical parameter.

2.6 Data Management

SOP's for the determination of Granulometry, Density (true and effective), water solubility, dispersibility and Zeta potential measurement were developed and up loaded to CIRCABC C-NANoREG results > NANoREG developed_improved SOPs and Methods > WP2 developed_improved SOPs and Methods .

The results of the characterization of the Nanoreg standard powders are up loaded to the TNO Nanoreg database under the chapter "Characterisation (https://diamonds.tno.nl/nanoreg2015/overview/index)

3 Deviations from the work plan

The work was completed as described in the DoW. Challenges and delays were met due to reorganization due to AIDICO leaving the project. For the remaining partners, delays were caused by instrumental down-time and the need to develop new characterization procedures rather than modify existing OECD TGs.

4 Performance of the partners

All partners contributed to the planned work as identified in the text. All partners contributed with additional work to cover up for AIDICO, who had to leave the NANoREG project at an early stage, and to develop proposals for new TGs suitable for MNM characterization.

5 References / Selected sources of information (optional)

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6 List of abbreviations (optional)

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7 Annexes

7.1 Excel file for the determination of agglomerate density

Density Calculations

Reference

$$\rho_{EV} = \rho_{media} + \left[\left(\frac{M_{ENM} - M_{ENMsol}}{V_{pellet} SF} \right) \left(1 - \frac{\rho_{media}}{\rho_{ENM}} \right) \right] \quad (5)$$

DeLoid et al. 2014

Input parameters

$M_{ENM} = c_{ENM} * V$

Density Di water (at 20°C)	0.998205	g cm^{-3}	
Density NP	3.6079	g cm^{-3}	measured density
Volume (tube)	1	mL	
SF (random stacking)	0.634		

c_{ENM} [g/mL]	ϑ_{ev} for ISDD	Vpellet (mL)	ϑ_{ev}	Comment	Date
	NM in DI water				
4.50E-03	**4.5 mg/mL**	0.003725	2.3764684540		
		0.003125	2.6410950372	Aurélie	
		0.003475	2.4756240982		
		0.003205			
		0.0037		Sophie	
		0.00385			
		0.003305			
		0.0033		Sophie	
		0.003305			
	4.5 mg/mL	0.003443333	2.498		

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7.2 Example of the determination of the Dispersibility: NM-103

INPUT:

- **Cell B1**: Name of the powder and other important information.
- **Cells C5 – L10**: The measured agglomerate diameter in nm. The values have to be measured following the SOP's and has to be given in DLS z-average.
- **Cell C15:** BET in m^2/g (you can use the BET values given in the Technical Data Sheet or measured following the corresponding SOP).
- **Cell C16**: Density in $g/cm^{3.}$
- **Cell C18** Diameter of the primary particles in nm (you can use the D_{TEM} values given in the Technical Data Sheet or measured following the corresponding SOP). This value is only for comparison. The diameter of the primary particle measured by TEM or calculated from BET and Density must be similar.

OUTPUT

- **Cells C11-L11:** Mean diameter of the agglomerates (nm) for each time point of dispersion.
- **Cells C12- L12**: Standard deviation for each time point of dispersion.
- **Cells C13-L13:** Standard deviation in % of the mean diameter for each time point of dispersion.
- **Cells C23-L23**: Dispersibility for each time point of dispersion calculated from D_{BET}/D z-average (Dz-avarage is taken from Cells C11-L11).
- **Cells D24-L24**: Dispersibility rate (1/min) is the rate how fast the agglomerate size is changing.
- **Cells C25-L25:** The agglomerate factor is the invers of Dispersibility. Agglomerate factor of 1 means primary particle size = agglomerate size, 10 means the agglomerate diameter is 10 times large than the primary particle size.
- **Cell F27:** Dispersibility after 30 min ultra sound treatment. This value has to be given in the Result template "Dispersibility "of Nanoreg. For your own purpose, you can change the dispersion time in cell E27 to see the effect of dispersion time on de-agglomeration.

Figures

- **Right side top**: Your measured data plus the calculated mean diameter.
- **Right side middle**: The calculated Dispersibility (**Cells C23-L23**).
- **Right side bottom:** Dispersibility rate (Cells D24-L24).
- **Bottom left:** Dispersibility, double logarithmic presentation, used for the calculation of the "dispersibility" Cell F27
- **Bottom right:** Dispersion rate in double logarithmic presentation (values not used so far, but could be helpful for a further classification of the materials)

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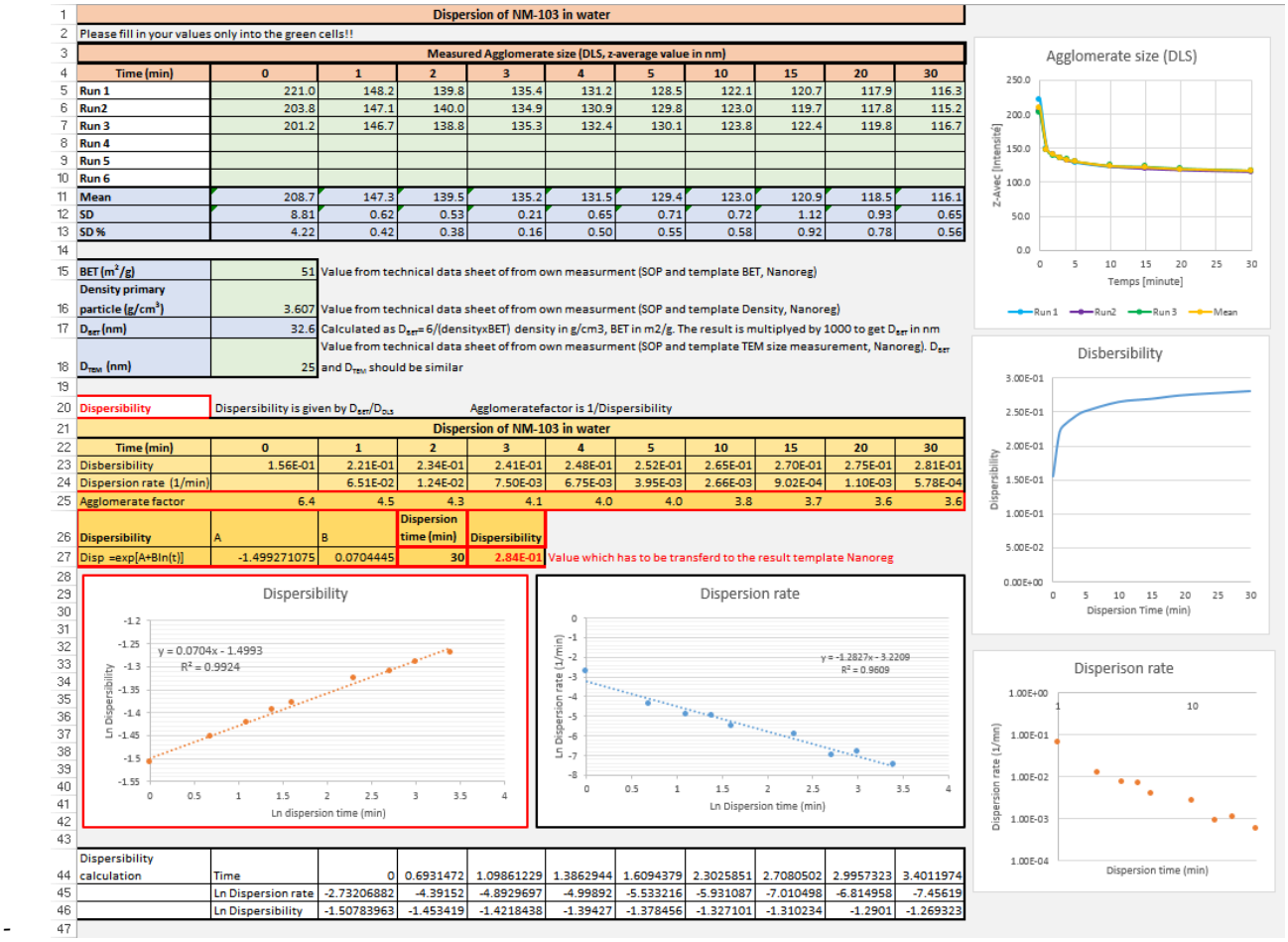

Dispersion of NM-103 in water

Please fill in your values only into the green cells!!

Measured Agglomerate size (DLS, z-average value in nm)

Time (min)	0	1	2	3	4	5	10	15	20	30
Run 1	221.0	148.2	139.8	135.4	131.2	128.5	122.1	120.7	117.9	116.3
Run2	203.8	147.1	140.0	134.9	130.9	129.8	123.0	119.7	117.8	115.2
Run 3	201.2	146.7	138.8	135.3	132.4	130.1	123.8	122.4	119.8	116.7
Run 4										
Run 5										
Run 6										
Mean	208.7	147.3	139.5	135.2	131.5	129.4	123.0	120.9	118.5	116.1
SD	8.81	0.62	0.53	0.21	0.65	0.71	0.72	1.12	0.93	0.65
SD %	4.22	0.42	0.38	0.16	0.50	0.55	0.58	0.92	0.78	0.56

BET (m^2/g)	51	Value from technical data sheet of from own measurment (SOP and template BET, Nanoreg)
Density primary particle (g/cm^3)	3.607	Value from technical data sheet of from own measurment (SOP and template Density, Nanoreg)
D_{BET} (nm)	32.6	Calculated as D_{BET}= 6/(densityxBET) density in g/cm3, BET in m2/g. The result is multiplyed by 1000 to get D_{BET} in nm
D_{TEM} (nm)	25	Value from technical data sheet of from own measurment (SOP and template TEM size measurement, Nanoreg). D_{BET} and D_{TEM} should be similar

Dispersibility — Dispersibility is given by D_{BET}/D_{DLS} — Agglomeratefactor is 1/Dispersibility

Dispersion of NM-103 in water

Time (min)	0	1	2	3	4	5	10	15	20	30
Disbersibility	1.56E-01	2.21E-01	2.34E-01	2.41E-01	2.48E-01	2.52E-01	2.65E-01	2.70E-01	2.75E-01	2.81E-01
Dispersion rate (1/min)		6.51E-02	1.24E-02	7.50E-03	6.75E-03	3.95E-03	2.66E-03	9.02E-04	1.10E-03	5.78E-04
Agglomerate factor	6.4	4.5	4.3	4.1	4.0	4.0	3.8	3.7	3.6	3.6

Dispersibility	A	B	Dispersion time (min)	Dispersibility	
Disp =exp[A+BIn(t)]	-1.499271075	0.0704445	30	2.84E-01	Value which has to be transferd to the result template Nanoreg

Dispersibility calculation	Time	0	0.6931472	1.09861229	1.3862944	1.6094379	2.3025851	2.7080502	2.9957323	3.4011974
	Ln Dispersion rate	-2.73206882	-4.39152	-4.8929697	-4.99892	-5.533216	-5.931087	-7.010498	-6.814958	-7.45619
	Ln Dispersibility	-1.50783963	-1.453419	-1.4218438	-1.39427	-1.378456	-1.327101	-1.310234	-1.2901	-1.269323

7.3 Granulometry

Table 7-1 Code and description of nanomaterials characterized by DLS measurements (Embrapa Instrumentação)

NM Code	Description
NM200	*Amorphous SiO_2*
JRCNM01101a	*ZnO*
JRCNM01001a	*TiO_2*
13463-67-7	*TiO_2 H2*
NM-300K	*Colloidal Ag*
NM-302	*Ag Nanorods*
NM-411	*Carbon nanotubes*
NFC Fine Coarse	*Cellulose nanofibers*
NFC Medium Coarse	*Cellulose nanofibers*

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Table 7-2 Description of methodologies used to disperse NMs (Embrapa Instrumentação).

NM Code	Description	Solvent	Dispersion 1 Mass (mg) /dispersion time (min)	Dispersion 2 Dilution (Vol/Vol) / dispersion time (min)	Additional information
NM-200	*Amorphous SiO_2*	*Deionized water*	*1.0 mg / 10 mL 3 min*	*0.10 mL / 10 mL 3 min*	*Previous de-agglomeration using mortar and pestle*
JRCNM01101a	*ZnO*	*0,1% (wt./vol) BSA in deionized water*	*1.0 mg / 20 mL 3 min*	*0.2 mL / 10 mL 3 min*	*-*
NM-300K	*Coloidal Ag*	*Deionized water*	*1.0 mg / 10 mL 3 min*	*0.10 mL / 10 mL 3 min*	*-*

An overall improvement of the Z-average size data can be verified from Table 7-3. Also, it was observed the Z-average values of ***JRCNM01101a***; ***NM-200*** and ***NM-300K*** (Colloidal Ag) slightly decreased with increasing sonication time. On the other hand, the ***13463-67-7*** NM presented an increase of size over increasing sonication time. Probably, the sonication treatment removed the coating of the particles which led to particles agglomeration.

Table 7-3 Summary of Dynamic Light Scattering (DLS) data for different Nanomaterials (NMs).

NM Code	Protocol (sonication time)	Z-average size (d.nm)	PDI
JRCNM01101a *(TiO2)*	*20s**	*812*	*0.34*
	1 min	*551*	*0.42*
	2 min	*528*	*0.34*
	3 min	*510*	*0.36*
NM-200 *(Amorphous SiO2)*	*20s*	*2359*	*1*
	1 min	*707*	*0.71*
	2 min	*535*	*0.68*
	3 min	*650*	*0.75*
13463-67-7 *(TiO2 H2)*	*20s**	*314*	*0.19*
	1 min	*551*	*0.35*
	2 min	*529*	*0.37*
	3 min	*492*	*0.38*
NM-300K *(Colloidal Ag)*	*20s**	*245*	*0.30*

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	1 min	*189*	*0.33*
	2 min	*108*	*0.40*
	3 min	*110*	*0.43*
NM-302 ***(Ag Nanorods)***	*20s**	*1894*	*1*
	1 min	*2225*	*1*
	2 min	*2505*	*1*
	3 min	*2313*	*1*
NFC Fine Coarse	*20s**	*2709*	*0.74*
	6 min	*2138*	*0.95*
NFC Medium Coarse	*20s**	*2945*	*1*
	6 min	*1051*	*0.763*

*Data from the protocol of 20s of sonication are that presented in the previous 6th Six-Month Technical Report.

Furthermore***, NM302, NFC Fine Coarse*** and ***NFC Medium Coarse*** are elongated and micro-sized NMs as observed by SEM data. The size of these materials exceeds the size scale accessed by DLS. Untypical correlation function obtained for ***NM-302***, ***NFC Fine Coarse*** and ***NFC Medium Coarse*** are plotted together with that obtained for ***JRCNM01101a*** to show the low quality of these materials to be studied by DLS.

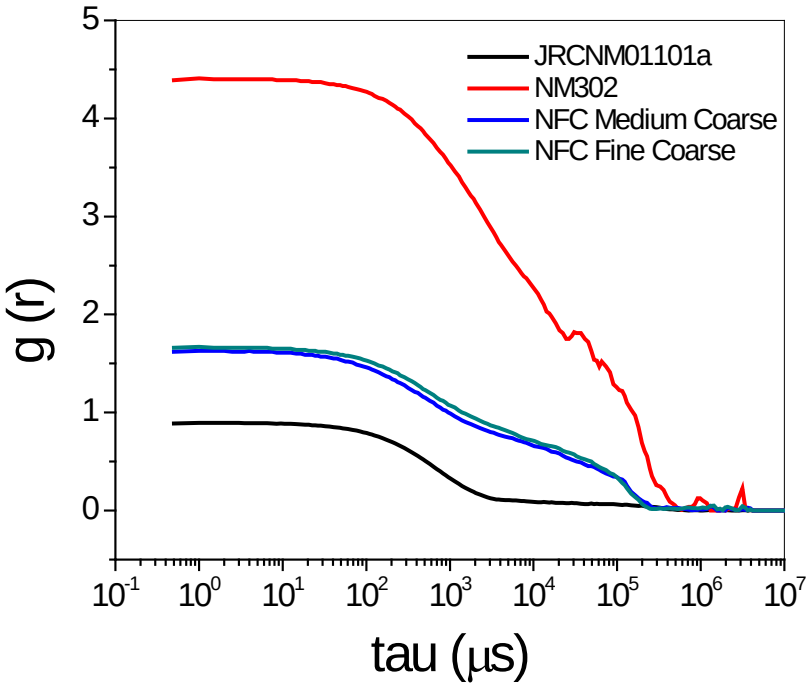

Figure 7-1 Correlation function obtained for NM-302, NFC Fine Coarse and NFC Medium Coarse and JRCNM01101a materials.

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Figure 7-2 presents the original size distribution obtained by DLS. It is observed for most NM dispersions that the change of tip dimensions and increase of sonication time from 20 s to at least 1 min led to an overall homogeneity and narrowing of the particle size distributions. In only a few cases, the modification of the PROSPECT dispersion protocol did not lead to significant improvement of the Z-average data. This is particularly seen for the cellulose nanofibers samples (NFC fine coarse and NFC medium coarse), which were too large to be properly measured by DLS.

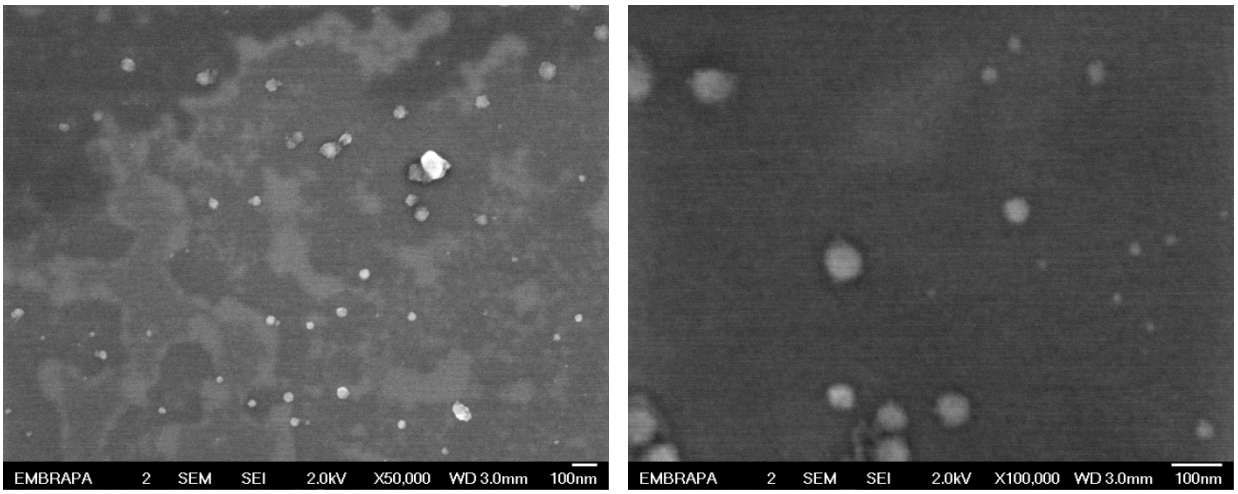

Figure 7-2 FEG-SEM images of magnifications of 50,000X (left) and 100,000X (right) of NM-200 **– Amorphous Silica.**

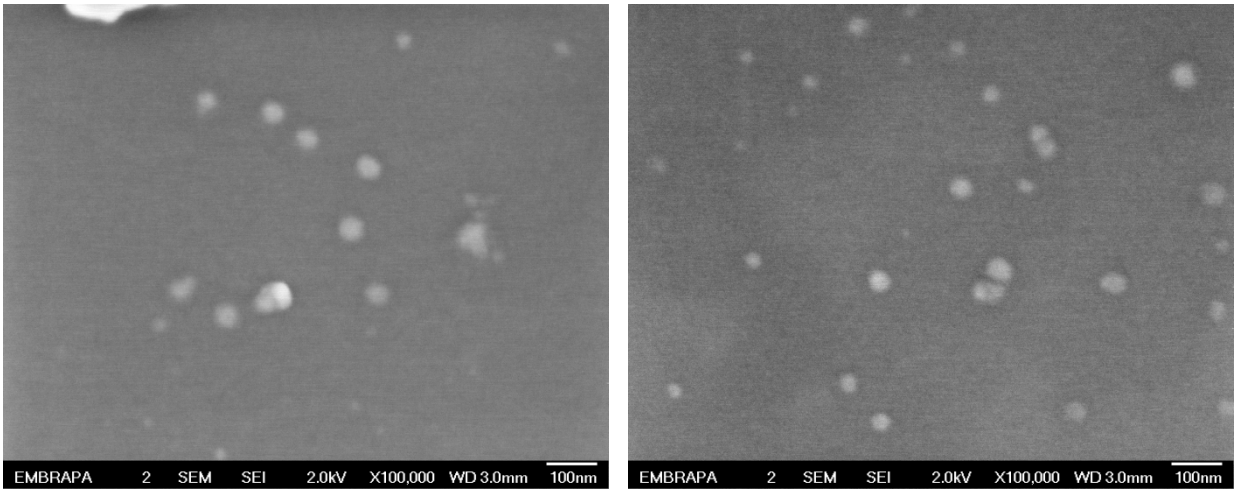

Figure 7-3 FEG-SEM images of magnifications of 100,000X (left) and 100,000X (right) of NM-200 **– Amorphous Silica.**

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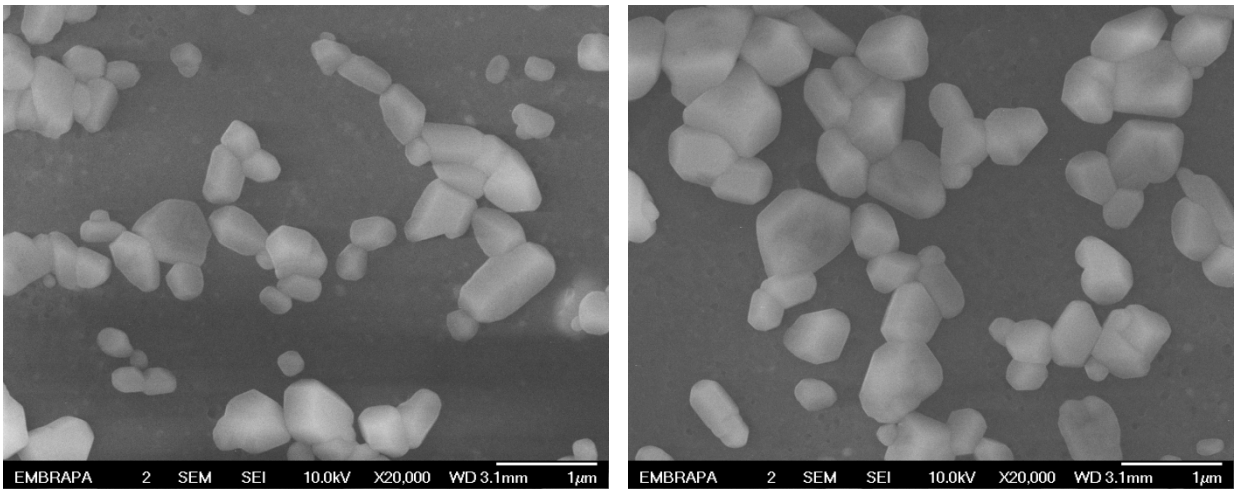

Figure 7-4 FEG-SEM images of magnifications of 20,000X (left) and 20,000X (right) of JRCNM01101a – ZnO.

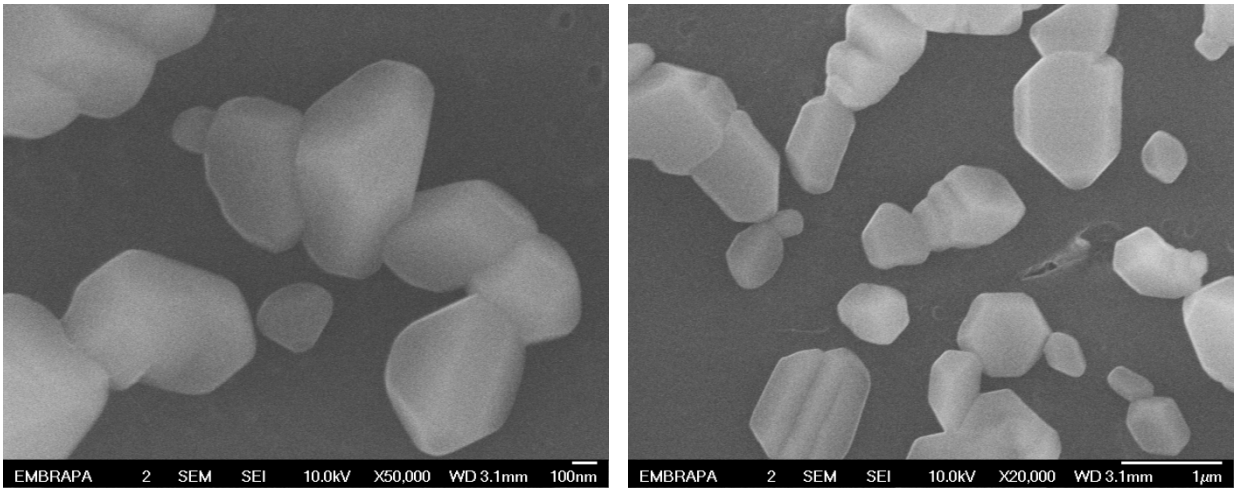

Figure 7-5 FEG-SEM images of magnifications of 50,000X (left) and 20,000X (right) JRCNM01101a – ZnO.

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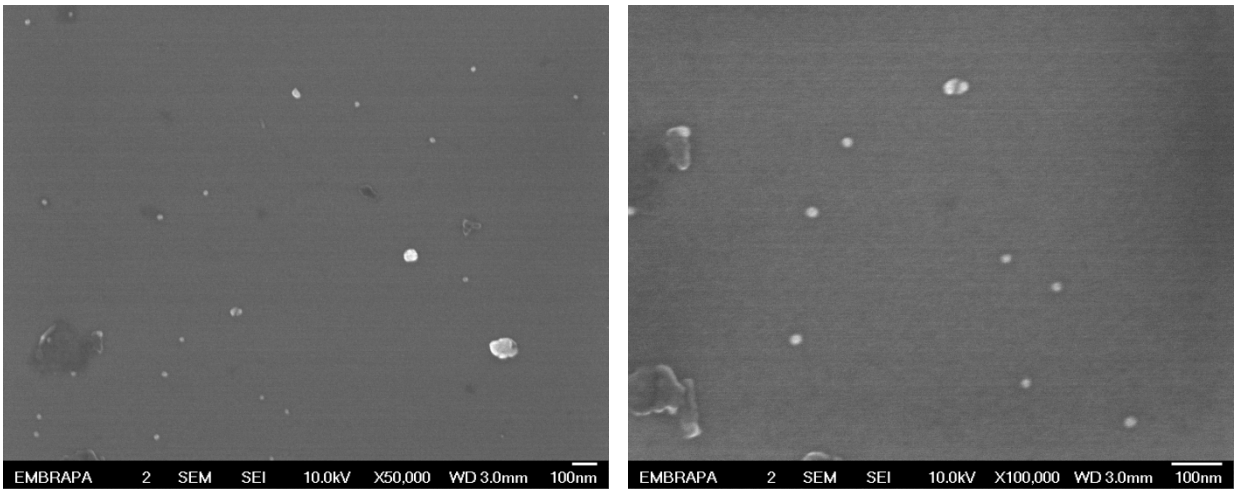

Figure 7-6 FEG-SEM images of magnifications of 50,000X (left) and 100,000X (right) of NM-300K – Colloidal Ag.

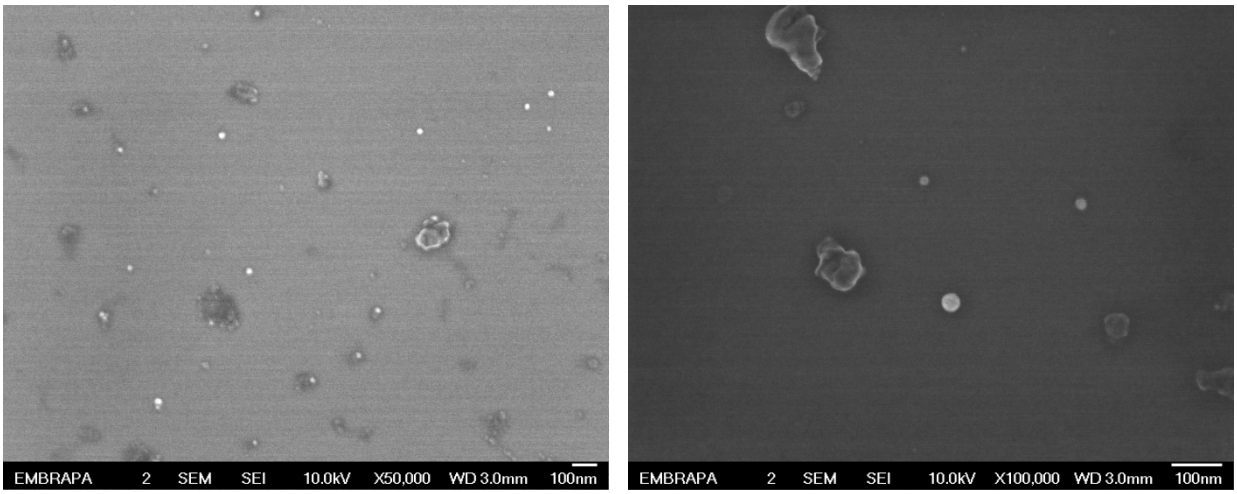

Figure 7-7 FEG-SEM images of magnifications of 50,000X (left) and 100,000X (right) of NM-300K – Colloidal Ag.

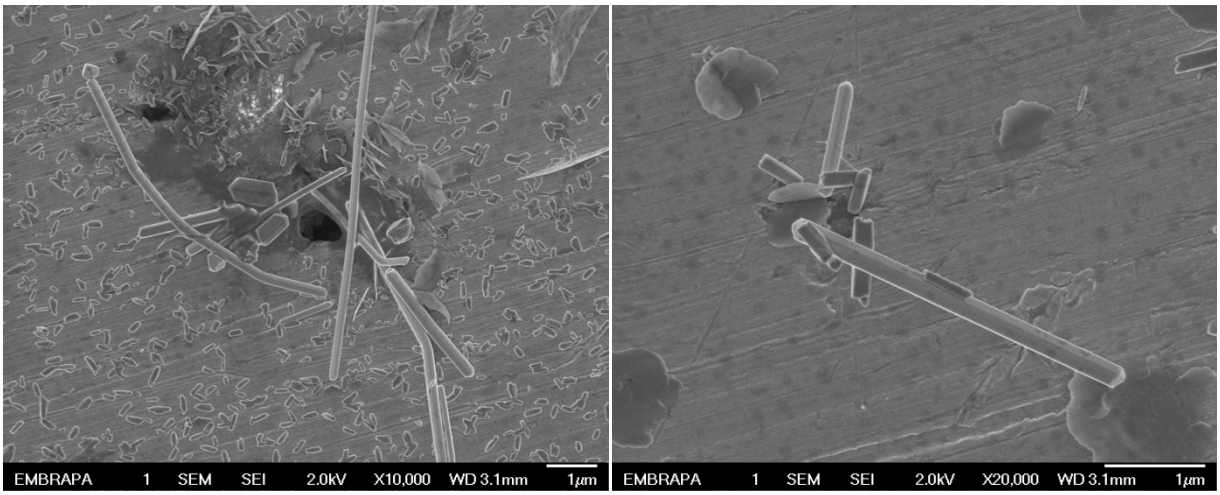

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Figure 7-8 FEG-SEM images of magnifications of 10,000X (left) and 20,000X (right) of NM-302K – Ag Nanorods.

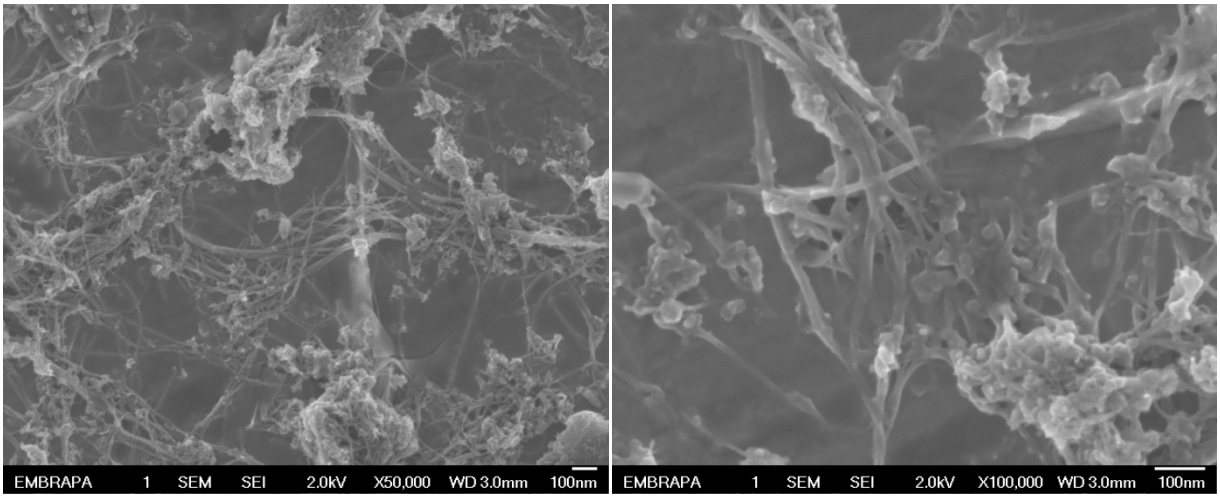

Figure 7-9 FEG-SEM images of magnifications of 50,000X (left) and 100,000X (right) of NM411 – Carbon Nanotubes.

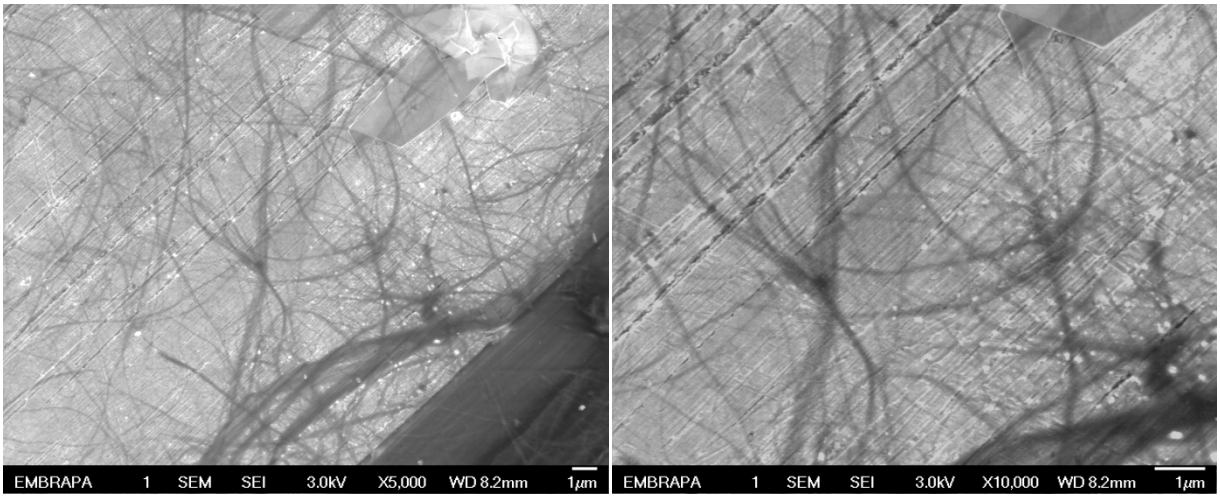

Figure 7-10 FEG-SEM images of magnifications of 5,000X (left) and 10,000X (right) of NFC – Medium Coarse.

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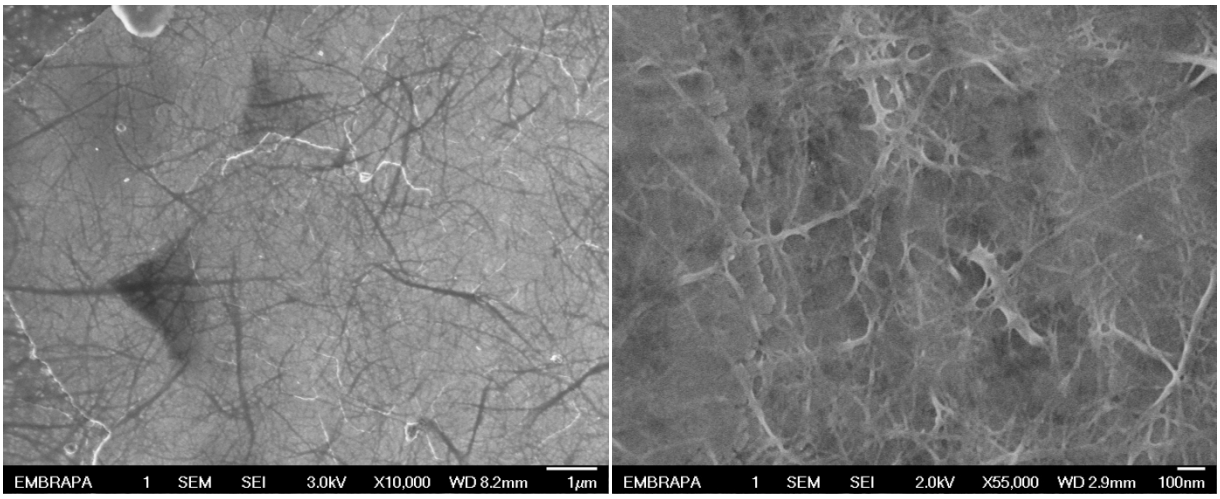

Figure 7-11 FEG-SEM images of magnifications of 10,000X (left) and 55,000X (right) of NFC – Fine Coarse

Figure 7-12, 7-13 and 7-14 present size distributions of the NMs, NM-200 - Amorphous Silica, NM-300K - colloidal Ag, JRCNM01101a - ZnO, NM-302 - Ag nanorods, NM-411 - Carbon Nanotubes, NFC Medium Coarse and CNF Fine Coarse, respectively. For the Amorphous Silica and the colloidal Ag, histograms were built over the measurements of the spherical particles diameters, thus dispersed single particles could be identified as spheres and their sizes could be measured. In the case of the JRCNM01101a (ZnO), nanoparticles could be dispersed and individually identified. Their morphology was not spherical, but rather elliptical or oval. The dimension reported is related to the greater diameter, identified as the particles' length. Samples NM-302, NM-411 and both CNF present fibrillary morphology, so their measurements described as diameter represent the smaller dimension, and the length, the greater dimension, and their size distribution are presented in Figures 7-15 to 7-19.

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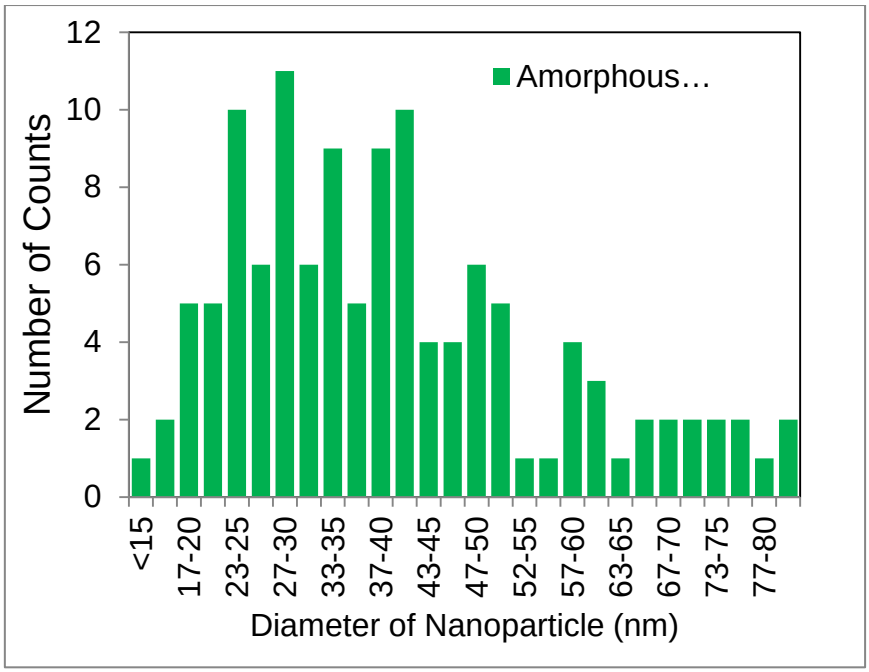

Figure 7-12 Particle size distribution of NM-200 - Amorphous Silica - obtained by FEG-SEM.

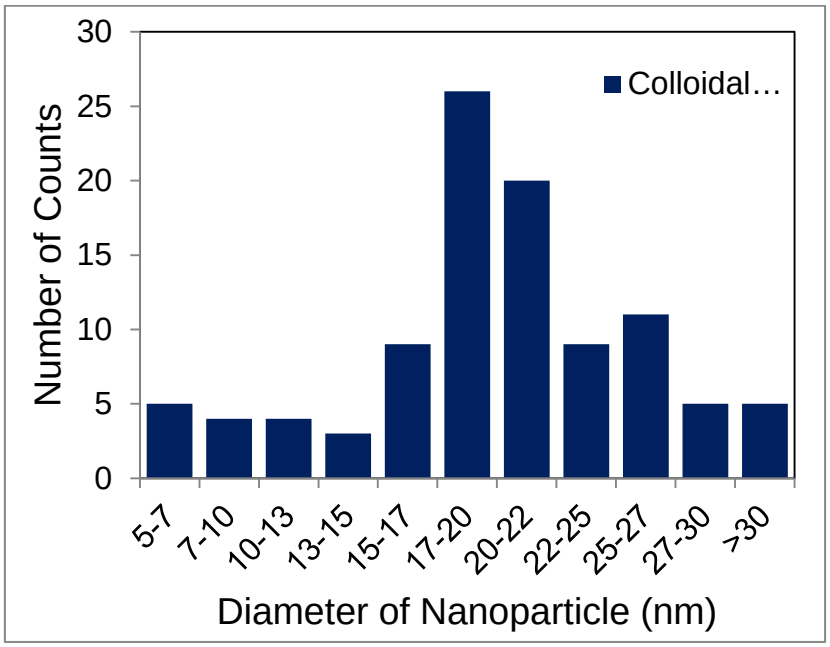

Figure 7-13 Particle size distribution of NM-300K - colloidal Ag obtained by FEG-SEM.

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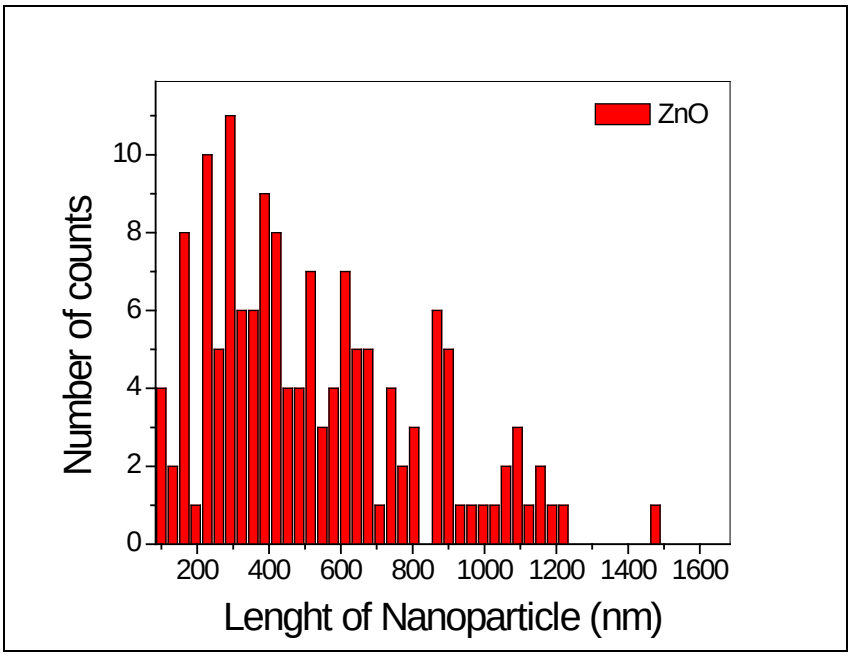

Figure 7-14 Particle size distribution of JRCNM01101a – ZnO - obtained by FEG-SEM.

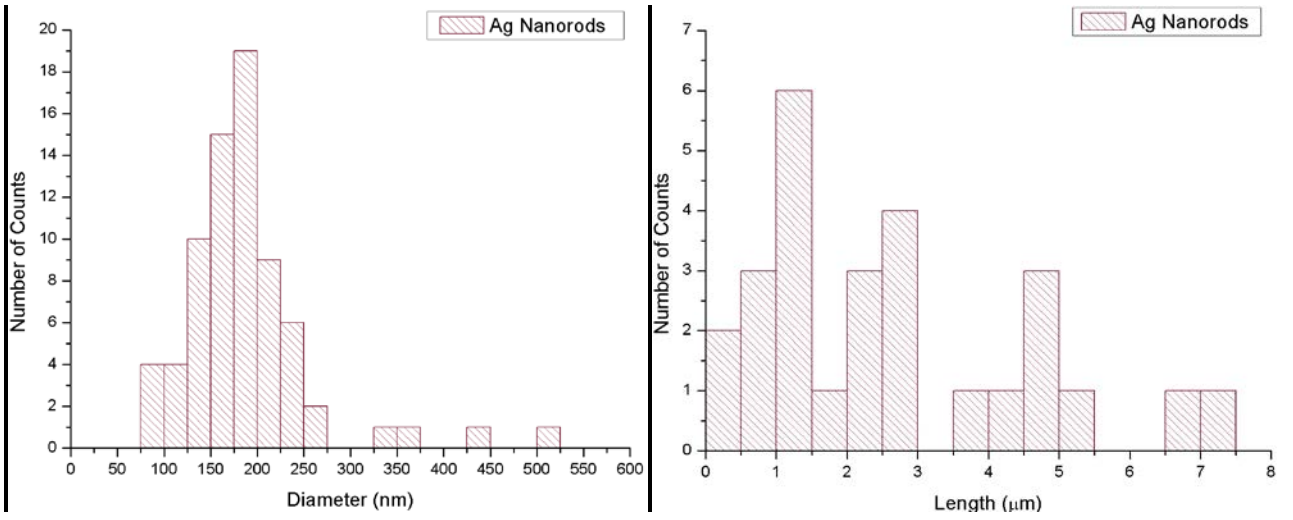

Figure 7-15 Particle size distribution of NM-302 - Ag nanorods - obtained by FEG-SEM. (Average Diameter of 190±60 nm and Lenght of 3±2 μm).

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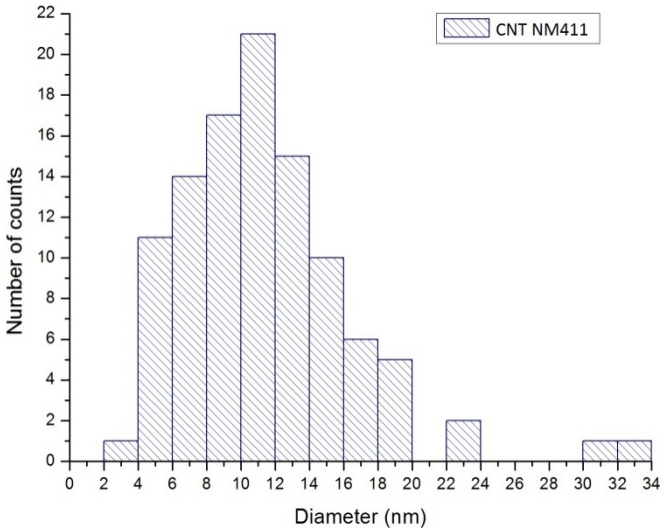

Figure 7-16 Particle size distribution of NM-411 - Carbon Nanotubes (CNT) - obtained by FEG-SEM. (Average Diameter of 11±5 nm and Length of several microns).

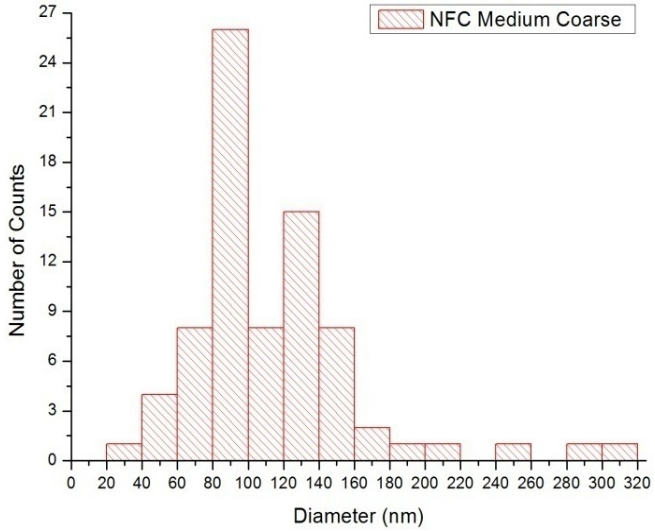

Figure 7-17 Particle size distribution of NFC Medium Coarse - obtained by FEG-SEM. (Average D=116±66 nm and Length of several microns).

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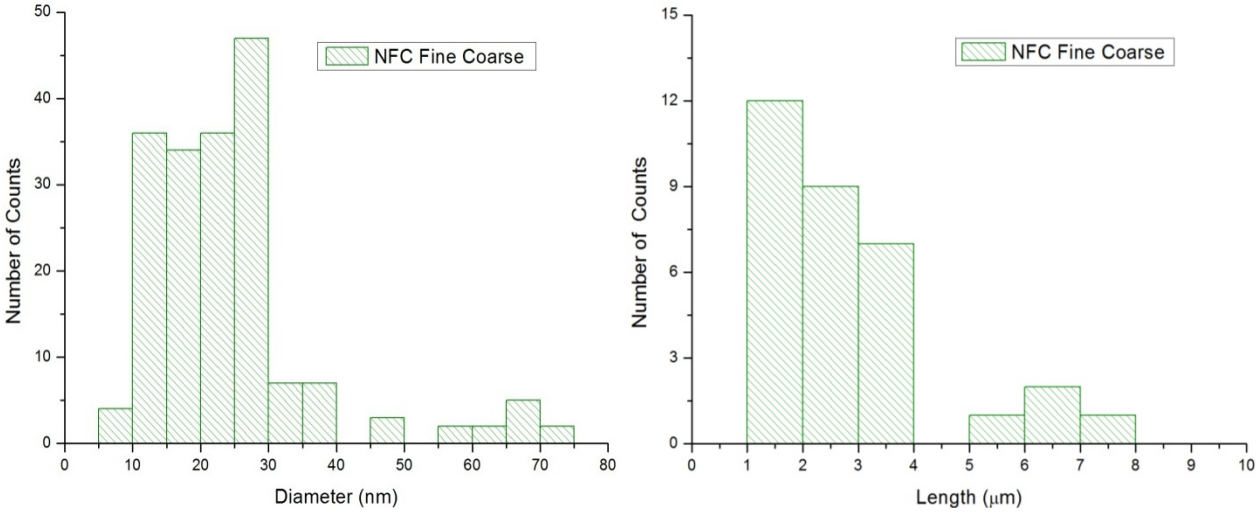

Figure 7-18 Particle size distribution of NFC Fine Coarse - obtained by FEG-SEM. (Average D=24±11 nm and Length L=3±1 μm).

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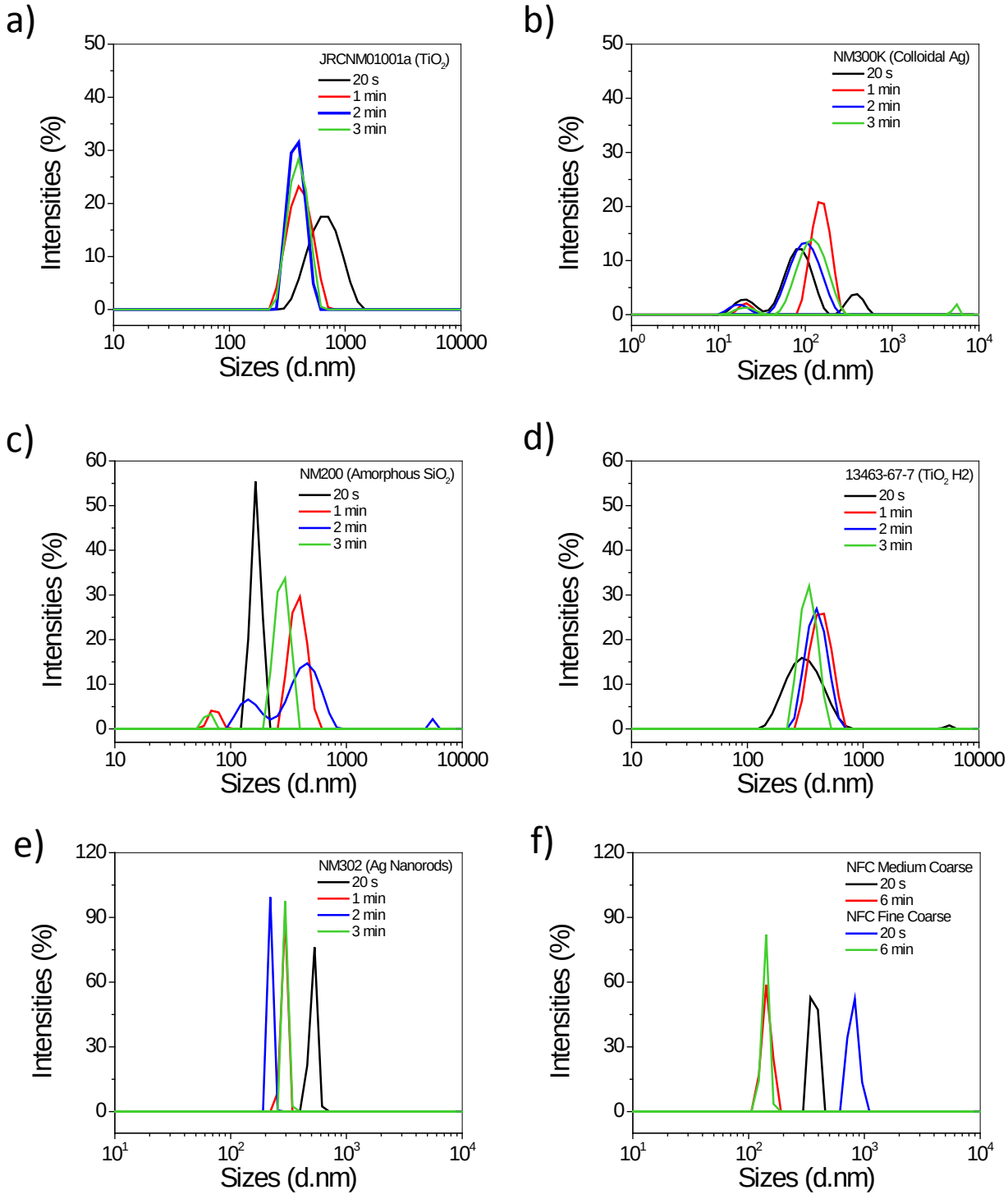

Figure 7-19 Particle size distributions of a) JRCNM01101a; b) NM-300K; c) NM-200; d) 13463-67-7 e) NM302 and f) NFC Medium Coarse and NFC Fine Coarse obtained by a different dispersion procedure (change of tip dimensions and sonication time).

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