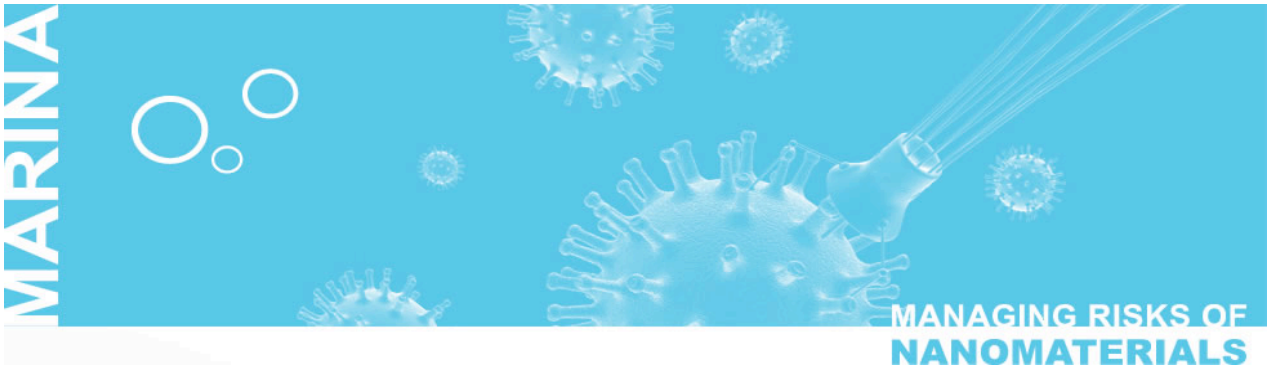

Report for

MARINA

Managing Risks of Nanomaterials

Grant Agreement Number 263215

Good Practice Guide for assessment of nanomaterials in situ during and after biological assays to be posted on the MARINA Hub

Work & main achievements

FP7 MARINA aims to harmonise research activities by developing a coherent set of common activities/tools. The establishment of a common research infrastructure is essential in order to establish a more cohesive research. It is the goal of this best practice to identify and provide common practices, with suitability to different studies i.e. nanospecific applications. We present basic lab practices and approaches, covering topics such as: measurement of good practice, terminology of physicochemical properties of toxicological relevance, how to sample, etc. This is a consensus-based "Best Practice" and thus a product of FP7 MARINA (WP4) and NANOVALID efforts.

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1.0 Introduction

Engineered nanomaterials represent a growing class of material being introduced into multiple business sectors, but there are increasing concerns surrounding the potential harmful effects of engineered nanomaterial imposed on health and the environment. The need to develop a sound scientific infrastructure for assessment of risk is clear but scientific findings have not yet provided any clear answers on the toxicity of nanomaterial [1].

Unlike chemicals, measuring the physicochemical characterisation of nanomaterials is not straightforward. From a scientific perspective, nanomaterials cannot be considered as a homogeneous group and this means that obtaining reliable data is not easy to achieve. Often, this leads to a situation in which experimental data gets reported without proper understanding of the associated errors, how all of these errors combine and propagate to the final result. Sources of errors may arise from a number of factors including: polydispersity of the nanomaterial sample, the biological environment that the nanomaterial is dispersed in and the fitness for purpose of analytical methods.

In relation to the polydispersity of nanomaterial, it has been argued by Baalousha and Lead[2] that most nanomaterials tested are too polydisperse. Materials close to monodispersity are needed in order to have better reliability of result findings associated with studying environmental behaviour, dose, structure–activity relationships and mechanisms of toxicity. With respect to the analytical techniques currently available, it is difficult to measure accurately, a highly polydisperse sample. A recent study conducted by Anderson et al. [3] show that complex particle size distributions i.e. away from the simple monomodal distribution results in large data variability. Furthermore, light scattering based methods such as Particle Tracking Analysis and Dynamic Light Scattering were only able to detect a single population of particles corresponding either the largest or smallest particles in a multimodal sample. Clearly, the inadequacy of the instrumental methods to characterise nanomaterials is a huge barrier in this field, as previously echoed by others in the field [1,2].

The complex biological matrix in which the particles are dispersed in can also pose problems where measurement is concerned. Nanomaterial-media interactions can be dynamic e.g., particles can agglomerate and sediment out. This may pose further problems where measurements are concerned e.g. unstable dispersion being more difficult to measure. Due to the inherent analytical challenges, many researchers often report the physicochemical properties of the “pristine” particles e.g., in the absence of the actual biological test media. In fact, few studies have assessed the potential transformation of nanomaterials in an environmental or mammalian system[4,5].

The problems identified above imply that careful thought should be given to potential sources of error, bias and variation in measurements. This guide thus aims to serve as a starting point for researchers i.e. a Best Practice that addresses generic issues, such as what we mean by good measurement practice. Other topics covered here include: terminology associated with physicochemical properties, powder and liquid sampling protocols, nanomaterial dispersion,

method development and validation.

2.0 Good measurement practice

Seemingly contradictory findings between studies are a major issue in nanotoxicology, sometimes attributed to the lack of comparability between assay methods[1]. If our final goal is regulatory certainty then having confidence in the data is imperative. Eurachem[6] has underlined six important principles of analytical practice:

1) *Analytical measurements should be made to satisfy an agreed requirement i.e. to a defined objective*
2) *Analytical measurements should be made using methods and equipment which have been tested to ensure they are fit for purpose*
3) *Staff making analytical measurements should be both qualified and competent to undertake the task (and demonstrate that they can perform the analysis properly)*
4) *There should be a regular independent assessment of the technical performance of a laboratory*
5) *Analytical measurements made in one location should be consistent with those made elsewhere*
6) *Organisations making analytical measurements should have a well-defined quality control and quality assurance procedures.*

Out of the six principles listed above, principle 2 that involved validation has seldom been carried out to the fullest extent. To date, no clear documents discussing validation in the context of nanomaterial testing exists. Even in established fields, where guidelines on validation exists e.g. pharmaceutical methods (United States Pharmacopeia (USP), International Conference on Harmonisation (ICH) and the Food and Drug Administration), clarity on what type of studies do exists but there is great diversity on how they are to be performed. In nanotoxicology, methods are often developed but rarely validated and yet every effort should be made toward validation, as only when the conditions of the method validation are met, a higher metrological standard of measurement can be considered e.g. to make measurements traceable, conduct analyses to estimate uncertainty and the propagation of uncertainty.

Making traceable measurements is not trivial. Traceability has been defined by Eurachem/CITAC guide[7] as "*property of the result of a measurement or the value of a standard whereby it can be related to stated references, usually national or international standards, through an unbroken chain of comparisons, all have stated uncertainties".* The generic traceability framework is broken down into two main activities i.e. the calibration and the development of an uncertainty budget. Calibration is defined as *comparison of an instrument against a reference or standard, to find any errors in the values indicated by the instrument*[8]. Uncertainty of measurement is the *quantified doubt about the result of a measurement*, which can be established by performing an uncertainty budget i.e.

a summary of the uncertainty calculations[9,10].

The main steps in establishing uncertainty budget are as follows (for further details on how to conduct a certainty budget, the reader is referred to other relevant documentations [11]):

a) *Define the measurand i.e. what is being measured.*
b) *Identify the major components contributing to measurement uncertainty*
c) *Quantify the contribution of each major component as standard deviations i.e. standard uncertainties*
d) *Statistically combine the contribution for each major component and compute the combined uncertainty*
e) *Compute the expanded uncertainty using an appropriate coverage factor, k.* This is done if we want to re-scale the result so that the if we wish to have an overall uncertainty stated at another level of confidence e.g. from 68% to 95 % level of confidence
f) *Report the results as the best estimate, along with the expanded uncertainty or a confidence interval*.

The topic of traceability will not be covered in details here as in relation to nanotoxicology, making traceable measurement is considered to be too high a goal due to the analytical challenges stated above, with an incomplete traceability chain being likely e.g. calibration being carried out under conditions too different from the application and calibration covering only a part of the measuring system.

3.0 Terminology: defining the measurand

The first step in measuring is to define the measurand as explicitly as possible. For physico-chemical property measurements (important to support toxicological findings), two guidance documents of relevance were issued by ISO and OECD. **Table 1** gives a summary of physicochemical properties considered of relevance and the terminology used by ISO and OECD[12,13].

Table 1: Terminology: physicochemical properties by ISO vs. OECD.

ISO physicochemical properties	OECD "end-points"	Definition; unless state otherwise the following definitions has been extracted from a recent ISO document[14].	Measurand; unless stated otherwise, the following definitions has been extracted from the corresponding ISO document[14].
Particle size and particle size distribution	Particle size distribution – dry and in relevant media Representative TEM images	*"The physical dimensions of a particle and, for collections of particles, the distribution of the sizes of the particles determined by specified measurement conditions"*	*"Equivalent spherical diameter, for particles displaying a regular geometry (unit m); the length of one or several specific aspects of the particle geometry, (unit m) the particle size distribution, the number of peaks and their width are a set of values, often displayed as a histogram, which for each of a number of defined size classes which shows the quantity of particles, being either the number of particles, or the cumulative length, area, or volume of these particles or the signal intensity they produce"*
Aggregation/ Agglomeration state in relevant media	Agglomeration/ Aggregation Representative TEM images	*Aggregate is "strongly bonded or fused particles where the resulting external specific surface area might be significantly smaller than the sum of known specific surface areas of primary particles". Agglomerate is "collection of weakly or loosely bound particles or aggregates or mixtures of the two in which the resulting external specific surface area is similar to the sum of the specific surface areas of the individual components"*	*"Particle size (unit, m); number of aggregate (or agglomerate) particles in comparison to the total number of primary particles, unit (number/number); number of primary particles in the aggregate (or agglomerate), unit (number/number); distribution of number of primary particles per aggregate (or agglomerate)."*
Shape	Representative TEM images	*"A description of the contour or outline of the surface of the nano-objects or collection of nano-objects, aggregates, agglomerates, that make up the material under investigation"*	*"Size-independent descriptors of shape (examples are ratios of extensions in a different direction such as aspect ratio, unit (m/m) or fractal dimension); distribution of values of the size-independent shape descriptors"*
Surface area/mass-specific surface area/volume-specific surface area	Specific surface area Porosity	*This is the "quantity of accessible surface of a sample when exposed to either gaseous or liquid adsorbate phase. Surface area is conventionally expressed as a mass specific surface area or as volume specific area where the total quantity of area has been normalised*	*"Specific surface area is defined as surface area of a substance divided by its mass, unit [m^2/g]; or surface area of a substance divided by its volume, unit [m^2/cm^3]. The research should also consider reporting results in both m^2/g and m^2/cm^3. "*

		either to the sample's mass or volume"	
Composition	Crystallite size. Crystalline phase.	*"Chemical information and crystal structure of the entire sample of nano-objects including: a) composition b) crystalline structure including lattice parameters and space group, and c) impurities, if any"*	*"The number and identity of elements alone or in molecules (can be expressed as a chemical formula with a specific stoichiometry; crystalline state; crystallographic structure; chemical state of atoms/elements; molecular structure-conformation including dextrorotatory and levorotatory (handedness); spatial distribution of the above items."*
Surface chemistry	Surface chemistry, where appropriate Redox potential Radical formation potential Photocatalytic activity Octonal- water partition coefficient i.e. to what degree colloidal suspended particle in the aqueous phase can also be suspended in a non-aqueous phase (such as octanol)	*"Chemical nature, including composition, of the outermost layers of the nano-objects and their aggregates and agglomerates greater than 100 nm"*	*"Elemental and molecular abundance unit [mole/mole], including thickness for fixed layers or [number of molecules/surface area] or [number of molecules bound /theoretical number of molecules bound with perfect reaction or perfect packing] for chemically reacted species that do not form a distinct phase; reactivity: standard chemical reaction rate concepts [mole/(dm^3/s)] preferably of a species of toxicological interest or its surrogate. Measurement of reactivity is very specific to the measurement of the species to which it is reactive (such as reactive to water) and typically involves measuring products or by-products of that reaction."*
Surface charge	Zeta-potential (surface charge)	*"Electrical charge on a surface in contact with a continuous phase"*	*"Net number of positive and negative charges per unit particle surface area, unit [Coloumb/m^2]; zeta potential, unit [V]"*

Solubility/Dispersibility	Water Solubility/ Dispersibility	*"Solubility is the degree to which a material (the solute) can be dissolved in another material (the solvent) so that a single homogeneous phase results. Dispersibility is the degree to which a particulate material (the dispersed phase) can be uniformly distributed in another material (the dispersing medium or continuous phase) and resulting dispersion remains stable (for example one hour or one minute)"*	*For solubility this is the "maximum mass or concentration of the solute that can be dissolved in a unit mass or volume of the solvent at specified (or standard) temperature and pressure, unit [kg/kg] or [kg/m^3] or mole/mole]".* *For dispersibility, this is "the maximum mass or concentration of the dispersed phase present in a unit mass of the dispersing medium (solvent) or in a unit volume of the dispersion (solvent plus dispersed phase) at specified (or standard) temperature and pressure, units [kg/kg], [kg/m^3], or [mole/mole].*
N/A	Dustiness	*This is defined as the "propensity of a material to generate airborne dust during its handling, and provides a basis for estimating the potential health risk due to inhalation exposure".*[15]	*"The measurand of interest is the degree to which a given nanomaterial can remain in the air column before settling. This would require investigation and characterisation of interactions of nanomaterials with other common airborne particulate matter."*[15]
N/A	Pour density	*This is the "apparent density of a bed of material formed in a container of standard dimensions when a specified amount of the material is introduced without settling".*[15]	*"Determination of bulk density."*[15]

The first major difference between the two guidelines is the use of OECD "endpoints" as oppose to ISO "properties". Another difference is that OECD has a longer list with sixteen endpoints as oppose to ISO's eight properties. However, a number of the OECD endpoints can be put in categories under a particular ISO property. These include:

a) ISO surface chemistry that includes OECD's surface chemistry (where appropriate), photocatalytic activity, octanol-water partition coefficient (where relevant), redox potential and radical formation potential.
b) ISO composition that includes OECD's crystalline phase and crystallite size
c) ISO surface area/mass specific and surface area/volume-specific surface area that includes OECD's specific area and porosity.

OECD has one unusual endpoint i.e. Representative TEM pictures. Unlike other endpoints listed, it is specific to an analytical technique. The use of TEM to acquire representative images can thus be placed under several ISO properties e.g. particle size/size distribution and shape. There are some endpoints specified by the OECD i.e. dustiness and pour density, which cannot be associated with any ISO properties. These properties (although not included in ISO) are still considered to be highly relevant, as it relates to the properties of airborne dust and thus to the workplace hazard and risk. Overall, improved synchronisation between OECD and ISO is still needed but one thing is clear: it is important for researchers to define the measurand when reporting.

4.0 Method development and validation

Validation is not trivial and it hard to tell when method development ends and when validation begins. It can be considered as an interactive process and for this purpose it will not be differentiated here. Only a simple skeleton of what the process can entail will be presented here and the reader should be referred to relevant documentations for further details [6,7,16,17].

Proposed Sequence of Steps for Validation of an Analytical Method:

a) Understand the stated objectives for carrying out the analysis or test.
b) Establish the analytical criteria, to ensure that the method developed will be fit for purpose. The analytical criteria will be dependent on the nanomaterial sample and the nano-specific application. Potentially this can include the need to: be specific, selective, accurate, repeatable/reproducible, robust/rugged (e.g. not sensitive to operator and day-to day variabilities), have fast analysis time, minimal training for operators, availability and accessibility of the method, etc.
c) Develop the method by choosing the best technique. Consider parameters such as: sample type (matrix) and size, data required (qualitative/quantitative), expected level of analytes, likely interferences etc. Always conduct literature research to see if a suitable method already exists and modify, if necessary.

d) Refine method to demonstrate fit for purpose for the analytical criteria established
e) Establish a steering committee to nominate management team, as part of a pre-validation study. This study should involve 3 established/competent laboratories with registered with a recognised validation authority (RVA) e.g. the European Centre for the Validation of Alternative Methods (ECVAM). Here, *Lab 1* is designated as the laboratory responsible for method development. *Labs 2 and 3* are needed to identify any necessary refinements of the amended protocol.
f) Pre-validation study. Phase 1: Protocol refinement, to be conducted between Lab 1 and protocol refinement lab (designated *Lab 2*).
g) Pre-validation study. Phase 2: Protocol transfer. *Lab 1 and 2* to collaborate with protocol transfer to *Lab 3*.
h) Pre-Validation. Phase 3: Protocol performance. This will comprise of conducting a blind study involving two or more laboratories, to include *Lab 2 and 3*.
i) Pre-validation. Phase 4: Subsequent action to be discussed. All parties involved in the study would discuss the outcome of the pre-validation study with RVA to establish subsequent actions e.g. commissioning a formal validation study or advising the need for further method development or recommending that no further work on the method be undertaken.
j) Validation. Carry out a formal validation trials with one or more other RVAs or other appropriate sponsors.

5.0 Protocol: Sampling

What is sampling?

According to ISO/IEC 10725 [18], sampling is defined as *a procedure whereby a part of a substance, material or product is taken to provide for testing or calibration a representative sample of the whole. Sampling may also be required by the appropriate specification for which the substance, material or product is to be tested*. In other words, the goal of sampling is to select and obtain a test portion of the material in some manner, such that the sub-sample is representative of the larger amount of material. Sampling is often a major source of error and if representative sample is not obtained, then the subsequent analysis will give a wrong figure[19].

Two types of sampling errors are possible:

a) Segregation errors
This occurs when particles are exposed to gravitational, rotational, vibratory or aeration operations (or other types of mechanical motion), usually resulting in fine particles migrating to the bottom and larger particles being concentrated at the top[20]. This type of error is dependent on the previous powder history and often occurs with free or easily

flowing powders having a significant range of particle size. Such errors can be minimised by suitable mixing and building up the sample from a large number of increments.

b) Statistical errors
This is type of error is caused by observing a sample instead of the whole population. Although this type of error cannot be prevented, it is likely that the size of statistical errors can generally be controlled by taking a large enough random sample from the population[20].

Sampling powders

Different methods are available for powder sampling with the main purpose to collect the gross sample and reduce its size for subsequent analysis[20,21,22]. Table 2 shows the different techniques (their advantages and disadvantages) and corresponding reproducibility i.e. the observed r.s.d. between the different sub-samples generated from the primary sample. This was work done by Allen and Khan[23], using sand-sand mixture (in particular a mixture of coarse sand (420 – 500 μm in size) and fine sand (120 – 250 μm in size), with volume fractions of 60% and 40 % respectively. The coarse and fine binary mixtures were prepared by sieving and blending), the different sampling methods were evaluated and compared. Results indicate that spinning riffler seems to be the most reliable method for sampling as it seems to show little operator bias[24]. Although recommended, the riffler is not suitable for every nanomaterial, as it is a pre-requisite that the powders must be free-flowing. If a spinning riffler is to be used, then it must be validated for different nanomaterial in accordance to appropriate ISO guidelines[25], as outlined below:

a) Before purchasing a riffler, ensure that the design is such that the hopper results in a constant/non-segregating and even mass flow of the sample material. No material is to be lost outside the sample holders (or retained between the sample holders) or trapped within the hopper, vibratory feeder, dividing ring or any part of the sampler.
b) Before using a spinning riffler, ensure that the sampler is clean.
c) During sampling, ensure that the material is carefully mixed before being placed in the feed hopper.
d) Actuate the vibratory feeder and leave to operate until the entire sample has been divided.
e) Sampling of new materials should be validated. The simplest validation is mass validation. First, measure the mass of the gross sample together with the masses of all the increments; repeat this three times. Then calculate the mean loss of the material. If the mean loss of material is larger than 1 %, then the riffler is not working properly and should be corrected before use.

Table 1: A Summary: Comparison of Powder Sample Reduction Techniques[21,23]

Sampling Device	Description of technology	Advantages	Disadvantages	r.s.d (%)
Scoop Sampler	This technique is the simplest for sample division and involves an operator, using a scoop, to extract laboratory samples from some portion of the bulk sample.	Reliable for sample that are homogeneous and exhibit poor flow characteristics	Not suitable for heterogeneous sample. All of the bulk material does not go through sampling process. Operator dependent; operator decides where to scoop and what quantity to extract. Sampling more likely to be atypical due to segregation of the material	5.14
Cone and Quartering	This technique involves placing the sample on a flat surface in the form of a conical heap. The heap is then spread out and fattened into a circular cake, which is then divided into approximately equal quarters. One pair of opposite quarters is removed, combined and formed into a new cone for the process to be repeated (with the other two quarters discarded). The process is repeated as many times as is necessary to obtain a sample of the required size.	Good for powders with poor flow characteristics (and minimal segregation)	Operator dependent; errors can occur due to differences in the manner the heap is formed and sub-divided	6.81
Table Sampler	This utilises tilted surface (in which there is a series of holes and splitting prisms) over which a powder sample is allowed to slide. The prism break the stream into fractions and some of the powder will fall through the holes (and then discarded). Ultimately, at the bottom of the plane, a decreased quantity of sample is collected.	Ability to separate large quantity of material	It is necessary that the incoming powder to be uniform and consistent. Hence, dependent on the initial feed being uniformly distributed and a complete mixing after each separation, a condition not general achieved.	2.09
Chute Riffler	This utilises chutes i.e. funnelling or channelling device, to divide the powder. Unlike the spinning riffler, the chute riffler has no moving parts.	Ability to reduce powder samples in half after one pass	The technique is subject to error and operator bias if segregation is allowed to occur in loading the bulk-sampling trough.	1.01
Spinning Riffler (also called rotary sample divider)	This utilises a series of smaller receivers (or collection tubes), mounted in such a way so as to collect a flowing powder stream over a very short time period. The powder flows from a "hopper" to a "vibratory chute" and then to a "receiver" that holds the containers, which are rotating in a circular motion at a constant speed. Spinning rifflers are available in different sizes, in which common commercial systems can provide samples ranging from about 0.5 g to 300 g[21].	Ability to do large quantity of powder efficiently.	Limited to free-flowing powder only	0.125

Protocol: Powder subsampling

a) Use clean instrumentation and containers
b) Do not overfill sample containers, preferably no more than three-quarters full
c) Sample when the material is best mixed e.g. in motion, if possible. The use of specially designed instruments like a rotary/spinning riffler is recommended. If using the scooping method, mix the sample beforehand and take several sub-samples across the bulk.
d) Spinning riffling is often suitable for the first sample reduction e.g. a step that divides kilograms to hundreds of grams. The next step is to subdivide in the order of a few grams (this is referred to as a "laboratory" size). Scoop sampling method should also be used when a riffler is not suitable e.g. sub-milligrams (or less) of samples of sample is required or when the researchers has no access to the spinning riffler.
e) If using a riffler, it is important to validate the riffler to show that this method is fit for purpose for the nanomaterial under investigation.
f) Store subsampled powder in properly sealed containers. Avoid extreme temperatures, pressures and light exposure. If possible, for powders store under inert gas, such as argon or nitrogen**.**

Protocol: Liquid subsampling

According to ISO14488[25], there are two possible options for subsampling suspensions i.e. using either a pipette or by multiple capillary tubes. The latter method involves flowing the sample through a set of capillary tubes (capillary tubes typically have a bore size of 0.7 mm) spaced symmetrically in a vessel; this method is only suitable if there is a requirement to sample multiple samples simultaneously.

Out of the two methods, the pipette method is simpler and is less prone to contamination. For this:

a) Ensure that the particles within the dispersion are sufficiently mixed.
b) During sampling, agitate the suspension and quickly withdraw aliquots containing the required test portion using a suitable pipette. Use appropriate volume and orifice diameter. It is recommended that the largest particle diameters do not exceed 40% of the pipette diameter.
c) Immediately, transfer the aliquot into a suitable pre-cleaned (glass) container. Use disposable pipettes to avoid contamination.

6.0 Nanomaterial Dispersion

Dispersion of powdered nanomaterial into a liquid suspension often involves three key steps [26]:

a) Wet the nanomaterial powder. The purpose of this step is to substitute solid-air interface with solid-liquid interface, such that the particles are

sufficiently “wetted”. The efficiency of wetting will depend on the comparative surface tension properties of the nanomaterial and the liquid media, as well as the viscosity of the resultant mix. ISO [27] recommends that the wetting step should be achieved by mixing the powder with several drops of liquid media, to form a thick paste.

b) De-agglomerate using a de-agglomeration tool. Sufficient shear energy is needed to break down (loosely bound) agglomerates of the powdered material.
c) Stabilise the dispersion. The goal here is to achieve and maintain stability. Stability is dominated by the choice of the liquid dispersant. Hence, the addition of surfactants for example can lead to marked changes in its interfacial properties and thus stability [27].
d) Characterise the dispersion quality using appropriate methods. This can be as simple following sedimentation kinetics (using turbidity meter). Other properties for measurement to include: particle size, zeta-potential, etc. Measurement of several properties and the use of different analytical techniques are advisable. During the process of developing method, always to refer to standard guidelines whenever possible e.g. dynamic light scattering according to ISO 22412:2008.

To date no clear guidelines are set by internationally recognised bodies as to how powdered nanomaterials should be dispersed but clearly the choice should be driven by the need to prepare high quality and reproducible dispersions. Due to the wide range of available dispersion systems it is imperative to have a set of guidelines (SOPs) that can be followed.

Table 3 shows a summary of commercially available de-agglomeration tools; the table provides a brief description of the background technology, the corresponding advantages/disadvantages and the relative amount of energy provided by the tool.

Table 2: Comparison of different de-agglomeration tools

De-agglomeration Tools	State of Nanomaterials	Principle of Operation	Advantages	Disadvantages	Shear Energy Provided/ References
Mills (to include ball, stirred media, centrifugal and jet mills)	Mainly suitable for dry/ wet powders	Involves ultrafine grinding process	Useful for large doing large batches	Slow/ inefficient Grinding motion can lead to significant breakdown of nanomaterial architecture Can be difficult to clean; contamination likely	Medium [28]
Stirring (magnetic or overhead stirring)	Nanomaterial in liquid media	The use of magnetic stir bar or an overhead-stirring paddle, having rotational speed that is sufficient to create a vortex. Overhead stirring has a much higher speed than the magnetic counterpart	Cheap/ affordable	Inefficient Rarely results in de-agglomeration and often-employed in order to improve homogeneity of dispersion.	Low. [29].
High speed Homogeniser	*Nanomaterial in liquid media*	The use of a rotor stator generator probe; the rotor acts as a centrifugal pump to re-circulate the liquid and suspends the solids through the generator, where it will subjected to shear, impact collision and cavitations	Suitable for large liquid sample up to 2500 ml	Never tested for nanomaterial dispersion	N/A

High Pressure Homogeniser	*Nanomaterial in liquid media*	Shear and cavitations provided via increase in the velocity of pressurised liquid streams in micro-channels	Highly efficient	Nanomaterial architecture can be altered; increase of temperature in the dispersion likely. Expensive	High [29]
Ultrasound Sonicating Bath	Nanomaterial in liquid media	The use of ultrasound waves and cavitations (i.e. the formation, growth and implosion of bubbles in liquid) activity in a bath	Cheap/ Affordable	Bath format less effective (less shear) compared to probe format. Can alter nanomaterial architecture Increase in temperature likely if dispersion is sonicated for long period. Highly variable performance at lower end of the market	Medium [29].
Ultrasound probe sonication or ultrasonic disruptor	Nanomaterial in liquid media	Similar to ultrasonic bath but aims to deliver more energy density in smaller volume in comparison to the corresponding bath format	Highly efficient	Probe tip disintegration can contaminate samples. Can alter nanomaterial architecture; temperature increase (even for a few minutes) in dispersion highly likely. Highly variable performance at lower end of the market.	High [29]

Certainly, the choice of the de-agglomeration tool will undoubtedly affect the properties final dispersion. Figure 1 shows the DLS particle size distribution (by intensity) of CeO_2 (50 mg/L) in DI water, when dispersed using an ultrasonic probe, with an exposure time of 1 minute. Results show a particle size distribution between 68 - 615 nm in size. The plot also shows the effect of altering the dispersion protocol step, when either an overhead stirrer or homogeniser was employed instead of ultrasonic probe. Results indicate a much broader particle size distribution, with particle sizes as big as 1 µm; the much bigger size particles found in the dispersions using these tools can only be explained by the much lower shear energy provided (to result in insufficient de-agglomeration/de-aggregation) if compared to the ultrasonic probe. The DLS instrument here was used to characterise the quality of the dispersions made by using different de-agglomerating tools. Although, the use of sonication methods such as ultrasonic probe is efficient in breaking up agglomerates, these methods can also be disruptive, sometimes resulting in undesired physical changes e.g cutting long nanotubes to shorter tubes. Hence, the sonication procedure should be optimised and any potential contamination resulting from the sonication step identified e.g. deterioration (through time) of the ultrasonic probe tip.

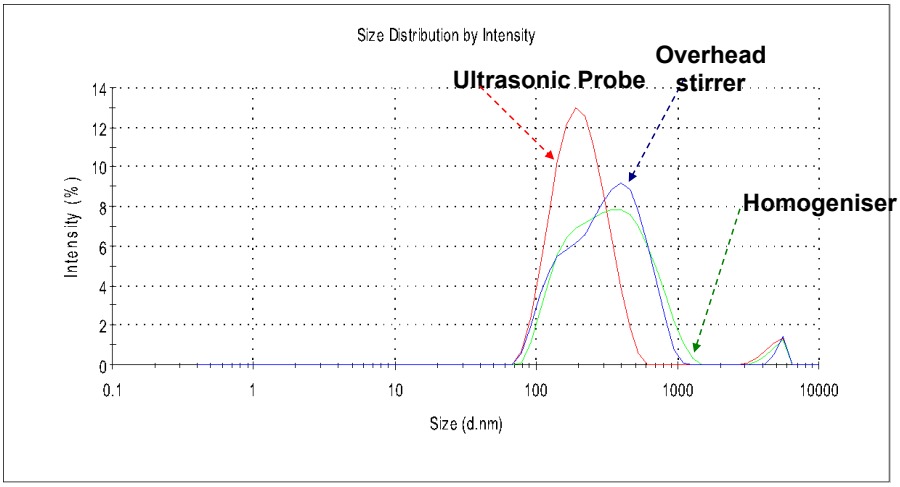

Figure 1: DLS Particle size distribution of CeO_2 dispersed in DI water using different de-agglomeration tools, using a dispersion protocol developed for PROSPEcT i.e. UK's contribution to the global OECD testing of engineered nanomaterials.

Efforts to harmonise the dispersion protocol have taken place in F7 MARINA WP 3 Task 3.5 and WP 4 Task 4.2 but this task is not trivial, as there are numerous factors that can affect variability of dispersion quality. A systematic study has been conducted in WP 3 Task 3.5, to understand the degree of variability that can arise from variations within one protocol, investigating various effects e.g. age of dispersion, sonication exposure time, sonication when in the presence/absence of an ice bath, powdered material subsampling, particle concentration and whether a pre-wetting step is included in the protocol. The results have been written up and currently in press[30]. Overall, the study indicated that the largest source of variability is associated with changes in particle concentration, followed by effects of sonication and subsampling. The findings suggest that for the purpose of data comparability, dispersion protocols must be reported in great detail. For data comparability a detailed dispersion protocol is needed; a video recording either to replace or support additional written instructions is advisable.

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