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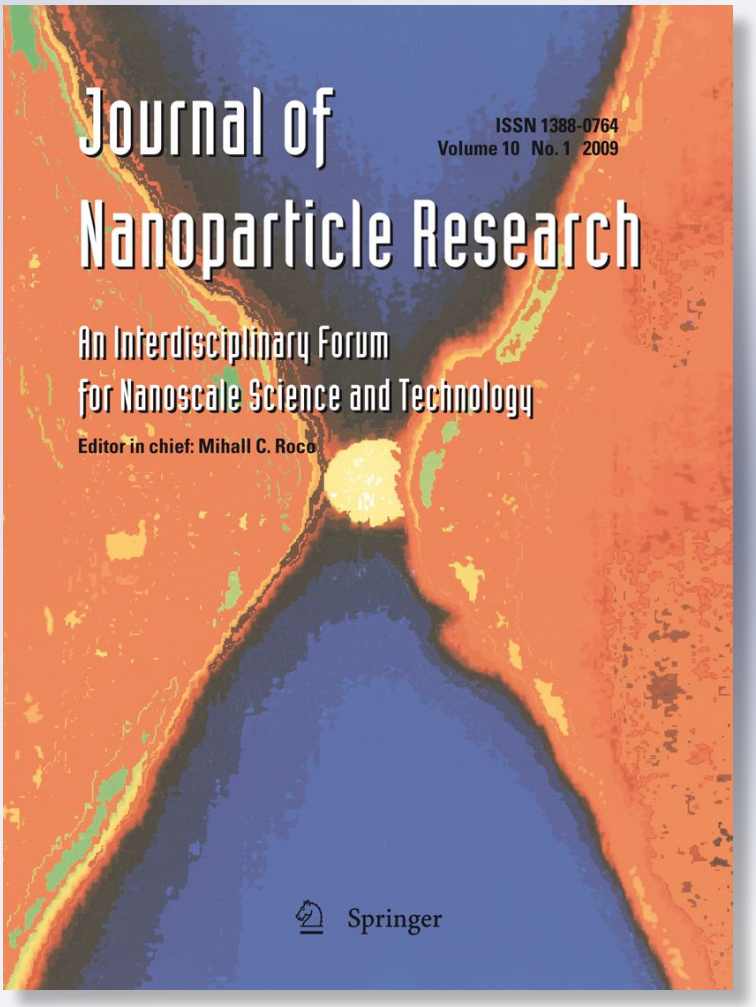


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PERSPECTIVES

# Interlaboratory comparison of size and surface charge measurements on nanoparticles prior to biological impact assessment

**G. Roebben · S. Ramirez-Garcia · V. A. Hackley · M. Roesslein · F. Klaessig · V. Kestens · I. Lynch · C. M. Garner · A. Rawle · A. Elder · V. L. Colvin · W. Kreyling · H. F. Krug · Z. A. Lewicka · S. McNeil · A. Nel · A. Patri · P. Wick · M. Wiesner · T. Xia · G. Oberdörster · K. A. Dawson**

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**Abstract** The International Alliance for NanoEHS Harmonization (IANH) organises interlaboratory comparisons of methods used to study the potential biological impacts of nanomaterials. The aim of IANH is to identify and reduce or remove sources of variability and irreproducibility in existing protocols. Here, we present results of the first IANH round robin studies into methods to assess the size and surface charge of suspended nanoparticles. The test materials used (suspensions of gold, silica, polystyrene, and ceria nanoparticles, with [primary] particles sizes between 10 nm and 80 nm) were first analysed in repeatability conditions to assess the possible contribution of between-sample heterogeneity to the between-laboratory variability. Reproducibility of the selected methods was investigated in an interlaboratory comparison

G. Roebben (✉) · V. Kestens
Institute for Reference Materials and Measurements, Joint Research Centre of the European Commission, Retieseweg 111, B-2440 Geel, Belgium
e-mail: gert.roebben@ec.europa.eu

S. Ramirez-Garcia · I. Lynch · K. A. Dawson
Centre for BioNano Interactions, University College Dublin, Belfield, Dublin 4, Ireland

V. A. Hackley
Material Measurement Laboratory, National Institute of Standards & Technology, 100 Bureau Drive, Stop 8520, Gaithersburg, MD 20899-8520, USA

M. Roesslein · H. F. Krug · P. Wick
EMPA, Lerchenfeldstrasse 5, 9014 Street, Gallen, Switzerland

F. Klaessig
Pennsylvania Bio Nano Systems LLC, 3805 Old Easton Road, Doylestown, PA 18902, USA

C. M. Garner
Garner Nanotechnology Solutions, Pleasanton, CA 94566, USA

A. Rawle
Malvern Instruments Inc, 117 Flanders Road, Westborough, MA 01581-1042, USA

A. Elder · G. Oberdörster
Department of Environmental Medicine, University of Rochester, 575 Elmwood Ave., MRB Annex, 3-11001, Rochester, NY 14642, USA

V. L. Colvin · Z. A. Lewicka
Department of Chemistry, Rice University, MS-60, 6100 Main Street, Houston, TX 77005, USA

W. Kreyling
Helmholtz Zentrum Muenchen, Institute of Lung Biology and Disease, Ingolstaedter Landstr. 1, 85764 Neuherberg/ Munich, Germany

S. McNeil · A. Patri
Nanotechnology Characterization Laboratory, Advanced Technology Program, SAIC-Frederick, Inc, 1050 Boyles St., Bldg 469, Frederick, MD 21702, USA

between ten different laboratories in the USA and Europe. Robust statistical analysis was used to evaluate within- and between-laboratory variability. It is shown that, if detailed shipping, measurement, and reporting protocols are followed, measurement of the hydrodynamic particle diameter of nanoparticles in predispersed monomodal suspensions using the dynamic light scattering method is reproducible. On the other hand, measurements of more polydisperse suspensions of nanoparticle aggregates or agglomerates were not reproducible between laboratories. Ultrasonication, which is commonly used to prepare dispersions before cell exposures, was observed to further increase variability. The variability of the zeta potential values, which were also measured, indicates the need to define better surface charge test protocols and to identify sources of variability.

**Keywords** Nanoparticle · Particle surface charge · Interlaboratory comparison · Reproducibility · Polydispersity · Toxicology · Health and safety implications

## Introduction

### The International Alliance for NanoEHS Harmonization

Nanoparticles are objects with all external dimensions smaller than 100 nm (ISO 2009). Reports of nanoparticles crossing cellular and blood–brain barriers have raised concerns regarding potentially adverse effects (Oberdörster et al. 2007). Although nanotechnology is not only about using nanoparticles, uncertainties about the biological impacts of nanoparticles may well undermine the public perception of the safe and responsible development of nanotechnology in its entirety. By organizing interlaboratory comparisons, the International Alliance for NanoEHS Harmonization (IANH) is seeking to improve the biological test methods assessing whether nanoparticle hazards are genuine, whether they are chemical in nature, and whether they arise from scalable (for example, specific surface area) or from non-scalable nanoscale properties (properties that change suddenly at a certain threshold particle size).

The literature reports on ecological and human health effects of nanoparticles are often based on local laboratory protocols. Even if these have been carefully assessed for their within-laboratory repeatability, also over time, this does not necessarily guarantee consistency with testing by other laboratories. It is therefore desirable to understand the inherent uncertainty of the existing nanoparticle literature as well as to establish a basis for enhanced protocols. Burdening investigators with rigorous preparatory physicochemical testing only to find that the variability of the subsequent biological test is unaffected would be as counterproductive as not knowing the variability of either. The purpose of the IANH test program is therefore to improve measurement protocols through an iterative and collaborative process, to identify potential sources of variability, and to ultimately validate these protocols through interlaboratory comparisons conducted on an international scale.

### General methodology of the IANH interlaboratory comparisons

The IANH uses interlaboratory comparisons (ILCs) to identify and remove sources of irreproducibility in existing protocols. Each of the laboratories participating in the ILCs has published data using protocols that have been accepted in reputable peer reviewed journals. In the first set of ILCs, of which results are reported in this article, one objective was to determine variability in their reported results primarily in terms of organizing the effort, evaluating available reference materials and taking sensible precautions to use a single sample source.

Ideally, ILCs require test materials with the characteristics of a reference material (RM): The targeted properties should be homogeneous (between the samples sent to ILC participants) and stable (between the moment of preparation and the moment of testing, including transport periods) (ISO 2008a). Using homogeneous and stable materials effectively reduces the variability in test results, making it easier to

A. Nel · T. Xia
Division of Nano Medicine, Department of Medicine at UCLA, 52-175 CHS, 10833 LeConte, Los Angeles, CA 90095-1736, USA

M. Wiesner
Department of Civil and Environmental Engineering, Duke University, Box 90287 Hudson Hall, Durham, NC 27708-0287, USA

interpret measurement results and identify the sources of the observed variability (between laboratories, methods, types of material, etc.). However, worldwide only a few nanoparticle RMs are currently available, and the status of these RMs is limited to the properties for which their homogeneity and stability were assessed, which in most cases is limited to their mean particle size (Linsinger et al. 2010). The IANH approach is to prepare and provide a common set of well-characterized test materials for the ILCs and to use existing RMs for laboratory qualification purposes.

Where possible, method reproducibility is analysed using the ISO 5725-2 approach, which is based on arithmetic mean values and standard deviations (ISO 1994). Where the distribution of results from different laboratories indicates the presence of statistical outliers, and where the elimination of these outliers is not possible based on sound scientific-technical arguments, IANH uses robust statistics as described in ISO 5725-5 (ISO 1998). Robust statistics is based on median values, instead of arithmetic mean values, and on median absolute deviation (MAD) values, instead of standard deviations. (MAD is the median of all absolute values of the deviations between the individual measurement results and the median of all individual measurement results.) Median and median absolute deviation values are less sensitive to the presence of one or a few outlier results. The mean and median values and the corresponding standard deviation and MAD values were calculated for results obtained within laboratories ($MAD_r$) and between laboratories ($MAD_L$). Unless otherwise stated, approximate 95% confidence intervals were used as indications of dispersion throughout.

### IANH ILCs on particle size (distribution) and particle surface charge

To study the reproducibility of biological effects of substances, the chemical composition of the substances introduced to test systems must be the same. For the case of nanoparticulate matter, attention is also needed for the particle size distribution and particle surface characteristics, because they can influence biological effects (Rivera Gil et al. 2010), and because these properties are more likely to be altered during sample preparation compared with the particle's chemical composition. Therefore, participants in the IANH biological impact assessment ILCs must be able to characterize the nanoparticle dispersions in terms of size distribution and surface characteristics. To validate these characterization capabilities, the IANH distributed nanoparticle RMs as well as centrally prepared suspensions of commercial nanoparticles and nanoparticle aggregates. To determine whether additional variability is introduced by the common practice of dispersion sonication in preparation of samples for exposure to cells, the tested nanoparticle agglomerate/aggregate was characterized before and after sonication.

## Materials and methods

### Materials

#### *Reference materials*

The US National Institute of Standards and Technology (NIST) and the Institute for Reference Materials and Measurements of the European Commission's Joint Research Centre (JRC-IRMM) provided samples of their respective RMs for use in the first IANH ILCs.

NIST RM 8012 consists of nominally 30 nm citrate-stabilized gold nanoparticles in an aqueous suspension (NIST 2008). The Au mass fraction is nominally 0.005%. The particle size and size distribution was determined using six independent techniques encompassing dry-deposited, aerosol, and liquid-borne forms of the material. This RM is intended primarily to evaluate and qualify methodology and/or instrument performance related to the physical/dimensional characterization of nanoscale particles used in preclinical biomedical research. The material may also be useful as a test material in the development and evaluation of in vitro assays designed to assess the biological response (e.g., cytotoxity, hemolysis) of nanomaterials and for use in ILCs.

IRMM-304 is a quality control material (i.e., a RM with proven homogeneity and stability, with a non-certified, but indicative property value). IRMM-304 consists of silica nanoparticles (nominal particle diameter 40 nm) suspended in an aqueous solution (IRMM 2009). The suspending medium contains a small amount of NaOH as a stabilizing agent (pH = 9). The nominal particle mass fraction in the suspension is 0.25%. The intended use is to check the

performance of instruments and/or methods that characterize the size distribution of nanoparticles suspended in a liquid medium.

*Test materials*

Polystyrene (PS) and $CeO_2$ test materials were selected for their positive control potential (Xia et al. 2008) and market relevance, respectively. Bangs Laboratories PA02 N-8626 (coded here as BL-PS),[1] is a commercially available aqueous suspension of nominally 50 nm PS nanoparticles with amine functionalization of the surface. BL-PS was included as a potential positive control for nanoparticle-induced cytotoxicity (due to its positive surface charge it is expected to exhibit biological impacts (Xia et al. 2008)). The suspending medium contains a small amount of residual surfactant from the synthesis, which acts as a stabilizing agent. The nominal particle concentration in the suspension is 0.05 g/L.

Umicore D246[2] is a material consisting of aggregated ceria nanoparticles suspended in an aqueous solution at pH 4 (coded here as UMI-ceria). Figure 1 shows a complex aggregated and/or agglomerated structure of the material's primary $CeO_2$ particles. The suspending medium contains $10^{-4}$ mol/L nitric acid ($HNO_3$) as a stabilising agent. The nominal particle mass fraction in the suspension is 0.05 g/L. Strictly speaking UMI-ceria does not consist of nanoparticles, because the average aggregate size is between 100 nm and 200 nm (ISO 2009). It was argued however, that the material did have relevance for the IANH studies as the primary particle size (the size of the crystals that 'stick' together to form aggregates) is in the nanoscale (equivalent sphere diameter, calculated from the specific surface area measured using the BET method, is 30 nm; see also Figure 1).

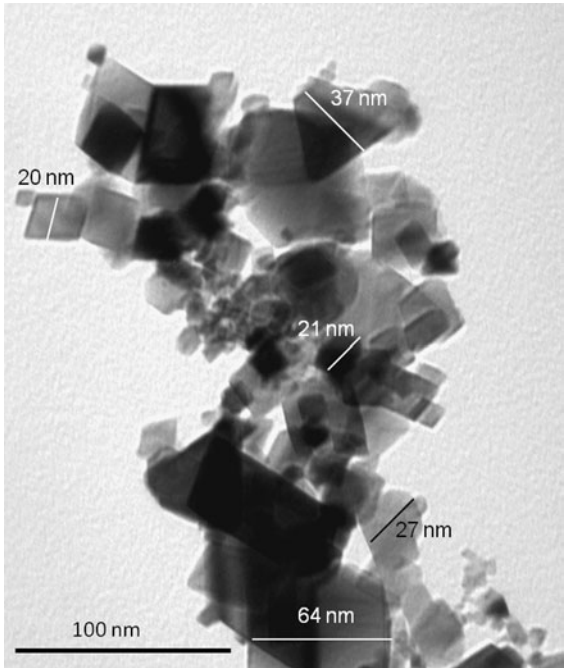


**Fig. 1** TEM image showing the aggregates and/or agglomerates of UMI-ceria primary particles

Homogeneity tests (conducted on a larger series of samples under repeatability conditions) were performed on the BL-PS and UMI-ceria materials, establishing the between-unit homogeneity, which is a key characteristic for materials used in an ILC. The results indicate a satisfactory relative standard deviation for the mean particle size as measured with the dynamic light scattering (DLS) method ($\bar{x}_{DLS}$, see next section) of about 5% for the BL-PS and about 6% for the UMI-ceria samples.

## Methods

*Particle size measurements*

A rapid and widely available technique for assessing nanoparticle size is dynamic light scattering (DLS), also referred to as photon correlation spectroscopy (PCS), or quasi-elastic light scattering (QELS). In its basic form, the method provides an estimate of the average hydrodynamic equivalent diameter (symbol $\bar{x}_{DLS}$) and of the width of the size distribution of the suspended particles undergoing Brownian motion (Cummins and Pike 1974). DLS is one of the few sizing techniques applicable to the nanoscale range

[1] Certain trade names and company products are mentioned in the text or identified in illustrations in order to specify adequately the experimental procedure and equipment used. In no case does such identification imply recommendation or endorsement by IANH or its member organizations, nor does it imply that the products are necessarily the best available for the purpose.

[2] Umicore produced the batch of particles used here specifically for EU FP6 project NanoInteract, and authorised its use in the IANH ILC.

for which international documentary standards exist (ISO 1996, 2008b; ASTM 2009). On the other hand, DLS is generally considered a "low resolution" method, and is subject to a strong dependence of scattering intensity on particle size (at laser wavelengths sufficiently larger than the particle size, the scattered light intensity $\sim$ (particle size)$^6$).

The IANH DLS test protocol was based principally on a NIST-NCL protocol (Hackley and Clogston 2007) that had been previously adapted for use in ASTM ILS-166, an ILC designed to produce a precision and bias statement for the ASTM E2490-09 standard DLS practice guide (ASTM 2009). The IANH protocol defined the sequence of size, pH and zeta potential measurements, and the precautions to be taken when preparing dilutions of the as-received test samples. It also incorporated specific instructions for the handling and use of NIST RM 8012-gold and IRMM 304-silica, as stated on the respective report of investigation (NIST 2008) or material information sheet (IRMM 2009).

A spreadsheet file was distributed electronically to participating laboratories to serve as a common data entry form. The details of the DLS instrumentation and data analysis approaches used are summarised in Table 1; details regarding the ultrapure water used in the procedures are reported in Table 2. Laboratories were asked to report the particle size values as intensity-weighted harmonic mean particle diameters, which is the most elementary of $\bar{x}_{DLS}$values. Several algorithms can be used to deduce $\bar{x}_{DLS}$ from a measured autocorrelation function (ACF). The most basic approach is the cumulants method. This method, described in ISO 13321 (ISO 1996) and ISO 22412 (ISO 2008b), assumes that the measured particle size distribution is monomodal, as it fits a single exponentially decaying function (corresponding with one particle size) to the measured autocorrelation function. Thus, the cumulants method produces, based on the translational diffusion coefficient information, a mean diameter ($\bar{x}_{DLS}$) and an estimate of the width of the distribution (PI, or polydispersity index), a parameter that is only meaningful where the sample's size distribution is unimodal. Other, more sophisticated approaches exist. However, the resulting diameter values are strictly defined by the instrument software method and not by an international documentary standard. The methods used by different instrument makers to solve the ill-defined mathematical problem of transforming the measured ACF to a particle size distribution, are most often proprietary methods. Therefore, to maximise between-laboratory comparability, the participating laboratories were requested to report results from the cumulants method. All labs used an instrument from the same manufacturer, except lab 10, which used an additional, second instrument, from a different supplier (results reported as 10b). Wider instrument variability

**Table 1** Details of the DLS instruments used by the laboratories participating in the stage 2 ILC

| Lab code | Detector angle (°) | Laser wavelength (nm) | DLS data analysis method | Cuvette material | Volume of sample in cuvette during measurement (mL) |
|---|---|---|---|---|---|
| 1 | 173 | 633 | Cumulants | Polystyrene | 2 |
| 2 | 90 | 633 | Cumulants | Quartz | 0.05 |
| 3 | 173 | 633 | Cumulants | Polycarbonate | >0.75 |
| 4 | 173 | 633 | Cumulants | Quartz | 0.045 |
| 5 | 173 | 633 | Cumulants | Plastic | 0.8 |
| 6 | 173 | 633 | Cumulants | Plastic | 0.5 |
| 7 | 173 | 633 | Cumulants | Plastic | 0.04 |
| 8 | 173 | 633 | Cumulants | Plastic | 2 |
| 9 | 173 | 633 | Cumulants | Plastic | 0.7 |
| 10a | 173 | 633 | Cumulants | Quartz | 1.5 |
| 10b | 177 | 650 | Cumulants | Quartz | 3 |
| 10c | 177 | 650 | Frequency analysis | Quartz | 3 |

**Table 2** Experimental details relevant to sample preparation and sonication

| Lab # | Water purification system[a] | Ultrafilter (pore size) | UV lamp (Y/N) | US dispersion system 1 | Frequency (kHz) | Power (W) | Probe | US dispersion system 2 | Frequency (kHz) | Power (W) | Probe |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Barnstead D50281 | Y | Y | Cole-Parmer 8890 Bath Sonicator | 47 | 80 | | Misonix S-4000 | 20 | Used: 100 (max = 600) | Misonix S1506 |
| 2 | Barnstead D50282 | Y | Y | Sonics Vibra Cell | 20 ± 50 | 20 | 3 mm Stepped microprobe Ti6Al4V | NEY Ultrasonik Cleaner 300 | | (1/2 Max) | |
| 3 | Milli-Q UV Plus | N | N | Fisher Scientific FS6 | 50/60 | 40 | | | | | |
| 4 | Aqua Solutions, Aqua-1 Type I Biological Grade Low TOC DI | Y | Y | Branson 1510 Bath Sonicator | 42 ± 6% | 70 | | | | | |
| 5 | Millipore Milli-Qsystem | N | N | Bandelin Sonorex T52 Bath | 35 | 60 | | Hielscher Ultrasound Technology UP50H | 20 | 50 | Sonotrode MS1 or MS7 |
| 6 | Millipore Milli-Qsystem | N | N | Branson 1510 Bath Sonicator | 42 ± 6% | 70 | | | | | |
| 7 | Aqua Solutions | Y | N | Branson 2510 Bath Sonicator | 42 ± 6% | 100 | | Sonics Vibracell VCX 130 | 20 | Used: 32.5 (max = 130) | |
| 8 | Millipore Milli-Q Academic, with syringe filter | Y (0.02 μm) | N | Branson 2510R-DTH Bath Sonicator | 42 ± 6% | 100 | | | | | |
| 9 | Millipore Milli-Q system | Y (Whatman Anotop 0.2 μm).[b] | Y | Branson 1510R-MTH Bath Sonicator | 42 ± 6% | 70 | | | | | |
| 10 | Millipore Milli-Q plus 185 | 0.2 μm | Y | 3510E-MTH Bransonic® Bath Sonicator | 42 | 100 | | | | | |
| 11 | Millipore Milli-Q Academic, with syringe filter | Y (0.02 μm) | Y | Bandelin SonorexSuperRK 156 BH | 35 | 150 | – | – | – | – | – |

[a] All laboratories used ultrapure water with a resistivity of 18 MΩ.cm or more

[b] Sample was measured before and after filtration

could have resulted in wider differences in reported results.

*Sonication*

The proposed sonication procedure was 30 min at 80 W using a bath sonicator at room temperature. The participating laboratories were explicitly asked not to use an ice bath to control temperature rising during sonication. Details of sonication devices used and reported by the laboratories are summarised in Table 2.

*Zeta potential and pH measurements*

The zeta potential and pH measurements were conducted in a more exploratory manner. The test protocol clearly requested to perform the tests on the as-received material (not sonicated, except for the $CeO_2$ dispersion, also to be measured after sonication). The zeta potential value to be reported had to be calculated using the Smoluchowski formula for thin double layers (which is the default setting for most commercial instruments). Laboratories were requested to also report relevant test parameters, such as details of the water purification system (Table 2). The water quality used for dilutions is indeed known to influence the characteristics of nanoparticle suspensions, especially zeta potential (Hackley et al. 1995). For the pH measurement, the pH-meter and probe models and the container type were reported (Table 3); for the zeta potential measurements, the sample volume, test temperature and the cell and electrode materials were reported (Table 4).

**Table 4** Details of the zeta potential instruments used by the laboratories participating in the Stage 2 ILC

| Lab code[a] | Volume of sample in cell during measurement (mL) | T (°C) | Cell material |
|---|---|---|---|
| 1 | 1 | 25 | Plastic |
| 2 | 1 | 25 | Polycarbonate |
| 3 | 0.75 | 25 | Polycarbonate |
| 4 | 0.8 | 20 | Plastic |
| 6 | 1 | 25 | Polycarbonate |
| 7 | 1 | 25 | Polycarbonate |
| 8 | 1 | 25 | Plastic |
| 10 | 0.75 | 25 | Plastic folded capillary cell (polycarbonate) |

[a] All laboratories used an instrument with a Au-plated Cu electrode

## Results and discussion

ILC stage 1

A preliminary ILC (Stage 1) was performed on the two selected nanoparticle suspension RMs (NIST RM8012-gold and IRMM-304-silica) to validate a DLS test protocol, and to serve as a test case for the operational procedures to support the more complex and extensive ILCs to follow. Resolving the few inconsistencies confirmed the need to have multiple measurements from each of the ILC participants and to clarify terms used in the protocol such as "replicate". Also, the need for temperature-sensitive indicators in the sample shipment packages became apparent. The results confirmed that NIST RM8012-gold and IRMM-304-silica can be used

**Table 3** Details of the pH meters used by the laboratories participating in the stage 2 ILC

| Lab code | Meter make/model | Probe make/model | Container type |
|---|---|---|---|
| 1 | Accumet XL-20 | Accumet Combi pH/ATC Polypropylene | Glass |
| 2 | Corning 140 | Orion911600 SemiPro | Polystyrene (15 mL) |
| 3 | Accumet | Accumet Combi pH/ATC Polypropylene | Glass |
| 4 | Thermo Orion | MV114-SC Malvern Comb Glass Electrode | Plastic |
| 5 | Sartorius PB11 | Sartorius PY-P11 | Plastic (Falcon) |
| 6 | Symphony | Glass | Plastic (Falcon) |
| 7 | Mettler Toledo Seveneasy | Inlab Versatile | Plastic (Falcon) |
| 9 | Thermo Orion | Orion pH Micro-Combination Electrode | Plastic |
| 10 | Metrohm | Metrohm 744, Universal Electrode (Solitrode with Pt1000) | 10 mL glass beaker |
| 11 | Metrohm | Metrohm AG Glass Electrode | Glass |

effectively as a quality check for laboratories using DLS to size suspended nanoparticles.

## ILC stage 2

### *Study design*

The Stage 2 ILC included additional laboratories expected to participate in subsequent in vitro and in vivo studies. DLS and zeta potential measurements were performed on BL-PS, on UMI-ceria, and also on IRMM-304-silica chosen as a quality control material. Based on the findings of the Stage 1 ILC, a number of logistic issues were modified. All samples were sent in insulated boxes with two temperature sensors: one indicating whether the contents were exposed to temperatures below 4 °C and another indicating if the contents were exposed to temperatures above 30 °C. Also, an even more detailed DLS test protocol was provided. The laboratories were asked to report values for the intensity-weighted harmonic mean particle diameter ($\bar{x}_{DLS}$) obtained via correlation function analysis (cumulants method). The 10 participating laboratories received one sample per test material and were asked to perform two independent, replicate measurements for each test material, i.e., to report results obtained on two separate aliquots taken from the distributed ampoules or vials; each aliquot was measured three consecutive times (3 repeats per replicate). Analysis of the incoming results showed that for the vast majority of laboratories, the variation between the repeat measurements on the same aliquot was larger than the variation between the two replicate results. This indicates satisfactory within-bottle homogeneity of the test materials, and it allowed treating the 6 results reported by each laboratory for each material, as independent values in the statistical analysis of the results.

Zeta potential measurements were also included in the test protocol for use in the laboratories where the necessary equipment was available. A specific test protocol was not imposed, but participants were asked to report on their equipment and methodology for the purpose of developing a working protocol.

### *Particle size results for monodisperse materials*

The $\bar{x}_{DLS}$ results obtained for IRMM-304-silica and BL-PS were analysed with both classical (average and standard deviation based) and robust (median and median absolute deviation based) statistics. When calculating the mean or median values of the laboratory means, results of all laboratories were used, including outlier values for which no technical reason for elimination could be identified. Figure 2 shows the DLS results obtained on the BL-PS test material. The results of each laboratory match with the mean and median values for both IRMM-304-silica and BL-PS, within the respective laboratory dispersion. For the IRMM-304-silica, the mean and median values also match the assigned value of the IRMM-304-silica, within its uncertainty (IRMM 2009). This demonstrates the satisfactory reproducibility of the $\bar{x}_{DLS}$ data, the suitability of the established test protocol, and the proficiency of the participating laboratories with the DLS method.

### *Particle size results for more polydisperse material*

The limited reproducibility of the $\bar{x}_{DLS}$ results for UMI-ceria, prior to sonication, is notable (Figure 3 a): 2 out of 10 laboratories report results that clearly do not match with the data of the other 8 laboratories. There are several factors to consider. Firstly, there is a difference between the rather monodisperse test materials (i.e., IRMM-304-silica, NIST RM8012-gold and BL-PS) and the UMI-ceria, which consists of nanoparticle aggregates that are larger than the primary particles seen in Fig. 1. Polydispersity measurements confirm that the UMI-ceria is significantly more polydisperse (average reported polydispersity index PI = 0.24) than the other tested materials (BL-PS: average reported PI = 0.07; IRMM-304-silica: average reported PI = 0.15; NIST RM 8012-gold: average reported polydispersity index PI = 0.17). Polydisperse samples are not well tolerated by the cumulants method for DLS data analysis, contributing to variability (Hassan and Kulshreshtha 2006). Secondly, $CeO_2$ has a much higher density than either $SiO_2$ or PS, and the nanoparticle aggregates are significantly larger than the particles in the other test materials. As a result, sedimentation was observed in the undiluted stock suspension, as well as in some of the diluted samples distributed to the participating laboratories. While these samples therefore had to be gently inverted to ensure sample homogeneity before DLS tests, sedimentation during the measurements may well have occurred. On the other hand, DLS

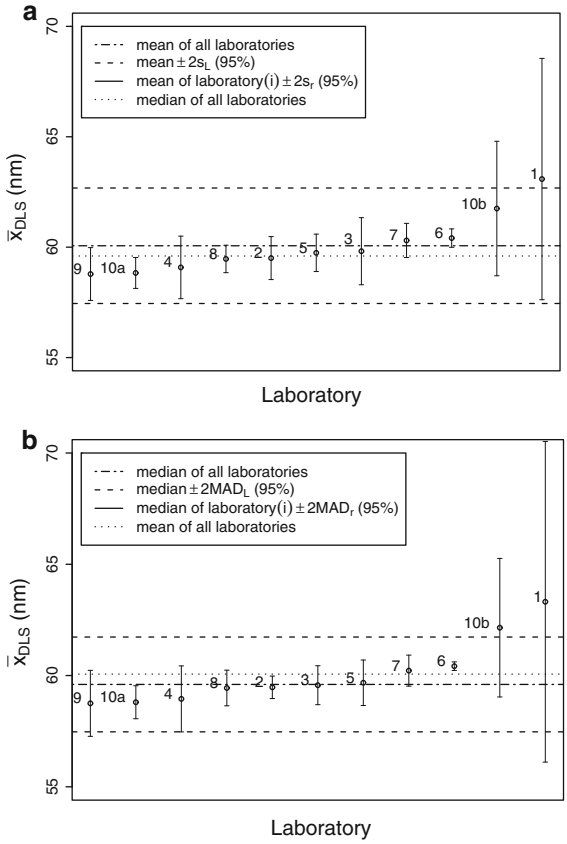


**Fig. 2** $\bar{x}_{DLS}$ results obtained on BL-PS; analysis based on classical (**a**) and robust statistical analysis (**b**)

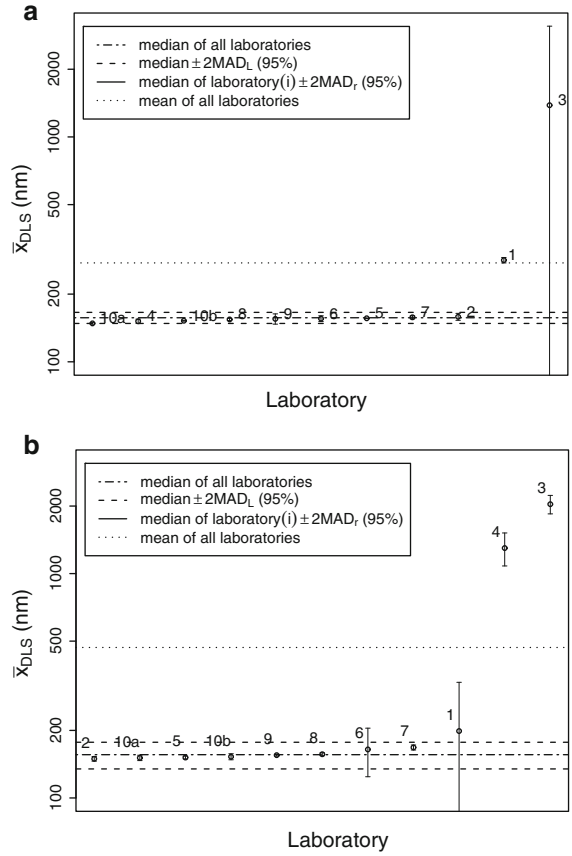


**Fig. 3** Robust statistics analysis of the $\bar{x}_{DLS}$ data obtained on UMI-ceria (**a**) prior to and (**b**) after sonication

results giving values on the order of micrometres suggest flocculation, and this would certainly degrade reproducibility.

Our results confirm that polydispersity and aggregation contribute to variability in particle size measurement results. In such case, it is considered good practice to investigate the size of the particle (aggregates) with multiple methods, based on different physical properties (sedimentation, Brownian motion, microscopy, …). While this will be subject of further IANH investigations, it must be recognised that, currently, the DLS method is the only widespread method for routine nanoparticle size analysis in clinical and toxicology laboratories. It would therefore be very useful to improve the comparability and reliability of the results of more sophisticated DLS analysis algorithms, which provide particle size distributions rather a single average value for an assumed monodisperse particle population.

When measurements were performed after sonication, reproducibility degraded further (Figure 3 b). While the median $\bar{x}_{DLS}$ value for UMI-ceria does not change significantly upon sonication (median $\bar{x}_{DLS}$ = 155 nm), the $MAD_L$ value more than doubled, from 6 % before sonication to 13 % after sonication. This observation suggests that sonication, routinely included in particle dispersion protocols, may be a significant source of variability. Several possible reasons have been proposed, one being the differences in the available sonication equipment (see Table 2). Even among the laboratories that used the suggested device (a bath sonicator at approximately 80 W), the sample containers and test volumes varied, as well as the energy distribution within the containers. It can be concluded that specifying the optimum sonication conditions is both critical and difficult to accomplish. If sonication is to be incorporated into protocols for biological testing, then further study must be made of

the possible effects and the validation of best practices (Bihari et al. 2008). Subsequent to the studies described here, more comprehensive recommendations on this issue, including a device-independent calibration procedure, have been reported (Taurozzi et al. 2010).

*Zeta potential*

Zeta potential measurements were performed by the majority of the 10 laboratories. A large between-laboratory variation is evident in Fig. 4, for the discrete nanoparticle test materials (IRMM-304-silica and BL-PS) and even more for UMI-ceria, the nanoparticle aggregate. The reliability of the median zeta potential values for IRMM-304-silica and, to a lesser extent, for BL-PS and non-sonicated UMI-ceria, is confirmed by the distribution of data points around the respective median values. This observation does not extend to the results obtained on sonicated UMI-ceria (Fig. 4 d). At the pH conditions encountered in this study, all $CeO_2$ particles should be positively charged (as the pH of the sample is below the isoelectric point, reported in the literature to range from 6.8 to 7.9) (Hsu and Nacu 2004). Yet, some labs report slightly negative zeta potential values and one lab reports a change from positive to negative upon sonication. It is suspected that the values greater than 30 mV are correct for the simple reason that the UMI-ceria material is deliberately manufactured to be a stable dispersion. These observations indicate that the routine measurement of zeta potential is not yet straightforward. Therefore results reported in the literature should be viewed with some reservation, and future efforts should focus on identification of possible laboratory artefacts requiring attention and development of a robust measurement protocol. This should, for example, include a mandatory pH measurement with a calibrated pH meter.

*Effects of sonication*

Both the particle size and the zeta potential of the cerium oxide material are affected by sonication. Sonication leads to localized heating of the suspension and might well result in the release of surface species (e.g., $NO_x$ groups) or the acceleration of an aging process leading to the final equilibrium surface composition as proposed by Vincent et al. (2010). Although precautions were taken to work concurrently with a single common sample, the variability of particle size and zeta potential results upon sonication point to a complex surface chemistry for a material like ceria that may assume multiple oxidation states. Sonication may well affect the Helmholtz layer and the zeta potential. Moreover, if the sample is aggregated and polydisperse, the zeta potential measurements will contain a very large uncertainty. For these two reasons it is not surprising to see differences in zeta potential before and after sonication. As this complexity was not anticipated, appropriate steps were not taken during sample selection, preparation and characterization to forestall chemical changes during sonication.

*Reference materials and robust statistics to reduce and deal with experimental scatter*

For several property/material combinations, large variability has been observed between laboratories. Since such scatter can arise from between-sample inhomogeneity, it often gives rise to discussions on the statements made about method reproducibility or laboratory proficiency. This IANH study used RMs and test materials with demonstrated between-sample homogeneity in terms of average particle size. The remaining between-laboratory variability therefore only reflects inherent reproducibility problems of the tested procedure or a lack of proficiency at one or several of the participating laboratories. If in the absence of sufficient technical arguments to delete outliers, the scatter of the validated data is high, then the evaluation of the data benefits from the use of robust statistical methods, as was shown in this report.

## Conclusions and outlook

The initial IANH ILC studies, the results of which have been presented in this paper, have been instrumental for planning and conducting the current, more complex IANH studies that involve in vitro and in vivo toxicity assays. Furthermore, a number of conclusions can be drawn and used to improve reproducibility in the measurement of nanoparticle properties:

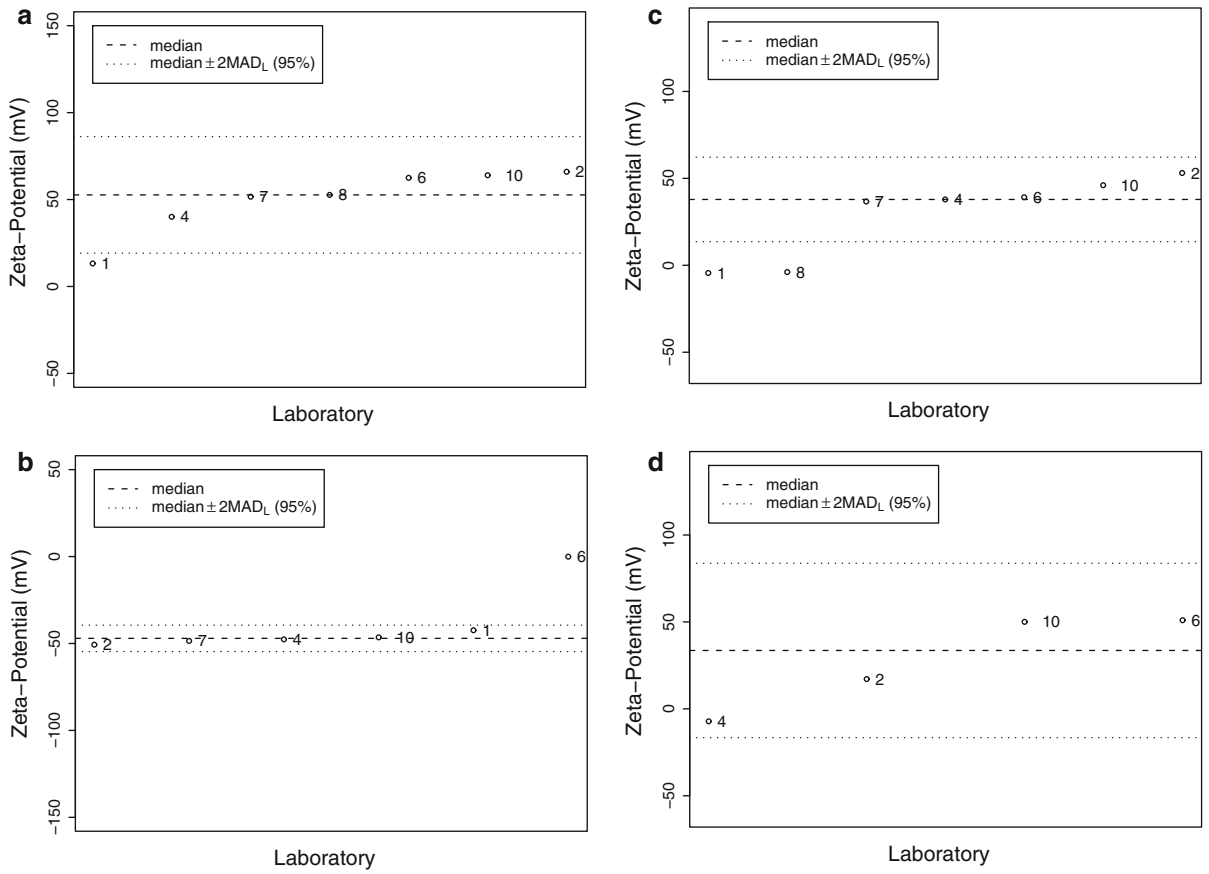


**Fig. 4** Zeta potential results on (**a**) BL-PS, (**b**) IRMM-304-silica, (**c**) UMI-ceria (before sonication), and (**d**) UMI-ceria (after sonication)

(i) The reported hydrodynamic equivalent diameter results confirm that polydispersity adversely impacts reproducibility of in situ particle sizing. This is particularly problematic for DLS due to this method's intensity-weighted results, which expresses as a strong sensitivity to the presence of small quantities of large particles or aggregates. The results also underscore the need for characterization procedures that are validated for the specific test material and which may include orthogonal techniques expressing different sensitivities (e.g., centrifugal liquid sedimentation, laser diffraction, small-angle X-ray scattering, etc.). From a practical perspective, the fact remains that most biologically oriented laboratories have access to DLS, and many do not have access to the other mentioned methods;

(ii) Routine sonication of nanoscale materials prior to testing is a likely source of variability in reported results, and requires standardization; dedicated studies have been initiated and an overview of this issue was recently reported (Taurozzi et al. 2010).

(iii) The proficiency of particle size measurement laboratories is not readily extended to zeta potential measurements. Additional development of suitable test protocols, documentary standards and reference materials for zeta potential analysis are required, particularly for industrially relevant complex powders and colloids. The latter implies, e.g., that also polydisperse or multimodal reference materials become available.

(iv) Present results illustrate the constructive role of RMs in ILCs, the usefulness of robust statistical

methods for data evaluation, and the need for ILCs even for what are considered routine laboratory measurements, to ensure comparability of subsequent biological assay data.

The nanoparticle test materials discussed in this paper were relatively well behaved and stable; however, dispersion of more diverse particles for biological testing is very challenging and will require well correlated measurements to determine that dispersions are equivalent at different sites. Under the best case scenario, in a round robin test, dispersions will be centrally prepared and distributed, but some dispersions may not be stable for the time required to perform multiple replicates of the biological test. In this case, the particle size distribution may also change during shipment or upon addition to the biological test medium, so characterization is needed to verify that the effective particle size distributions are equivalent. In other cases, dispersions may require sonication and dispersion at each site, so validation of dispersion size distribution will be necessary. Other dispersions may require deposition of a biomolecular coating, such as albumin, to stabilize their size and remove other ligands, if this can mimic the protein corona actually developed in vivo. Thus, it is crucial that each laboratory participating in the current and future IANH round robins has a measurement capability, that is correlated with other laboratories, to verify the size distribution of particles in a dispersion prior to commencement of the biological test.

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