Practical Guidelines for the Characterization and Quality Control of Nanoparticles in the Pharmaceutical Industry

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Practical guidelines for the characterization and quality control of nanoparticles in the pharmaceutical industry

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Abstract

Nanomaterials are used in a wide range of applications bringing completely new properties to a material or considerable improving pristine material property. In the medical domain where they are named nanomedicines, their usefulness was found to resolve drug delivery challenges and to improve performances of imaging-based diagnostic methods. Some carry activity on their own giving birth to new types of medicines. Whatever the application of the nanomaterial is for, a quality assessment is needed to ensure the repeatability and efficiency of industrial processes and in turn activity and safety of the product. This chapter was aimed to discuss the characterization of physicochemical parameters that can be used to define a nanomaterial. It gives basis in metrology and explains how it can be used to develop validated procedures for the characterization of the main physicochemical parameters that define NMs including their transfer to be used in many laboratories. Examples discussed in the chapter include the measurement of the size of NMs, the evaluation of the size distribution and of the zeta potential. The development of validated procedures for the characterization of NM is in its infant ages facing challenges that are discussed in this chapter.

Key Words

Characterization, size, zeta potential, size distribution, metrology.

Words for the index

Characterization, size, size distribution, zeta potential, measurement procedure, metrology, validation, precision, trueness, transfer, dynamic light scattering (DLS), electron microscopy (EM), atomic force microscopy (AFM), quality control, quality assessment, physicochemical parameter.

Abbreviations

ANOVA: Analysis of variance
AUC: Analytical ultracentrifugation
AFM: Atomic force microscopy
CD: Circular dichroism
CE: Capillary electrophoresis
CLS: Centrifugal liquid sedimentation
CRM: Certified reference material
DCS: Differential centrifugal sedimentation
DLS: Dynamic light scattering
DSC: Differential scanning calorimetry
ELS: Electrophoretic light scattering
EM: Electron microscopy
ES-DMA: Electrospray-differential mobility analysis
FFF: Field flow fractionation
GE: Gel electrophoresis
GUM: Guide to the expression of uncertainty in measurement
HDC: Hydrodynamic chromatography
ICH: International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use
IR: Infrared spectroscopy
ISO: International Organization for Standardization
ITC: Isothermal titration calorimetry
MS: Mass spectrometry
NIST: National Institute of Standards and Technology
NM(s): Nanomaterial(s)
NMR: Nuclear magnetic resonance
NP(s): Nanoparticle(s)
NTA: Nanoparticle tracking analysis
PALS: Phase analysis light scattering
PSD: Particle size distribution
RM: Reference material
SAXS: Small-angle X-ray scattering
SEC: Size exclusion chromatography
SEM: Scanning electron microscopy
SLS: Static light scattering
sp-ICP-MS: Single particle inductively coupled plasma-mass spectrometry
TEM: Transmission electron microscopy
TRPS: Tunable resistive pulse sensing
XPS: X-ray photoelectron spectroscopy
XRD: X-ray diffraction
ZP: Zeta potential

1. Introduction

Over the last decades, nanomaterials (NMs) have become extremely popular thanks to unique properties that can be exploited in different fields such as energy[1,2], transportation[3,4], industry[5,6], food[7], cosmetics[8] and medicine[9,10]. They can occur with different structures and be composed of various matter such as metals i.e. titanium oxide, gold, silver, platinum and ferric oxides, polymers, lipids, carbons including carbon nanotubes, graphene derivatives, nanodiamonds and fullerenes.

Many NMs have found interest in medical applications. Pharmaceuticals and medical devices based on the use of these technologies were called nanomedicines. They include various types of nano-objects which varies from their structure and composition. It was a rapidly growing field over the past two decades but several aspects on their definition remain under debate. There is a need to clarify the classification of the different types of nanomedicines occurring with complex structures[11]. Regarding the size, the definition given for a NM proposed by authorities in the early 2010 is too narrow to include all types of nanomedicines as it excludes many nanomedicines which size are larger (200-300 nm) than the upper limit given in the official definition based on at least one dimension lower than 100 nm for 50% of the number size distribution of NMs. Nevertheless, a consensus is established on the need to provide with relevant quality control procedures to assess product quality insuring repeatability and reproducibility of the safety and efficacy on a batch-to-batch basis. This can be achieved performing the characterization of NMs by the use of validated procedures under conditions compatible with quality control[12–17] or methods whose performances have been proven by interlaboratory comparisons[18–20] thus ensuring reliable results. The reliability of measurements can be ensured by defining a series of handling precautions and quality criteria for good measurements[12,13,21,22]. The selection of relevant methods to characterize properties of NMs should be performed by comparing available methods to provide reliable measurements[23–32]. It is noteworthy that the characterization of physicochemical parameters of NMs in general remains a difficult task even for parameters including the size of the nano-object and the distribution of size, the surface charge using automatic measurement instruments. Most characterization methods of NMs require a preparation of the sample that will be used to perform measurements with the specifically designed method. This can include a dilution of the sample or the realization of a dry depot on a substrate. Whatever the modalities for the preparation of the sample, efforts are needed to ensure that measurements will be representative of the original dispersions of NMs[12,13,21,22,33–35]. This chapter aims to give some practical guidelines to characterize nanomedicine-based pharmaceuticals in the quality control assessment perspective.

2. Characterization of materials

The characterization of NMs under conditions compatible with quality control is a societal task. NMs are characterized by two different types of parameters. For instance, the composition, the concentration, the structure and the surface functionalisation of the NMs are general parameters which are not restricted to NMs although methods for the determination of the concentration are very specific. Specific characteristics of NM include their size parameters, i.e. size, particle size distribution (PSD), agglomeration or aggregation state, their surface properties as surface charge through the evaluation of the zeta potential (ZP), reactive surface, surface area and porosity and their shape[36,37]. These characteristics should be characterized as suggested by the technical committee of International Organization for Standardization (ISO TC 229 - Nanotechnologies) and the OECD Working Party on Manufactured Nanomaterials. Modifications of size parameters and surface properties of nanomedicines can affect their biological fate hence biological efficacy and safety[38,39]. Size parameters and surface properties of NMs are among paramount factors to evaluate in order to assess repeatability and reproducibility and efficiency of industrial processes and product quality[40]. The Table 1 summarizes the different methods that are available to assess specific physicochemical parameters of NMs.

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Table 1. Specific physicochemical parameters used to describe properties of NMs[36,41].

Physicochemical parameter	**Definition**	**Method**	**Measurand**
Size, PSD and agglomeration or aggregation state	***Size***: Physical dimensions of NM evaluated with specific size measurement method with given experimental conditions. ***PSD***: Proportion of distinct populations with different NM sizes of a given dispersion of NMs. ***Agglomeration***: NMs bounded by weak interaction as Van der Vaals force and electrostatic interactions[42]. ***Aggregation***: NMs bounded by interaction with higher intensity such as covalent binding[42].	***Batch*** Acoustic techniques[32,43] DLS[13,23,26,28,29,44,45]* SLS[23,46] SAXS[31,47–49] XRD[31] ***Single*** ***- Direct method*** EM[23,26,29,31] AFM[23,50] ***- Indirect method*** NTA[23,26,29] TRPS[23,29,51] sp-ICP-MS[47,52] ES-DMA[53,54] ***Separative*** AUC[55,56] CE[57–59] DCS (known as CLS)[28,29] FFF[23,28,34,60] HDC[61] SEC[62]	 Volume-based diameter and PSD. Hydrodynamic diameter/Scattering intensity-based PSD. Gyration diameter (Rayleigh)/Scattering intensity-based PSD. Gyration diameter (Guiner)/Scattering intensity-based PSD. Scherrer's diameter/No PSD. Equivalent spherical diameter or Feret's diameter/Number-based PSD. Height or diameter from analysis of images in (x-y) dimension/Number-based PSD. Hydrodynamic diameter/Number-based PSD. Raw diameter/Number-based PSD. Height of intensity of detected pulse/Mass-based PSD. Mobility diameter/Number-based PSD. Sedimentation diameter/Density-based PSD. Apparent mobility (or electrophoretic mobility)/PSD depending on detector used. Sedimentation diameter/Extinction intensity-based PSD. Retention time/PSD depending on detector used. Retention time/PSD depending on detector used. Retention time/PSD depending on detector used.
Surface charge	***Evaluation of ZP***: Charged NMs are surrounded by electrical double layer formed with opposite charged ions (Stern layer	***Batch*** ELS[12,64]* Acoustic techniques[65,66] ***Indirect single method***	 Electrophoretic mobility. Electrophoretic mobility.

	with strongly bound ions) and diffuse layer with weakly bound ions) in ionic dispersant. Ions from diffuse layer are sharing from ions of bulk dispersant with movement of NM. The potential on shar surface corresponds to ZP[63].	NTA[30,67] TRPS[30,68,69] ***Separative*** CE[70–72]	Electrophoretic mobility. Electrophoretic mobility. Electrophoretic mobility.
Reactive surface	Surface of NMs available to interact with different mediums such as biological medium i.e. with biomolecules.	CE[73–75] DLS[76,77] ES-DMA[78,79] GE[80]***** ITC[81–83]*****	Apparent mobility (or electrophoretic mobility) or area. Hydrodynamic diameter. Mobility diameter. Degree of complement pathway. Released or absorbed heat from binding event providing thermodynamic parameters.
Surface area and porosity	Developed surface of NMs including surface area of open pores.	Brunauer, Emmett and Teller method[84]*****	Adsorption of gas molecules as N_2 on surface of NMs (adsorption isotherm).
Shape[a]	Geometrical description of NMs.	***Direct method*** EM AFM ***Indirect method*** AUC[85] SAXS[49]	 Aspect ratio described with dimensionless terms such as elongation ratio, flatness ratio, sphericity, circularity and rugosity. Shape factor. Shape form factor.

[a]Evaluation of agglomeration or aggregation state of NMs can be performed with methodologies applied to determine the shape of NMs.

*****Used in routine.

AFM: Atomic force microscopy, AUC: Analytical ultracentrifugation, CE: capillary electrophoresis, CLS: Centrifugal liquid sedimentation, DCS: Differential centrifugal sedimentation, DLS: Dynamic light scattering, ELS: Electrophoretic light scattering, EM: Electron microscopy, ES-DMA: Electrospray-differential mobility analysis, FFF: Field flow fractionation, GE: Gel electrophoresis, HDC: Hydrodynamic chromatography, ITC: Isothermal titration calorimetry, NM(s): Nanomaterial(s), NTA: Nanoparticle tracking analysis , PSD: Particle size distribution, SAXS: Small-angle X-ray scattering, SEC: Size exclusion chromatography, SLS: Static light scattering , sp-ICP-MS: Single particle inductively coupled plasma-mass spectrometry, TRPS: Tunable resistive pulse sensing, XRD: X-ray diffraction, ZP: Zeta potential.

It points out direct and indirect methods and those that can be applied in routine analysis. The Table 2 overviews the general physicochemical parameters that are used to describe the properties of NMs. It highlights the methods that can be applied to assess these general parameters. It is noteworthy that the evaluation of the concentration of NMs can be performed using the methods specific to the NMs. The application of any mentioned method of characterization in quality control analysis needs to be validated according to general procedures used in metrology in order to provide uncertainties associated to the measurement of the physicochemical parameter of NMs using a given method and applying a specific measurement procedure.

Table 2. General parameters used to describe properties of NMs.

Physicochemical parameter	Definition	Method
Concentration	Number of NMs per volume unit.	ES-DMA, NTA, TRPS, sp-ICP-MS
Composition	Chemical and molecular structure of NMs.	MS, NMR, sp-ICP-MS
Structure	Structure state.	IR, CD, DSC, NMR, SAXS, XRD
Surface functionalisation	Chemical and molecular structure at the surface of NMs.	IR, XPS

CD: circular dichroism, DSC: Differential scanning calorimetry, ES-DMA: Electrospray-differential mobility analysis, IR: Infrared spectroscopy, MS: Mass spectrometry, NM(s): Nanomaterial(s), NMR: Nuclear magnetic resonance, NTA: Nanoparticle tracking analysis, SAXS: Small-angle X-ray scattering, sp-ICP-MS: Single particle inductively coupled plasma-mass spectrometry, TRPS: Tunable resistive pulse sensing, XPS: X-ray photoelectron spectroscopy, XRD: X-ray diffraction.

3. General consideration to achieve quality control analysis and metrology: validation and transfer of analytical procedures

The characterization of NMs is necessary to describe the properties of the NMs composing nanomedicines thus achieving safety-efficiency and batch-to-batch consistency. In practice, very few methods are available to achieve the characterization of nanomedicines on a routine basis considerably limiting the number of parameters that can be included in quality control assessment. It is noteworthy that almost all methods are indirect methods which means that the parameter measured by the instrument is then used to calculate the property desired to determine. Models developed to convert the measure into the measurand can be quite complexed restricting the application of the technique to the characterization of a narrow range of NMs. Standardisation of size measurement methods is paramount to provide results that are comparable between laboratories. For instance, the widely used method for size determination by dynamic light scattering (DLS) can be applied on spherical NMs with a narrow size distribution. Results are biased while the polydispersity increases, and the method is inappropriate to characterize the size of non-spherical particles. Measurements should be performed under conditions compatible with quality control that requires the use of standardized procedures. The procedures should be validated, and uncertainties should be evaluated with a reference NM closed to that which will be analysed. Moreover, instruments must be qualified using appropriate reference NMs including materials from National Institute of Standards and Technology (NIST) when available. In the quality control assessment procedure for the analysis of a NM, the reference NMs should be analysed before and after the analysis of "unknown" NMs by the same validated measurement procedure.

The evaluation of physicochemical properties of NMs should be performed under conditions which are compatible with quality control to provide reliable characterization. Reliability of results can be appreciated with associated measurement uncertainty determined through the validation of analytical procedures. The validation of analytical procedures consists in providing guarantees with certified reference material (CRM) or reference material (RM) that analytical procedures are sufficiently acceptable, reliable and adequate for elements of their scope[86–88]. Moreover, laboratories should prove that their analysts are able to perform analytical procedures with similar results[88–90]. So, there is a need to provide guidelines to ensure quality control and thereby to evaluate safety and toxicity of NMs. Draft guidance documents are provided for manufactured NMs, indicating various methods that can be applied to evaluate these parameters[37,91]. Nevertheless, no indication is given to validate and transfer analytical procedures applied to the characterization of NMs and to provide uncertainty closed to results[37].

Although many parameters can be used to define one NM, only few are really accessible for a routine analysis using marketed instruments or having been the subject of standards from International Organization for Standardization (ISO) description as size[92–108], surface charge[109–111], shape[99,100,112], surface area[113,114] and reactive surface[115].

3.1. Validation and transfer of analytical procedures

Whatever the type of analysis, it follows a well-established analytical procedure describing in detail all steps needed to carry out a given analysis. All analytical procedures will follow a life cycle which includes a validation stage and a transfer stage as illustrated in Fig. 1. The validation is achieved applying strict metrology concepts which aims to prove that the analytical procedure is sufficiently acceptable, reliable and adequate for the elements of its scope[86–88]. The validation is generally achieved using CRM or RM. It consists of performing numerous measurements of these materials following the described procedure. The results are then analysed with appropriate statistical analytical methods. The Guide to the expression of uncertainty in measurement (GUM) outlines statistical methodologies to interpret raw data of validation as analysis of variance (ANOVA)[116]. The different parameters evaluating performances of analytical procedures were summarized in Table 3. The validation of analytical procedure permits to assess to the associated expanded uncertainty expressing reliability of results provided with validated analytical procedure[116]. CRM is a material that is metrologically characterized with valid procedure for one or more specified properties[117]. Analysis certificate providing value of specified property with corresponding uncertainty and metrological traceability is produced with CRM. RM is a homogeneous and stable material towards one or more specified properties[117]. It is adequate for its used in process of measurement of specified property. When it is possible, it is important to validate the analytical procedure with a material certified for the analytical method that will be used. The number of CRM and RM available to validate methods of characterization of NMs is limited. Size CRM generally consist in monodispersed NMs. Only one consists in bimodal dispersion of silica nanoparticles (NPs) certified at 18.2 and 84 nm with electron microscopy (EM) (ERM-FD102). There is only one available CRM with assigned SI-traceable values of positive electrophoretic mobility (NIST Standard Reference Material® 1980, value: 2.53 ± 0.12 $\mu m.cm.V^{-1}.s^{-1}$). It is noteworthy that there is another CRM with a negative value of ZP (ERM-FD100, value: 43.0 ± 21.8 mV[118]). However, the uncertainty of the certified value of ZP of this standard is about 50 % of the certified value. Other CRMs are currently under development[119]. Polystyrene latex particle based-standard is commercially available but it is not a CRM (DTS1235 from Malvern, value: 42.0 ± 4.2 mV).

Besides having appropriate CRM or RM, validation also needs to investigate adequate parameters. No official specific guidelines were yet established to perform the validation of a measurement procedure characterizing NMs. The guidelines Q2(R1) from International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH guidelines Q2(R1)) was established only for the validation of most common types of analytical procedures including identification tests, quantitative tests for impurities' content, limit tests for the control of impurities and quantitative tests of the active moiety in samples of drug substance or drug product or other selected components in the drug product[86]. Other types of analytical procedures such as dissolution testing of drug products and the evaluation of particle size of drug substance have not been addressed in this document This guideline mentioned that the validation of these analytical procedures is equally important to those listed herein and may be considered in subsequent documents. Although this guideline did not provide any specific information on how validation of NM characterization procedures should be carried out, concepts to achieve such validations can be drawn from it. The selection of studied parameters should be adapted on a case by case basis.

Other official documents propose some lines to perform validation of measurement procedures applicable to the characterization of NMs. Standards from ISO suggest to study trueness and precision i.e. repeatability and intermediate precision of procedures used to evaluate ZP of NMs with ELS coupled to phase analysis light scattering (PALS)[110] and precision i.e. repeatability and reproducibility of procedures applied to evaluate size of NMs by DLS[95]. However, no indication about number of samples needed to study each parameter and statistical methodologies to interpret raw data was given in ISO standards[95,110]. The Nanomedicine Characterization Laboratory (Frederick, MD, USA) proposes standardized procedures to evaluate size of NMs with DLS[120], atomic force microscopy (AFM)[121], transmission electron microscopy (TEM)[122], scanning electron microscopy (SEM)[123]and Electrospray-differential mobility analysis (ES-DMA)[124] or to evaluate ZP[125]. It was reported that procedures used for evaluating size of NMs by DLS[120] and procedures applied to size evaluation of NMs with SEM[123] should be validated. Last decade, Shekunov *et al.* and Gaumet *et al.* were the first to carry out reflexion about the reliability of results for NMs characterization through size measurement with acceptable trueness[38,39].

A measurement procedure validated in one laboratory can be transfer to other laboratories through a transfer approach. The aim is to demonstrate that the procedure validated by the sending laboratory can be applied in the other laboratories, named receiving laboratories, with the same performances. It must prove that receiving laboratories are able to carry out analytical procedure by providing similar results as the sending laboratory[88–90]. Approaches that can be used to achieve the transfer of an analytical procedure are described by Food and Drug Administration (FDA)[89] and the USP Pharmacopeia[90]. They are also described in Handbook of modern pharmaceutical analysis[88]. The different approaches that can be included in a transfer of analytical procedure are summarized in the Table 4. Their selection to achieve the transfer of a given analytical procedure depends on risk assessment, complexity, criticality and aim of the analytical procedure. In general, during the analytical stage, each laboratory including the sending laboratory and all receiving laboratories analyze the same batch of samples. Data obtained from the different laboratories are compared and confronted to acceptance criteria that are defined depending on the method. It is noteworthy that no specific information is provided to perform transfer of physicochemical characterization procedures of NMs. The selection of suitable approach to transfer such procedure should be adapted on a case to case basis.

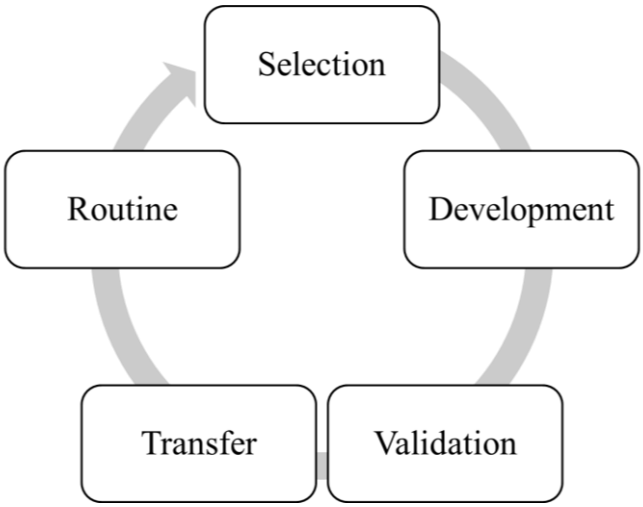

Fig. 1. Life cycle of analytical procedure.

Table 3. Overview of parameters used to describe performance of analytical procedures[86–88].

Parameter	**Definition**
Specificity	Ability of analytical procedure to perform unambiguously analysis of substance in the presence of impurities, degradation products or matrix.
Linearity	Ability of analytical procedure to provide results directly proportional to the concentration of substance in samples for a given range of concentrations.
Trueness	Difference between the average value provided by a large series of test results and the accepted value i.e. conventional true value or accepted reference value highlighted systematic errors (bias).
Precision	Degree of dispersion of a series of test results provided with multiple sampling of same homogeneous sample carried out under stipulated experimental conditions pointed random errors. Three distinguished levels: ➢ *Repeatability (or intra-assay precision):* repetition performed with same experimental conditions including method, instrument, laboratory and analyst over short period of time i.e. same day. ➢ *Intermediate precision (or within laboratories variations):* repetition carried out by varying factors as day, analyst or equipment within the same laboratory. ➢ *Reproducibility (or inter-laboratories variations):* repetition performed in different laboratories.
Range	Interval whose boundaries are defined by lowest and highest concentrations of substance and for which appropriate level of trueness, precision and linearity of analytical procedure have been proved.
Detection limit	Lowest quantity of substance that can be detected but not necessarily quantified as exact value.
Quantification limit	Lowest quantity of substance that can be quantified with acceptable trueness and precision.
Robustness	Ability of analytical procedure to remain non-affected by small deliberate variations in experimental conditions.
System suitability testing	Developed tests to control equipment, electronics, stability of sample or analytical operations.

Table 4. Overview of approaches used for the transfer of analytical procedures[88–90].

Approach	Definition
Comparative testing	Analysis of defined number of samples from the same batch performed by sending and receiving laboratories.
Interlaboratory covalidation	Participation of receiving laboratories in part of process of validation of the analytical procedure such as precision study i.e. investigation of reproducibility.
Revalidation	Partial or complete validation of analytical procedure by the receiving laboratories. *Used when variations in analytical procedure are provided or no suitable samples are available.*
Verification	Demonstration of performance of receiving laboratories by comparison between results obtained by the receiving laboratories and certified results provided with certificate of CRM or by the sending laboratory or by checking conformance of results provided by receiving laboratories with respect of performance criteria.
Application	Demonstration of performance of receiving laboratories by application according to control test procedure by checking conformance of results provided by receiving laboratories with respect of performance criteria defined in test procedure.
Transfer waiver	Receiving laboratories are considered to be able to perform the analytical procedure without investigation of their performance.

3.2. Qualification of instrument

The qualification of an instrument is achieved to provide documented evidence that the instrument performs with specification. According to the ISO standard and the Good Manufacturing Practices, the instruments should be calibrated or checked by appropriate methods with suitable control samples as traceably calibrated materials at defined periods[126,127]. There are different stages of qualification covering the life of an instrument from its design to its utilisation in routine (Fig. 2).

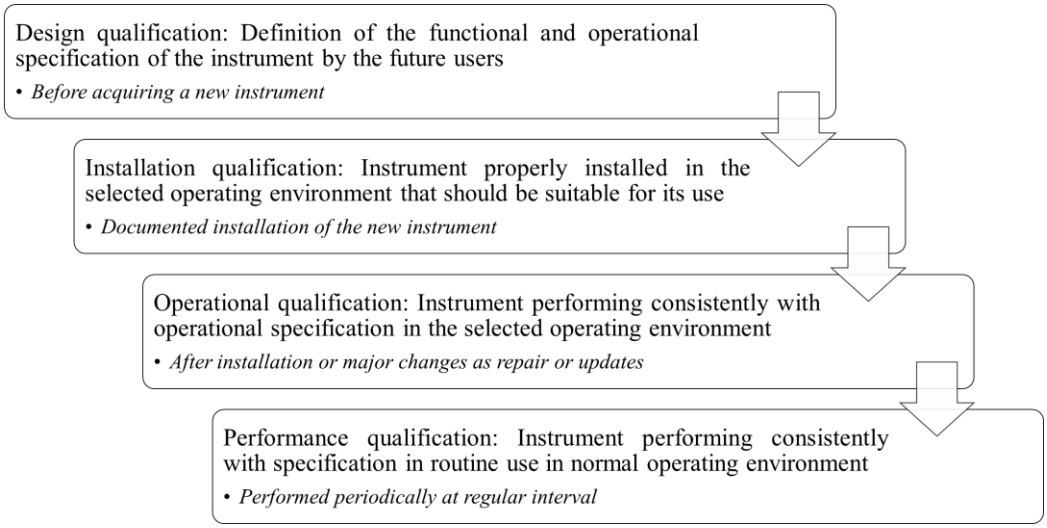

Fig. 2. Stages of qualification of an instrument.

This aspect was introduced in the ISO standard devoted to the measurement of size of NMs by DLS[95]. The ISO standard mentions that the qualification of the instrument should be performed after installation (operational qualification) and at regular time intervals (performance qualification) with a dispersion of materials with certified size. CRM with values assigned for DLS using the same algorithm to determine the size of the CRM should be used to carry out the qualification of the instrument. It is mentioned that the chemistry and the morphology of the NMs constituting the CRM should match the test samples as closely as possible. It is noteworthy that, alternatively, certified dispersions of polystyrene latex with narrow size distribution with average particle diameter as evaluated by DLS or EM can be used for the qualification of instrument. The qualification of the instrument can be evaluated either from five repeats measurements of size of CRM by comparing the difference between the measured average and the certified values and the expanded uncertainty closed to the measured average value[128] or from three repeats measurements of size of CRM carried out before and after the measurement of the size of unknown NMs, the size of the CRM should be within the range of size determined during the validation of the procedure used to evaluate the size of unknown NMs[13,44]. If the qualification fails, it can indicate a mistake in the preparation of the dispersion or the instability of the dispersion or the failure of the instrument.

4. Validation of procedures evaluating physicochemical parameters of NMs: Examples

4.1. Size measurement by dynamic light scattering

DLS is a major technique used to measure size of NMs. This method is very popular thanks to the existing of easy to use affordable marketed measurement instruments. DLS was also implemented to achieve continuous measurements using a glass capillary mounted in classical laboratory instrument[45]. Results provided with this method are reliable considering NMs of homogenously distributed size having a narrow size distribution[13,44]. However, this technique should be applied with cautious when characterizing the size of unknown NMs as bias on measurements can be introduced in the case of non-homogenous in size dispersions or of dispersions showing a wide or complex polydispersity[23,26,28,29,129,130].

Very few works have reported size results with associated measurement uncertainty ensuring reliable characterization of size of NMs by DLS[13,17,44,131]. The preparation of the sample to perform size measurement by DLS is particularly a critical step[13,17,22,44,129,132]. The presence of dust may compromise the size measurement of NMs. It is necessary to prepare diluted samples of NMs with freshly filtered dispersants with 0.22 µm filter and flasks with caps should be pre-rinsed with filtered ultrapure water and stored in a dust-free environment. Bias can be introduced with the quality of measurement macrocuvettes. Cuvettes showing defects on the optical faces must be discarded while they can represent 85% of the units in a box depending on suppliers and quality. The measurement cuvettes should be cleaned with filtered ultrapure water and stored in a dust-free environment until use. The measurement cuvettes should be used only once to avoid cross-contamination. The volume of sample introduced in the macrocuvette should be sufficient to permit the passage of the Laser into the sample. The larger the volume is, the longer equilibration time is to let the sample to reach the temperature of measurement. Indeed, the temperature of the sample during measurement is paramount to control to provide with reliable size results as the measured parameter is the diffusion coefficient from which

the size is calculated using the Stokes and Einstein equation. Artifacts due to degassing of the samples may be created with high difference between the temperature of the sample and the temperature of measurement. The equilibration time should be long enough for the sample to achieve the temperature of measurement. A minimum of 1 min per degree of difference should be considered for a volume sample of approximately 1 mL. Optimal concentration of the dispersions of NMs to carry out size measurement should be optimized for the intensity of the signal to be within the range recommended by the supplier of the instrument used. For this purpose, the curve representing the intensity of the signal as a function of the concentration of NMs should be established and the optimal concentration is selected on the linear part of this curve[133].

Some quality criteria should be defined and followed to ensure reliable results[13,22]. For size measurement by DLS, the quality of the correlogram reflecting the probability to find the NMs at the same place after a few times and the count rate curve corresponding to the number of photons collected by the detector associated to each run during measurements can be followed during the size measurement. After measurement, the raw correlogram, the intercept describing the amplitude of the correlogram that is closed to the signal-to-noise ratio, the mean count rate and the cumulant fit error can be inspected. The cumulant fit error is the closeness of agreement between the experimental raw correlogram and the calculated correlogram by means of the Cumulant method described in the ISO standard[95].

It is noteworthy that an ISO standard dealing with good practice for DLS measurements is under development[134].

The selection of the CRM or RM is crucial. NIST Traceable Particle Size Standards consisting in polystyrene latex standard with SI-traceable certified values by TEM can be used to validate the developed procedures. These CRM are spherical NPs known to not swell in aqueous dispersions and appeared quite monodisperse as acknowledged by the low PDI (PDI < 0.05) and available from 50 to 900 nm. These CRM should be diluted in NaCl 10 mM for suppressing the electrical double layer and ensuring that the measured hydrodynamic diameter was the same as the expected by TEM as described in the ISO standard[97]. Other CRM with traceable mean diameter of 20, 30 and 40 nm by DLS are available.

The developed procedures should be validated by studying robustness, precision i.e. repeatability and intermediate precision and trueness to evaluate the expanded uncertainties of the procedures. The robustness is investigated by varying experimental parameters that may influence measurements of size of NMs permitting to provide indication on the reliability under normal conditions of use of the proposed procedures. This study is a preliminary step before transferring methods to other laboratories or performing collaborative studies. The repeatability is performed by measuring size of the CRM carried out successively in the same day and the intermediate precision by measuring size of the CRM performed in different days. In the experimental nested design proposed by Varenne *et al.* to investigate the precision of the procedure, three samples of diluted CRM at optimal concentration were analyzed per day[13]. Each sample was analyzed in triplicate i.e. three successive size measurements were performed on each sample. This experimental nested design permits to investigate the influence of the factors days, samples and replicates that are considered as random (Fig. 3). The raw data were interpreted by means of ANOVA permitting to investigate the variability between days, between samples variability analyzed on the same day (within days) and between replicates variability of a sample (within samples). Appropriate statistical models were developed to interpret the raw data. According to the ISO standard[95], the relative uncertainties of repeatability and reproducibility should be below than 2 and 5 % respectively. This ISO standard mentions any information about the evaluation of intermediate precision to evaluate the influence of factors as the instrument and/or the analyst or over longer period of time (i.e. typically on different days)[95]. It is suggested to investigate the trueness of developed procedure. However, no limit was provided for the relative uncertainty of trueness[95]. According to the

literature, the limits of the relative uncertainties of intermediate precision and trueness may be set at to 5 and 10 % for intermediate precision and trueness respectively.

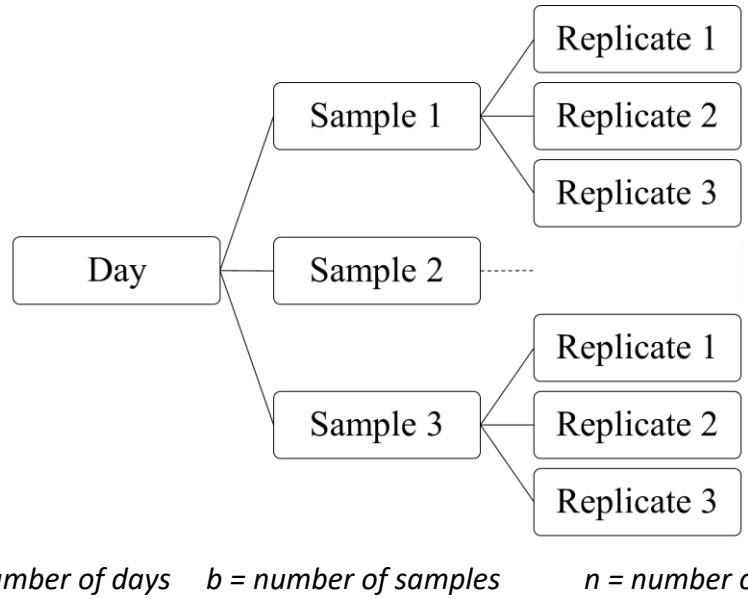

Fig. 3. Experimental nested design to investigate precision of procedure. The factors days, samples and replicates are studied, and the symbols a, b and n correspond to the number of levels of a nested factor within the factor above ranked.

Qualified size measurements should be provided to characterize unknown NMs by DLS under quality control conditions. The procedure proposed by Varenne *et al*. includes (i) the control of the absorption spectrum of NMs for ensuring that no absorption band appears at the wavelength of the Laser source of the measurement instrument, (ii) the evaluation of the optimal concentration of the dispersions of NMs and (iii) the measure of the size of unknown dispersions of NMs at the determined optimal concentration under operational or performance qualification of the instrument[13,44]. It means that the size of CRM which size and nature is closed to the size of the investigated NMs should be measured before and after the evaluation of the size of investigated NMs permitting to evaluate size of monodispersed NMs under conditions compatible with quality control assessments.

Validated size measurement procedures using DLS proposed by Varenne *et al.* are suitable to measure size of a wide range of NMs including polymer NPs, liposomes and inorganic NPs as silica NPs[13,44]. However, it was found unsuitable to evaluate the size of NPs having a high density such as anastase TiO_2 and magnetic NPs which sizes are in the upper limit of the measurement instrument[131].

4.2. Evaluation of the particle size distribution

Evaluating the PSD of a dispersion of NMs is a difficult issue. Several size measurement methods present major inherent limitations that hamper reliable determination of the PSD of NM dispersions that have a wide or complex PSD. Besides, there is only one multimodal CRM (ERM-FD102) including silica particles of two sizes, 18.2 and 84 nm certified by EM. However, this CRM is not certified to be used for the determination of PSD. Without an appropriate reference dispersion of NMs, the performance of a method applied to measure PSD cannot be evaluated. No official procedure has been proposed to characterize of the PSD of NMs. The scientific community recommended to apply two methods at least based on two different physical principles. One the

method should be based on a direct size measurement method including image analysis of particles obtained from AFM, SEM or TEM or it should include a separative size stage combined with batch size measurement method as detector[23,60,135].

It is noteworthy that the DLS method needs to be used with caution while applied to characterize size and PSD of unknown NMs although this technique is widely used in routine. The intensity of the scattered light is proportional to the power six of the radius of NMs. Thus, the intensity of the scattered light due to the large NMs can cover the signal produced by the smaller NMs of the dispersion. Important bias were reported with this method when it is applied for the determination of the size and PSD of NMs having a wide or complex size distribution although it is reliable while applied to the characterization of NMs having a narrow size distribution[23,136]. Direct size measurement methods include EM and AFM[23,135,137]. The size of the NMs is measured directly from images obtained for the NMs. The preparation of samples for observations by EM and AFM consists in the spreading of the NMs on a sample holding[33,35]. This preparation is critical for the quality of the subsequent image analysis process used to determine PSD and may requires that a specific procedure may be developed for each NM[138]. NMs of the dispersion must be randomly distributed on the surface of the sample holder (carbon grid or mica substrate). It is also preferable that NMs will be well individualized to avoid distortions due to the proximity of neighbour NMs (Fig 4. (A)) and to facilitate image processing measurements. It may be difficult to obtain a random deposition of NMs on sample holder from a dispersion of NMs having a high PSD as a segregation according to the NM size may occur as illustrated in the Fig. 4 (B). For this reason, it is recommended to evaluate the PSD performing orthogonal measurements with different methods[23,26,28,29,60,62].

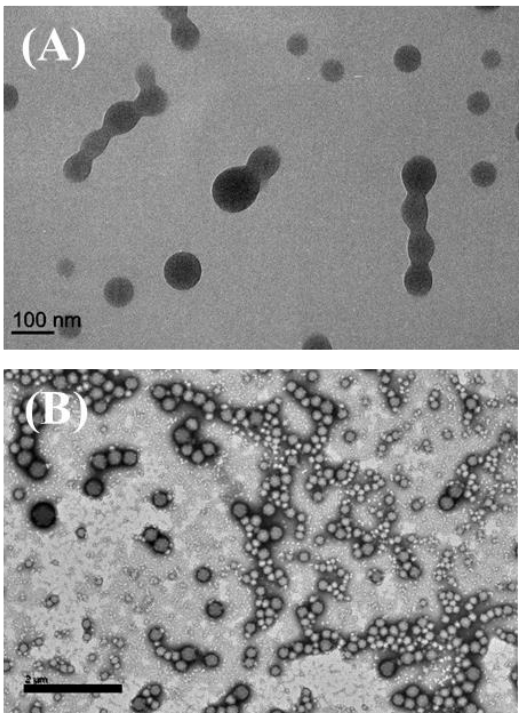

Fig. 4. Electron micrograph of unstained poly(isobutylcyanoacrylate) NPs deposited on a formvar-carbon coated cupper grid for EM. (A) Projected image of single particles appeared circular suggesting that the particles were spherical. In contrast, particles included in aggregates appeared distorted due to the close contact with their neighbors. Scale bar: 100 nm. (B) Segregation according to particle size occurred during sample preparation of a highly polydisperse dispersion of the NPs. Scale bare: 2 µm. Evaluation of shape, size and PSD by EM requires that NPs will be well individualized on the sample holder and randomly distributed over the surface of the sample holder.

To evaluate PSD from direct methods, size measurements must be performed on a sufficiently large number of NMs. The debate around the number of NMs that should be considered remains open. The ISO standard suggests that the size of one thousand individual NMs should be measured that seems not always possible to achieve due to sample preparation constrains[139]. A much lower number of NMs was considered in different works. Song *et al.* studied the PSD of a dispersion of synthetic gold NPs consisting in one population of size with a polydisperse distribution and showed that the PSD provided by counting a few hundred NPs was similar to the one produced by the analysis of one thousand NPs[137]. Varenne *et al.* investigated the PSD of a multimodal dispersion of polymer NPs from the "real-life" obtaining similar PSD from three independent evaluation performed by measuring samples including around three hundred NPs[138]. A number of at least five hundred NPs was considered in an interlaboratory comparison of the evaluation of the PSD of NPs performed by TEM indicating a good performance of the method considering this number of NPs[135]. Rice *et al.* have found that the best model to use to interpret raw data evaluating the PSD was the log normal reference model as it provided with the lower relative standard errors (RSEs) compared with other size distribution reference models tested in their work while determining the PSD of their NM[135].

4.3. Evaluation of zeta potential using electrophoresis light scattering

Reliable evaluation of ZP of NMs by electrophoretic light scattering (ELS) also requires the validation of measurement procedures. An ISO standard gives guidelines for good practices in the evaluation of ZP[140]. Another ISO standard indicates the thresholds for the relative standard uncertainties of repeatability, intermediate precision and trueness that should be used for the validation of procedures to evaluate ZP[110].

A similar strategy than that applied to validate procedure of size measurements may be applied[12,21,64,131]. In short, as for size measurements performed by DLS, the preparation of samples to evaluate ZP by ELS is a key step[12,21,64,131]. The presence of dust in samples can be avoided preparing dilutions with freshly filter dispersants with 0.22 µm filter just before use. All flasks with caps devoted to the preparation of dispersant and samples are needed to be pre-cleaned with filtered ultrapure water and stored in a dust-free environment. Selection of high-quality measurement cell is needed as optical defects including scratches and/or apparent impurities in the polycarbonate faces may interfere with optical measurements. Beside cell cleanliness appearance, electrodes should be homogenous and well attached on both the inside and outside of the cell measurement to insure a homogeneous electric field. The cells including caps should be rinsed with appropriate filtered solvent and stored in a dust-free environment before using. The cells should be used only once to prevent the cross-contamination. The temperature of the sample is a critical parameter. Large differences between the temperature of the sample and the temperature of measurement may generate artefacts during measurements due to the degassing of the samples.

Optimal concentration of the dispersions of NMs to evaluate ZP should be evaluated using methods based on the equilibrium dilution procedure mention in the ISO standard[111]. This procedure consists in maintaining the composition and the concentration of dispersant identical between diluted samples.

Quality of data may be appreciated by means of defined quality criteria achieved during measurement and on the raw data[12,21]. For example, the phase plot showing phase difference between the measured frequency and the reference frequency as a function of time and the count rate curve giving the number of photons detected by the photomultiplier associated to each run can be inspected during measurement. The final phase plot, the frequency plot corresponding to the Fourier Transform analysis of the slow field reversal part of the analysis used to evaluate ZP distribution and the mean count rate can be controlled on the raw data.

Experimental measurement procedures established to evaluate ZP of a NM must be validated using reference NMs. Only two were developed so far. One CRM is available with assigned SI-traceable values of positive

electrophoretic mobility (NIST Standard Reference Material® 1980). It is noteworthy that this CRM tend to adsorb on the intern surface of measurement cells made of polycarbonate[12]. For this reason, measurement cells should be preconditioned with the dilute dispersions of NMs before introducing fresh samples and carrying out the analysis as explained in the notice of use. To validate procedures for NMs with a negative ZP, it necessary to use one negative ZP RM classified as a transfer standard. This type of standard has been referenced to an accepted standard by the scientific community as there is no CRM with acceptable uncertainty of the certified value of ZP[118].

The procedures should be validated by investigating robustness, precision i.e. repeatability and intermediate precision and trueness to determine the expanded uncertainties of the procedures. The same experimental design than the one presented in Fig. 2 may be used to investigate the precision of the developed procedures. The repeatability can be determined with successive evaluation of ZP of the RM in the same day while the intermediate precision can be assessed by carried out evaluation of ZP of the RM for various days. The ISO standard gives thresholds for the relative standard uncertainties of repeatability, intermediate precision and trueness (10, 15 and 10% respectively)[110].

Qualified evaluation of ZP can be performed following the same procedure than the one described for the measurement of size of NMs. According to the mode used to perform the evaluation of ZP, the proposed procedures by Varenne *et al.* can be applied to the characterization of NMs including polymer NPs and liposomes, but were not appropriate to evaluate the ZP of dense NPs such as titanium dioxide NPs[12,64,131]. In any cases, the evaluation of ZP of a NM is not trivial as many parameters can influence the final results[141]. A series of advices on how to interpret and report measurements of ZP was proposed based on the evaluation of the ZP of metal NPs dispersed in complex media of relevance for studies on nanotoxicology and environmental interactions[141].

4.4. Transfer

Once validated in one laboratory, it has to be demonstrated that the validated procedure can be applied in other laboratories with the same performances. A transfer of the procedure is needed to prove that the results of measurements are similar in all laboratories. Such transfer were achieved for very few procedures applied to the characterization of NMs[19,129,132,142,143]. For instance, procedures to characterise size and ZP of NMs by DLS and ELS respectively were transferred from one sending laboratory to other laboratories[142]. Two situations were considered. In the first case, the sending and receiving laboratories were equipped with the same measurement instrument (same wavelength of the laser source). A comparative test performed on the same batch of CRM or RM was proposed to show that performances of the receiving laboratories were similar to those of the sending laboratory taking into account handling precautions, crucial factor highlighted by the validation carried out by the sending laboratory and measurement quality criteria. In the second case, the sending and receiving laboratories were not equipped with the same instrument (different wavelength of the laser source). It was then suggested to perform a partial validation to prove the ability of receiving laboratories to perform the procedures. This partial validation was based on the study of the precision i.e. repeatability and intermediate precision and the trueness to assess the expanded uncertainties of the procedure. To achieve the transfer of a procedure, it is important that all partners carry on measurements of the same batch of CRM or RM. Results of measurements obtained by the different laboratories are compared using statistical analytical methods. A development of appropriate methods was proposed in the work of Varenne *et al.* based on the β-expectation tolerance interval method and ANOVA[142].

5. Conclusion

Main issues found for the characterization of NMs were considered in the present chapter. It discussed validation of analytical procedures based on metrology approaches to be applied to assess quality analysis of NMs. The reflexion associated basis in metrology and their application to method of characterization of the main physicochemical parameters that are used to define NMs. This analysis pointed out the urgent need to standardize, validate and transfer analytical procedures applied to characterize NMs. This is paramount to ensure reliability of results obtained from quality assessment of NMs which, in turn, is needed to ensure their safety providing proof of the repeatability and efficiency of industrial processes producing NMs-based products. Today, physicochemical characterization of NMs associated with metrology remains a challenge for future development in all application fields. Quality assessment of NMs is still in its infant age. Efforts are on the way to provide with more official guidelines to perform validation and transfer of measurement procedures and develop appropriate RM including CRM. Besides, several validated measurement procedures and results from interlaboratory measurement comparisons were published in the literature that can now serve as basis to go further setting up quality control procedures for NMs.

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