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Abstract

Particle size distribution (PSD) is an important factor determining the efficiency of industrial manufacturing processes for nanomaterials, assuring the reproducibility and safety of the final product. Among the instruments that have been developed to determine size and PSD of nanoparticle dispersions, the easiest to handle are based on indirect measurements; therefore, it is recommended to use at least two approaches to evaluate the PSD. This work evaluates the possibility of using direct size measurement methods based on the analyse of images of multimodal dispersion of nanomaterials by electron microscopy. Samples for measurement of the PSD were prepared by different deposition methods from a multimodal dispersion of poly(isobutylcyanoacrylate) nanoparticles. Grids prepared by flotation showed particle agglomeration and segregation between large and small particles and was found unsuitable for obtaining relevant measurement of the PSD. In contrast, spin-coating produced a homogenous and random deposition of well isolated

particles on the substrate used to prepare the samples for electron microscopy. This deposition method was suitable for evaluating the PSD of this highly heterogenous dispersion. Deposition strategies are therefore essential to provide a statistically representative sample for PSD measurement of nanomaterial-based products using a direct measurement method.

Key words

Particle size distribution, Single size measurement method, Microscopy, Flotation, Spin-coating, Metrology.

Abbreviations

AFM: Atomic force microscopy
CV: Coefficient of variation
DLS: Dynamic light scattering
EM: Electron microscopy
FFF: Field-flow fractionation
IBCA: Isobutylcyanoacrylate
ISO: International Organization for Standardization
NTA: Nanoparticle tracking analysis
PIBCA: Poly(isobutylcyanoacrylate)
PSD: Particle size distribution
SEM: Scanning electron microscopy
SLS: Static light scattering
TEM: Transmission electron microscopy
TRPS: Tunable resistive pulse sensing

1. Introduction

Among the many physicochemical parameters defining nanomaterials, particle size distribution (PSD) is frequently used to determine the repeatability and efficiency of industrial manufacture processes and their products [1]. PSD indicates the proportion of different particle sizes making up the product. In the field of nanomedicine, PSD is a key attribute of the nanomaterial. It influences the *in-vivo* fate and the biological activity and safety of this kind of pharmaceutical product [2–7]. In particular, the PSD of nanocarriers used

for drug delivery influences their pharmacokinetics, tissue distribution and clearance and, therefore, the control of PSD and the reliability of it evaluation are paramount for the effective clinical applications of drug delivery systems [3]. This is required not only to control batch to batch consistency of the process of fabrication of the nanomedicine but also to ensure the safety and efficacy of the formulation.

Various approaches can be used to evaluate PSD, including batch-mode or single particle size measurement and separative methods coupled with batch-mode particle size measurement methods as detector [8–12] (Table 1). Direct single particle size measurement methods are based on the analysis of images of nanomaterials and measure the size of each particle seen on the images. All other single particle measurement methods are indirect. They consist of measuring an intermediate property from which the size is deduced by the application of a known relationship or model. In contrast with batch-mode methods which provide an evaluation of the size of the whole population of particles composing the nanomaterial, single particle measurement methods perform a measurement on every nanoparticle in the sample. In order to give an accurate description of the sample, size measurements should be provided using direct single size measurement methods.

The determination of the particle size and PSD of dispersions of nanomaterials obtained in real-life situations may be challenging as pointed out by several studies comparing the relevance of measurement strategies applied to artificially prepared dispersions mixing particles of different sizes [13–16] or real dispersions of nanomaterials [8,17–19]. Batch-mode dynamic light scattering (DLS) is the most frequently used method in routine applications because affordable instruments are available on the market. However, although this method is suitable for evaluating the PSD of monomodal dispersions of nanomaterials [8,20,21] it does not accurately characterize polydisperse preparations. This is because the intensity of the light scattered by a particle in a nanomaterial is proportional to the power of six of the diameter, hence the intensity of the signal produced by the Brownian motion of the smallest nanomaterials may be hidden by that of larger particles. The evaluation of the PSD of a polydisperse dispersion can be dramatically biased as demonstrated in previous work [7,8,14,16,22,23]. Thus, it is recommended to determine PSD including either a single particle size measurement or a separative method coupled with batch-mode size measurement methods to determine the particle sizes [8,14].

Particle-by-particle or single particle size measurement methods include direct size measurement of particles from images obtained by electron microscopy (EM) and atomic force microscopy (AFM). These methods have been rarely used to measure the size and PSD of nanomedicine so far, although they have been widely applied for qualitative description of the morphology of particles composing nanomedicines. They can be applied to a wide range of materials within the nano and micrometer size range, in contrast to batch-mode and separative particle size measurement methods (Fig. 1). Direct measurements using EM and AFM can be applied to measure the PSD of nanomaterials with a wide range of compositions, as well as indirect single size measurement methods like nanoparticle tracking analysis (NTA) and tunable resistive pulse sensing

(TRPS). This is in contrast to the indirect single size measurement methods based on single-particle inductively coupled plasma-mass that are only applicable to metallic nanomaterials.

Measuring the size and PSD of nanomaterials by direct measurement methods is challenging. It consists of measuring the size of individual particles on relevant images of the nanomaterials obtained by EM or AFM as described in the standard 13322-1 of the International Organization for Standardization (ISO) [24]. Appropriate reference materials should be used to assess the quality of images defined as the number of pixels per nanoparticles and certified reference materials with a distribution expressed as the D90/D10 ratio above 1.5 should be used for qualification of the instrument [24].

The application of the ISO standard 13322-1 necessitates a random and representative deposit of particles on the sample holder for preparation of samples for EM and AFM [24]. This standard suggests practices for sample preparation including (i) the sample splitting and reduction (ii) the cleanliness of the dispersant liquid and requirements in terms of refractive index (particle-free, transparent and with a refractive index different from the refractive index of the particles for image contrast), (iii) the quality of particle deposition on the sample holder with a minimum number of nanoparticles touching others allowing size measurement on isolated nanoparticles to avoid introducing bias and (iv) the minimum number of nanoparticles that must be measured to obtain a statistically representative evaluation of the PSD. According to this last recommendation of the ISO standard, measurements should be performed on a minimum of one thousand particles, but this number is still under discussion. Recent studies have shown that the required number of measurements performed on single particles depends on the complexity of the PSD of the nanomaterials dispersion and the required level of accuracy of determination [24–27]. The preparation of the sample is a critical step of the measurement procedure. The sample should be sub-divided from the whole sample in a manner that ensures that the test sample is representative of the whole sample. Thus, during sample preparation, all nanoparticles should have the same probability of being deposited on the substrate in order to carry out relevant measurements of PSD by microscopy. The ISO standard 14488 that describes sampling and sample splitting practices can be followed to prepare suitable samples to be used in AFM and EM [28]. When a reference sample with similar PSD and flowability in comparison to the investigated nanomaterial sample is available, it should be used to evaluate the quality of the sample splitting/preparation method.

Careful sampling and sample splitting practices described in the ISO standard 14488 should be followed to measure PSD of nanoparticles [28]. The sequence of sampling and sample division result in a test sample for a defined bulk dispersion. The test sample should be as much representative as possible to that of the initial one. So, the method of preparation requires that all nanoparticles should have the same probability of being deposited on the substrate to carry out relevant measurements of PSD with microscopy.

For a defined dispersion of nanoparticles, there are critical size classes for which sampling error has a significant influence of the properties of the dispersion, according to the ISO standard 14488. The total error of sampling and sample splitting corresponds to the combination of the fundamental error (independent of the discrete nature of nanoparticles with different nanoparticles) and the segregation error (related to the

degree of segregation, systematic deviation between the provided PSD for identical dispersion). The dispersion of nanomaterials should be stable (no sedimentation of nanomaterials) during the sampling. The sample splitting can be performed by various methods, including sample splitting by pipette or using multiple capillary tubes. The quality of the sample splitting should be validated using appropriate reference material with similar PSD and flowability compared to the investigated nanomaterials.

Preparation of samples of nanomaterials for EM can be achieved by different methods. The easiest ones that are generally used for nanomedicines consist in direct deposition of an aliquot of the dispersion on the substrate or on the flotation of the substrate over a drop of the dispersion of nanomaterials. No specific material or training is required. Samples prepared by these methods are generally suitable for a qualitative observation of nanomedicines. The high quality of samples required to perform size and PSD determination by EM is rarely obtained in this way and more sophisticated deposition methods should be used to obtain a uniform and thin-layer deposit. For example, the spin coating method is increasingly used to prepare samples of nanomaterials for microscopic observations when simpler methods are unsuitable. This method was originally developed for rigid substrates such as silicon or mica as used for SEM (scanning electron microscopy) or AFM but has not yet been adapted to prepare grids for transmission electron microscopy (TEM). Special equipment is required with which the sample deposition is achieved by the action of a centrifugal force. As well as producing a deposit of well individualized particles on the sample holder, the deposit must also be representative of the PSD of the whole sample. It is noteworthy that particle sizes evaluated by SEM would be close to those of the real diameter of the particles contained in the dispersion.

No major difficulty was reported out in the literature considering dispersion samples with a low polydispersity index. A few studies have assessed the uncertainty of size measurement by EM with reference materials with narrow or broad size distribution [26,29,30]. The work of Dudkiewicz *et al.* mentioned that the number of nanoparticles counted was a minor source of measurement uncertainty in comparison with the source issuing from the sampling, the preparation of the sample and the analysis of the images [26]. The preparation of the samples is a crucial step to provide a statistically representative sample of the dispersion of nanoparticles; hence EM requires a well dispersed nanoparticle population on the substrate. However, these studies were devoted to the analysis of monomodal dispersions of nanoparticles [26,29,30].

In contrast, segregation in size was observed with polydisperse dispersions; this being a major issue leading to biased measurement of PSD of the corresponding dispersions of nanomaterials. In practice, the absence of bias in the evaluation of PSD by direct measurement methods should be assured by the use of appropriate reference materials according to usual best practice described in the ISO standards [24,28]. Most available standards for EM are designed to be used with monodisperse dispersions. For polydisperse dispersions of nanoparticles, only one reference material is available, consisting of a bimodal dispersion of SiO_2 certified at 18.2 and 84 nm by SEM (provided by Joint Research Centre, ERM-FD102). It can be used to validate the method applied to a narrow range of nanomaterials of similar size and nature, which is highly insufficient considering the tremendous diversity of nanomaterials. For instance, this reference material is not

appropriate to validate the methods applied to nanomedicines composed of liposomes, lipid nanoparticles and polymeric nanoparticles. Another approach based on an orthogonal measurement of the size and size distribution with other methods is needed to validate the evaluation of the size and PSD of a heterogeneous dispersion of such nanomaterials using a direct measurement method. The only available approach will be to use data obtained from indirect methods to validate those obtained by the direct size and PSD measurement method: in contrast to the usual procedures suggested by Caputo *et al.* [4]. This will be the only strategy able to prove that the deposit of nanoparticles is random and thereby the relevance of the deposition methods employed. It is noteworthy that the ISO standard 13322-1 suggests investigating the accuracy of the developed method for size measurement using microscopy [24].

Faced with the difficulty of performing size and PSD measurements on polydisperse nanomaterials using direct methods, the present work was designed to evaluate the potential of two methods of sample preparation for EM for their ability to provide relevant measurements of a polydisperse dispersion of taken from the large family of poly(alkyl cyanoacrylate)- based nanomedicines [31–33] and to propose an approach to validate the adopted strategy. The dispersion was composed of dextran-coated poly(isobutylcyanoacrylate) (PIBCA) nanoparticles of different sizes as reported in a work in which their size was determined by different methods [8]. In this work, samples were prepared with a simple deposition method by flotation for TEM analysis and using the spin coating method applied to the preparation of samples for SEM. The relevance of the results of size and PSD determination were explored by comparing the results obtained from the analysis of the electron micrographs with the results of measurements performed with indirect methods including TRPS and NTA.

Table 1. Overview of size measurement methods [8–12].

Method	Measurand	Produced PSD
Batch		
Acoustic techniques	Volume-based diameter	Volume-based PSD
Dynamic light scattering (DLS)	Hydrodynamic diameter	Scattering intensity-based PSD[a]
Static light scattering (SLS)	Gyration diameter (Rayleigh)	Scattering intensity-based PSD
Small-angle X-ray scattering	Gyration diameter (Guiner)	Scattering intensity-based PSD
X-ray diffraction	Scherrer's diameter	No PSD
Single		
Electron microscopy (EM)[b]	Equivalent spherical diameter (area-equivalent diameter) or Feret's diameter	Number-based PSD
Atomic force microscopy (AFM)[b]	Height or diameter issue from the analysis of images in (x-y) dimension	Number-based PSD
Nanoparticle tracking analysis (NTA)[c]	Hydrodynamic diameter	Number-based PSD
Tunable resistive pulse sensing (TRPS)[c]	Raw diameter	Number-based PSD
Single particle inductively coupled plasma-mass spectrometry[c]	Height of intensity of detected pulse	Mass-based PSD
Electrospray-differential mobility analysis[c]	Mobility diameter	Number-based PSD
Separative		
Analytical ultracentrifugation	Sedimentation diameter	Density-based PSD
Capillary electrophoresis	Apparent mobility (or electrophoretic mobility)	Depending on detection method[d]
Differential centrifugal sedimentation	Sedimentation diameter	Extinction intensity-based PSD
Field-flow fractionation (FFF)	Retention time	Depending on detection method[d]
Hydrodynamic chromatography	Sedimentation diameter	Depending on detection method[d]
Size exclusion chromatography	Retention time	Depending on detection method[d]

[a]Scattering intensity-based PSD can be transformed into volume or number-based PSD if the optical properties of the nanomaterials (nanomaterial refractive index and absorption) are known. [b]Direct method. [c]Indirect method. [d]The most frequently used detection methods are batch-mode DLS and SLS (see corresponding methods described above).

[insert Fig. 1]

2. Materials and methods

2.1. Materials

Isobutylcyanoacrylate (IBCA) was obtained from Orapi, dextran (69 kDa) and NaCl (purity ≥ 99.5%) from Sigma-Aldrich, cerium (IV) ammonium nitrate and nitric acid (purity within the range from 61.5 to 65.5 %) from Prolabo; perchloric acid and ethanol were purchased from Fisher Chemical and acetone from Acros Organics. All chemicals were used as purchased. Aqueous solutions were prepared with ultrapure water provided by a Millipore water system. This system also provided deionized water.

2.2. Methods

2.2.1. Preparation of poly(isobutylcyanoacrylate) nanoparticles coated with dextran

The dispersion of nanoparticles used on this work was prepared by emulsion polymerization of IBCA. following the procedure described in Varenne *et al.* [8]. Briefly, the polymerization medium composed of 9.3 mL solution of dextran (0.5%) in aqueous nitric acid 0.2 M was heated to 40°C and purged with argon for 10 min. The polymerization was initiated by adding 0.7 mL of cerium (IV) ammonium nitrate (8.10^{-2} M) in 0.2 M aqueous nitric acid and 0.5 mL of IBCA under vigorous stirring. The polymerization was pursued for 1 hour. After cooling in an ice bath, the dispersion was purified by dialysis against deionized water (SpectraPor® membrane with MW 100 kDa, Spectrum Laboratories). Dispersions with a concentration in nanoparticles at 51 ± 1 $mg.mL^{-1}$ were obtained as determined by gravimetry. They were stored at + 4°C until use. Further details on the preparation of the dispersion can be obtained in our previous work [8,34].

2.2.2. Transmission electron microscopy

2.2.2.1. Sample preparation

The dispersion of nanoparticles was diluted in ultrapure water at two concentrations of nanoparticles: 100 and 400 $\mu g.mL^{-1}$, to investigate the effect of the concentration on the quality of the deposition of these nanoparticles on grids. Nanoparticles were deposited on formvar/carbon coated cupper grid for electron microscopy purchased from Agar Scientific. Grids were floated over a drop of the diluted sample of

nanoparticles for 3 min. The excess of liquid was left to dry in the air in a dust-free space. When specified, the grids were then stained with phosphotungstic acid (1%) by flotation for 30 s. The excess of staining solution was blotted with a filter paper and the grid was left to dry in a dust-free place prior introduction in the electron microscope.

2.2.2.2. Measurements

TEM images were acquired with a JEOL JEM1400 electron microscope operating at 80 or 120 KeV upon indications given in the legends of the figures. Images were recorded with a camera ORIUS SC1000-A2. The analysis of the sample was performed by a systematic scan of the surface of the sample holder.

2.2.3. Scanning electron microscopy

2.2.3.1. Sample preparation

The sample preparation method used in this work was described by Delvallée *et al.* [35] for SEM measurements of gold and silica nanoparticles deposited on silicon substrates. This method is suitable for nanoparticles and substrates with different chemical natures. The nanoparticle dispersion was diluted with ultrapure water to reach a concentration in nanoparticles of 1 $mg.mL^{-1}$. Then, a volume of 100 µL of perchloric acid was added to 5 mL of diluted dispersion in order to reach an acid pH (pH = 2.2). The acidification was made to change the particle charge and hence to promote their adhesion on the substrate used for imaging with SEM. The dispersion was then sonicated for 20 min using a Bioblock Scientific Vibracell 75043 ultrasonication probe in a pulsed mode including cycles of 10 s ON/30 seconds OFF with an amplitude of 20 % to break down any remaining particle agglomerates. The probe was directly plunged into the dispersion. To limit the increase of the temperature of the dispersion during sonication, the bath with the probe and the tube containing the dispersion were constantly cooled with ice.

The silicon wafer (Agar Scientific) used as substrate was cleaned prior deposition of the nanoparticle samples. It was submersed for 20 min in acetone placed in an ultrasonic bath, followed by 20 min in ethanol placed in an ultrasonic bath (power: 75 W). Sample deposition on the clean and dried wafer was then achieved using a spin coater LabSpin 6 SUSS Microtec apparatus, applying the protocol recommended for aqueous dispersions of nanoparticles. The first step included a spreading phase performed with a rotational speed of 1000 rpm. for 1 min for aqueous solvent. The second step was a drying phase performed with a rotational of 8000 rpm for 10 s.

2.2.3.2. Measurements

SEM images were acquired with a Zeiss ULTRA-Plus equipped of a Field Emission Gun (FEG) microscope and in-Lens SE detector. All images were carried out through secondary electrons collected by InLens detector at a voltage of 2 kV and with a working distance equal to 4 mm (the calibration is checked and readjusted each year during the maintenance of the instrument by the supplier (Zeiss)). In these working conditions, the resolution of the microscope was 1.7 nm according to the supplier. The size of pixel of the images used to measure size and PSD (images at higher magnification) was 1.861 nm. Image analysis was performed with a specific semi-automatic Matlab routine developed in previous work [35]. This program allows the user to measure and count of exclusively isolated nanoparticles through a control interface. Each nanoparticle was segmented by means of « Active Contour » of Matlab. Each segmented nanoparticle was checked by the user to eliminate artifacts (dust, impurities, …) present on the sample holder and detected through the segmentation method. The source of uncertainty was the binarization of the image permitting to highlight the edges of nanoparticles (the diameter provided by this approach was different of 5.8% of simulated diameter of particles (deposit of particles consisting in adding gaussians of random diameters on plane substrate) as described by Delvallée [36]). The measurand for SEM measurements was the area-equivalent diameter of the particles. The mean value, σ value which corresponds to the width of the distribution around the mean, modal value and D-values such as D10, D50 and D90 according to the ISO standards 14488 and 9276-2 [28,37] were determined from the measurement of three PSD issued from the analysis of the same deposition. The three PSD were evaluated with three different sets of 327, 327 and 326 individual nanoparticles by performing a systematic scan of the surface of the sample holder. The evaluation of PSD was performed by measuring all nanoparticles from a series of images at different position selected randomly on the sample holder to provide a representative PSD of the nanoparticles. The number of measured nanoparticles per image was about 100. The uncertainty of size measurements by SEM was assessed by evaluating the repeatability (degree of scatter between a series of size measurements of the same dispersion of nanoparticles performed by the same analyst using the method with the same instrument in the same laboratory for a short period i.e. successive measurements [38–40]) of the proposed method for which samples were prepared with spin-coating method.

2.2.4. Atomic force microscopy

2.2.4.1. Sample preparation

A freshly cleaved mica plate was prepared to serve as the sample holder for AFM. Nanoparticle dispersions were adjusted to a concentration at 100 µg.mL^{-1} by dilution with ultrapure water. 100 µL of diluted dispersion were deposited on freshly cleaved mica plate and left to dry for 18 hours in a dust-free environment at ambient temperature and humidity.

2.2.4.2. Measurements

The AFM instrument used to observe the dispersion was a JPK nanowizard® Ultraspeed AFM from JPK instrument. Operating conditions were the following: force (80 - 90 % of free amplitude), cantilevers with tip curvature radius of ~ 10 nm (gold coating silicon PPP-NCHAuD from Nanosensors), spring constant: ~ 42 $N.m^{-1}$. Images were analysed using the JPK Data processing software (JPK instrument). PSD based on the evaluation of the widths determined at the full width at half maximum of height of peak provided by line profile measurement option to reduce the convolution effect on lateral size of nanoparticle and PSD based on the height of nanoparticles were determined from the measurement of 393 nanoparticles. The mean value, σ value, modal value and D10, D50 and D90-values were selected to characterize the provided PSD as suggested by the ISO standards 14488 and 9276-2 [28,37].

2.2.5. Other size measurements

All PSD were characterized by mean value, σ value, modal value and D-values such as D10, D50 and D90 according to the ISO standards 14488 and 9276-2 [28,37].

2.2.5.1. Tunable resistive pulse sensing

Size measurements were performed with a qNano instrument (Izon). The instrument was calibrated using polystyrene particles with a modal size of 115 nm and a concentration of 1.2 x 10^{13} $particles.mL^{-1}$ from Thermo Fisher Laboratories. Samples were diluted at a concentration of 51 $\mu g.mL^{-1}$ with phosphate buffer saline containing 0.03% of Tween 20 (Izon solution Q). PSD was evaluated with Izon Control Suite Software v 3.2 (Izon) on 545 nanoparticles.

2.2.5.2. Nanoparticle tracking analysis

Size measurements were carried out using a Nanosight NS300HSB instrument (Malvern) operating with a sCMOS camera, a laser source of wavelength of 405 nm, a syringe pump and the Software NTA v 3.0. Measurements were carried out under a regular flow to track all nanoparticles during the analysis. Samples were diluted at a concentration of 51 $ng.mL^{-1}$ with filtered ultrapure water (0.22 µm filter, Roth).

2.2.5.3. Batch-mode dynamic light scattering

Size measurements were performed with a Zetasizer Nano ZS (Malvern) with a fixed scattered angle of 173° using Zetasizer Software v 7.04. The wavelength of the laser source was 633 nm. Samples were diluted at a

concentration of 256 µg.mL^{-1} with filtered ultrapure water through 0.22 µm filter (Roth). 1 mL of samples was introduced into macrocuvettes with four optical faces (VWR) and placed in the instrument for equilibration time of 300 s to reach the temperature of measurement. All conditions for sample preparation and measurements were in accordance with our previously validated procedures [20,21]. Size measurements were performed in triplicate at 25°C.

2.2.5.4. Asymmetrical Flow Field-Flow Fractionation combined with batch-mode dynamic light scattering as detector

The size of nanoparticles was analysed using an asymmetrical flow field flow fractionation system (Eclipse 2 System, Wyatt Technology) combined with a detector based on dynamic light scattering equipped with a laser source of 633 nm and operating at a fixed scattered angle of 99° (18 angles multi-angle light scattering instrument DAWN-HELEOS II, Wyatt Technology). The channel was fitted with a 350 µm spacer and a regenerated cellulose membrane (cut-off of 10 kDa, Wyatt Technology). The carrier liquid consisting of deionized water containing 0.02% of sodium azide (Sigma-Aldrich) filtered with a vacuum filtration system (filter of 0.1 µm, Gelman) was delivered by an Agilent 1100 Series Isocratic Pump (Agilent Technologies). Samples were diluted at 510 µg.mL^{-1} with carrier liquid and mixed by means of a vortex at 2500 rpm for 10 s before analyse (Scientific Industries). Experimental conditions were as described in previous work [8]. The system was controlled by means of the Software Astra v 5.3.4.20.

2.2.6. Evaluation of zeta potential of PIBCA nanoparticles in different media

Zeta potential evaluation was performed with a Zetasizer Nano ZS (Malvern) equipped with a laser source of wavelength of 633 nm and operating at a scattered angle of 13° with Zetasizer Software v 7.04. Samples were diluted to 1 mg.mL^{-1} in an aqueous solution of 1 mM sodium chloride filtered through 0.22 µm filter (Roth) or to 102 µg.mL^{-1} in medium used for deposition of sample on silicon substrate for SEM size measurements. Samples was introduced in measurement cells (DTS 1070 from Malvern) and placed in the instrument for equilibration time of 300 s to reach the temperature of measurement. All conditions for sample preparation and measurements were performed according to our previously validated procedures [41]. Measurements were performed in triplicate at 25°C.

3. Results and discussion

The aim of this work was to evaluate the measurement of the PSD of a nanomaterial dispersion composed of polymeric nanoparticles that are polydisperse in size using direct measurements. There is a debate about the

number of particles that needs to be measured to obtain statistically representative and accurate size and PSD measurements [24–27]. However, the quality of the measurement is also greatly influenced by the quality of the sample, which in turn depends on the deposition of particles from the dispersions during sample preparation. A relevant evaluation requires that the particles are be homogenously distributed on the substrate without segregation according to their size. The distribution of the particles should represent the PSD of the whole sample, otherwise the size average and PSD will be biased. Reports in the literature have stressed that these two requirements may be difficult to fulfil for nanoparticles of heterogenous size [4,8,26]. Two methods of sample preparation of EM were used in this work. The first was an easy and very simple method based on the flotation of the TEM grid on a drop of the dispersion. The second was more sophisticated, consisting in spreading the dispersion as a thin layer on a substrate for SEM using a spin coating apparatus. With this second method, samples are generally prepared in acid media to promote the adhesion of the particles to the silicon wafer substrate. These acidic conditions were compatible with our sample of PIBCA nanoparticles based on their remarkable stability in the even more severe acid conditions used during synthesis and in experimental procedures described in previous works [42–45]. These nanoparticles are sterically stabilized mainly by dextran molecules forming a corona around the PIBCA core and did not show a dramatic change of zeta potential after dilution in the medium used for spin coating. The value of zeta potential was close to neutral in the perchloric acid medium (- 2.1 mV) and to that measured in NaCl 1 mM (- 6.1 mV). The sample was immediately deposited on the silicon substrate after acidification to prevent destabilization of the dispersion and the formation of agglomerates. It is noteworthy that measurements are performed on projected images resulting from the reflexion of electron by particles providing area-equivalent diameter of the particles with SEM. Particle diameters evaluated by SEM would be close to those of the real diameter of the particles contained in the dispersion.

Images of PIBCA nanoparticles contained in our polydisperse dispersion obtained from samples prepared by the flotation deposition method for TEM showed at least two populations based on the particle size (Fig. 2). Most particles were in clusters showing segregation between small and large particles for concentrated samples at 400 $\mu g.mL^{-1}$ (Fig. 2 (a) and (b)). At a lower concentration (100 $\mu g.mL^{-1}$), clusters of much smaller size and more individual particles could be observed but a clear segregation of the particles according to their size can be detected (Fig. 2 (c) and 2 (d)). The quality of samples obtained by the flotation method was unsuitable for measuring size and PSD. Firstly, the obvious segregation of the particles according to their size on the sample holder prevented a relevant determination of the PSD. Secondly, the size of particles cannot be measured within agglomerates as this can lead to errors, according to Delvallée *et al.* [35]. We also noted that many particles in clusters were distorted compared with the morphology shown by isolated particles. The projected images of well individualized particles showed circular objects (Fig. 2 (e)) but those of particles in clusters appeared distorted (Fig. 2 (f)). This can be explained by the composition and structure of the nanoparticles with a corona of a dextran hydrogel surrounding the PIBCA core. During the sample preparation procedure, the drying of this layer of hydrogel may not occur

symmetrically around the core of the nanoparticle when they are in clusters, leading to particles appearing not perfectly spherical as is the case when the particles are fully individualized. Image analysing software would not take into consideration this distortion, which can be identified as another source of error when attempting measuring the size and PSD of particles on such a sample. Such deformation does not occur with all types of particles, for example metallic nanoparticles would not show this, allowing analysis of the size of the particles in clusters with standard software, although this would not be recommended as it can be a source of error [35]. In contrast, and in agreement with the work of Delvallé *et al.* [35], we would not be able to evaluate nanoparticles with current softwares, neither the size of PIBCA nanoparticle nor their size. For this nanoparticle dispersion, TEM images obtained from samples prepared by flotation of the grid can be used for qualitative analysis of the morphology of the particles provided that they are well individualized. Furthermore, it revealed the complex PSD of the dispersion on a qualitative basis.

Representative images of particles of the dispersion obtained by SEM from sample deposited by spin-coating are presented in Fig. 3 (a). There was no clustering of nanoparticles on the image since the dispersion of PIBCA nanoparticles was immediately deposited on the silicon substrate after the acidification of the medium and nanoparticles appeared well individualized. No apparent segregation between small and large nanoparticles was observed, suggesting that the deposition of nanoparticles was random. The quality of the deposition appeared suitable for measurement of PSD. The three PSD given in Fig. 3 (b) were derived from the analysis of the same deposition from a systematic scan performed on different areas of the sample holder. These PSD were measured from the measurement of the size of 327, 327 and 326 individual nanoparticles. They showed an asymmetric gaussian shape with a major population at 53.7 ± 0.9 nm. Secondary peaks appeared between 85 and 220 nm and minor populations appeared between 250 and 350 nm. The shape of the PSD was consistent with those provided by previous measurements performed by single size measurement methods [8].

The performance of the method of measurement of PSD could not be evaluated according to usual best laboratory practice. This requires a suitable reference material which would be a dispersion containing particles with a high polydispersity in size, preferably in the size range shown by the sample. Most available standards that can be used for EM are samples of particles monodispersed in size. The only available reference material that includes a polydisperse dispersion of nanoparticles consists in a bimodal dispersion of SiO_2 certified at 18.2 and 84 nm with SEM (provided by Joint Research Centre, ERM-FD102). The population at 84 nm is in a minority compared with the population at 18.2 nm. With a simple PSD compared with that of our dispersion and a factor of 5 between particle sizes of the two populations, this reference material is unsuitable to evaluate the performance of the method used to determine the PSD of our dispersion of PIBCA nanoparticles.

In the absence of appropriate reference material, another strategy must be applied to assess the performance of the size measurement method by SEM applied to the determination of the PSD of the PIBCA nanoparticle dispersion considered in the present work. The quality of deposits on substrate is paramount for this

evaluation. Thus, PSD from the analysis of deposits obtained with various deposition methods should be compared to each other and to those produced with other direct or indirect single size measurement methods to highlight the relevance of deposition methods.

The mean value, σ value, modal value and D-values characterizing the distribution widths obtained for each set of nanoparticles counted for SEM measurements and for other size measurement methods are given in Table 2. The repeatability that corresponds to the degree of scatter between a series of size measurements of the same dispersion of nanoparticles under prescribed conditions was evaluated by three successive measurements of PSD on the same sample for the spin-coating method combined with SEM. To establish each PSD, some images were analysed at a randomly selected position on the sample holder to carry out the size measurements hence to provide a representative size distribution of particles. The pool of nanoparticles counted was similar between the three established PSD. As shown by the low coefficient of variation (CV) close to the all values, the measurement of the PSD of the dispersion of PIBCA nanoparticles was repeatable. Thus, the nanoparticles were randomly dispersed on the sample holder. This also indicated that the number of nanoparticles counted of 327 was sufficient to establish a repeatable PSD of the dispersion of the PIBCA nanoparticles.

The principal source of uncertainty of size measurements by SEM is the width of the electron beam. This contributes to 90% of uncertainty assessment as described by Crouzier *et al.* [46]. The total uncertainty was determined by combining the uncertainties of type A closed to the repeatability (7.2 nm for mean value) and uncertainties of type B that included all other sources of uncertainties such as the size beam (1.7 nm to which a contribution of 10% is added corresponding to the other sources). The contribution of uncertainties of type B was estimated at 2 nm. Thus, the total uncertainty was 7.5 nm. It is noteworthy that any threshold for the total uncertainty of the measurement is mentioned in the ISO standards [24,28]. Considering the work of Dudkiewicz *et al.* [26], the relative uncertainties of repeatability and day-to-day variation can be fixed at 10 and 20% respectively and the expanded uncertainty depends on the relative standard of intermediate precision (combining the relative uncertainties of repeatability and day-to-day) and the instrumental calibration. The uncertainty associated with our approach was consistent with the work of Dudkiewicz *et al.* [26].

Table 2. Mean value, σ value, modal value and D-values of distribution provided by size measurement methods.

Method	Counted nanoparticles	Mean (nm)	σ[b] (nm)	Modal[a] (nm)	D10[c] (nm)	D50[d] (nm)	D90[e] (nm)	Span[f]
Batch								
DLS 173° (volume-based PSD)	na	193	75.8	190	108	179	302	1.08
DLS 173° (number-based PSD)	na	135	47.0	106	87.8	122	200	0.92
Single								
AFM (width)[g]	393	138	65.9	95.0 [8]	87.6	122	196	0.89
AFM (height)	393	68.1	49.2	55.0	33.8	51.9	72.3	0.74
SEM	327 (set 1)	90.5	78.0	52.6	31.3	53.4	203	3.22
	327 (set 2)	85.2	73.0	54.4	31.1	45.9	198	3.63
	326 (set 3)	76.3	70.5	54.0	29.1	43.4	189	3.68
	Average	*84.0 ± 7.2*	*73.4 ± 3.8*	*53.7 ± 0.9*	*30.5 ± 1.2*	*47.6 ± 5.2*	*197 ± 7*	*3.51 ± 0.25*
	Repeatability	*CV = 8.5%*	*CV = 5.2%*	*CV = 1.8%*	*CV = 4.0%*	*CV = 11%*	*CV = 3.6%*	*CV = 7.2%*
	980 (sets 1 to 3)	84.0	74.0	54.3	30.6	46.6	201	3.66
NTA	n.s.	87.8	31.7	72.5	55.9	79.2	124	0.86
TRPS	545	135	n.s.	102	97.0	123	190	0.75
Separative								
FFF (coupled to DLS 99°)[h]	na	128	n.s.	91.5 [8]	77.9	101	202	1.23

[a]Modal: nanoparticle size most commonly found in the distribution. [b]Width of the distribution around the mean. [c]D10: 10% of nanoparticle size below this value. [d]D50: 50% of nanoparticle size below this value. [e]D90: 90% of nanoparticle size below this value. [f]Span is defined as (D90 - D50)/D10. [g]No nanoparticle with size below than 60 nm indicating that no artifact from the tip (tip curvature radius of ~ 10 nm) was observed in the AFM images. [h]Differential number fraction. na: not applicable. ns: not specified.

In the absence of a suitable standard, the performance of a given measurement method can be assessed by comparing the results with those obtained from the analysis of the same sample by other methods measuring PSD. In previous work, we have compared the size distribution profile obtained from measurements of the same dispersion using different methods with the aim of identifying methods able to decrypt the complex polydispersity of our PIBCA nanoparticle dispersion [8]. Relevant methods could be identified, including a direct measurement method based on AFM, indirect methods based on single particle measurements such as NTA and TRPS and FFF that includes a separative method coupled with a detector that measures the size by a batch-mode measurement method. According to this study, these methods were suitable for determining the size and PSD characteristics of our sample of PIBCA nanoparticles, but the quantitative analysis of the distribution curves was not complete. The full quantitative analysis of the distribution curves was achieved during this work to provide experimental data on size and PSD parameters of our dispersion obtained by different methods that could be compared with results obtained from the direct measurement performed here by SEM. Corresponding original data of size and PSD are included in Table 2.

The results obtained here from the analysis of images of the dispersion by SEM can also compared with those of the full analysis of the distribution curves obtained in a previous work to provide an orthogonal size measurement on the same dispersion by another analytical method. In this previous work, the PDS was evaluated by AFM, another direct method for the evaluation of PSD by measuring the width of nanoparticles. The widths were measured manually at the full width at half maximum of the peak height to reduce the convolution effect. No nanoparticle with a size below than 60 nm was observed, indicating that no artifact from the tip (tip curvature radius of ~10 nm) was measured in the AFM images. Indeed, this lateral diameter is larger than the real particle diameter because of the introduction of an unavoidable bias related to the convolution between the tip of the AFM apparatus and the particle that is measured [47]. An asymmetric gaussian distribution showing a similar shape was obtained with major population appearing at 95.0 nm, secondary peaks appearing at 135, 150, 175 and 195 nm and minor populations observed at 245, 275, 335, 445, 505, 550 and 575 nm [8]. The PSD was also established from the height of the nanoparticles. An asymmetric gaussian distribution showing a similar shape was obtained with a major population appearing at 55.0 nm, a secondary peak appearing at 95.0 nm and minor populations observed between 125 and 395 nm (Fig. 4 (a) and Table 2). The shape of the distribution was similar to the one obtained by width of nanoparticles. However, the modal size was shifted. This can be attributed to the compaction of the dextran corona. The gaussian curves obtained by SEM showed a similar asymmetric shape to the one obtained by AFM (width and height), NTA (Fig. 4 (b)), TRPS and FFF in our previous work [8]. This complex size distribution was also revealed from analysis performed by single particle size measurement method, including TRPS and NTA, and methods based on separation prior to size measurement by a batch-mode DLS method. Because the different methods yield different measurands, the absolute value obtained for the size cannot be compared directly [29,48]. This is the case even for a single method performing the

analysis by different means. For example, two values were obtained for the modal diameter determined by AFM depending on the method of analysis of the images of the particles.

In contrast, a comparison of the different parameters evaluating the PSD of the dispersion by the span of the values can be made. It is noteworthy that they were mostly consistent between AFM (height and width), NTA, TRPS and FFF. The data obtained from these methods support the relevance of the data obtained from measurements performed by SEM. The span close to the PSD provided by SEM was higher, highlighting the complexity of the PSD of the dispersion of PIBCA nanoparticles. The span values close to the distributions provided by DLS were also close to those obtained for AFM (height and width), NTA, TRPS and FFF. However, the shape of the number-based PSD is given in Fig. 4 (c). This PSD showed one single population that did not reflect the complexity of the PSD.

The distribution and particle size deduced from analysis of SEM images was consistent with those derived from measurements performed with other direct size measurement methods such as AFM (width and height) and indirect methods including TRPS, NTA and FFF coupled with batch-mode DLS [8]. This indicated that the deposit of nanoparticles obtained by spin coating on the sample holder used in SEM was random. Regarding the determination of the PSD by SEM, the number of nanoparticles counted of 327 was sufficient to establish a statistically representative and accurate measurement. The PSD obtained could be determined in a repeatable manner while the total uncertainty was within an acceptable range. Thus, counting a number of around 300 nanoparticles was sufficient to establish a statistically representative and accurate PSD giving similar PSD to that evaluated from the measurement of almost one thousand nanoparticles as recommended in the ISO 13322-1 [24] (Table 2). A similar observation was made in a previous work evaluating the PSD of a dispersion of synthetic gold nanoparticles showing moderate complexity including one population with a broad size distribution by Song *et al.* [25]. Together, those results suggest that relevant measurements of the size and PSD of a dispersion of nanomaterials can be achieved by a direct method of size measurement measuring a much lower number of particles than that required in the ISO 13322-1 [24] even when the particle size distribution of the dispersion is broad or rather complex.

The main difficulty found during this work was achieving a suitable deposition of the particles of the dispersion on sample holders used for TEM or SEM when determining the PSD of our polydisperse dispersion of dextran-coated PIBCA nanoparticles with a direct size measurement method. The main difficulty was avoiding segregation by size that was observed with our sample but cannot occur with monodisperse dispersions of nanoparticles. The easy-to-use sample preparation method by flotation to prepare grids for TEM was unsuitable, leading to agglomerated particles and a segregation of the particles according to their size. In contrast, spin coating used to prepare samples for SEM provided a sample with suitable characteristics for determining the PSD of the dispersion by SEM, as shown by the consistency of the results obtained by SEM with those produced by measurements of the same dispersion by other methods [8]. The spin-coating method was developed to deposit nanoparticles on rigid substrates such as silicon or mica for size measurement by SEM or AFM. This method has not yet been developed with grids used to

prepare samples for TEM. Although more sophisticated than other deposition methods, it is highly adaptable, and conditions can be tuned to prepare a suitable sample without formation of clusters and segregation between small and large nanoparticles. The spin coating method could be a fairly universal method applicable to all polymer-based nanoparticles. However, the conditions of its application will not be universal and will require adapting to the type of nanomaterial. For example, the pH of the medium in which the nanoparticles are dispersed will need to be adjusted to promote adhesion of the samples. Also, operating conditions of the spin coating itself will need to be tuned to obtain the required quality of sample. The random deposition of the particles on the sample holder could be confirmed from the results obtained from three sets of size measurements on different areas of the sample holder which yielded similar results of mean diameter and PSD. In the light of the present results, this method could be proposed as a general method for the preparation of nanomaterial samples for size measurements performed by SEM and probably also by AFM, but conditions for the preparation of the sample might need to be adapted according to the nature of the dispersion, heterogeneity of density of the particles and sample holder used. The success of the deposition method may also depend on the nature of the polydispersity of the particles composing the nanomaterial. The deposition method may also affect or induce fragmentation of agglomerated nanoparticles. It should be investigated with nanoparticles that are known to be agglomerated.

[insert Fig. 2]

[insert Fig. 3]

[insert Fig. 4]

4. Conclusion

The evaluation of statistically representative and repeatable PSD of unknown dispersions of nanomaterials such as nanomedicines is urgently needed to ensure the biological activity and safety of these formulations. This work has pointed out some difficulties linked to sample preparation to achieve size measurement of nanomaterials, including nanomedicines, by direct measurement methods. Obtaining a randomly distributed and representative deposition of the particles on the sample holder for electron microscopy was challenging and was not achieved using the simplest methods of sample preparation for EM observations. The spin coating method, which can be used to prepare samples for SEM or AFM, was found suitable for preparing a sample in which the particles of the dextran-coated PIBCA nanoparticles considered in this work were well individualized and randomly spread according to their size on the sample holder. The relevance of the deposition method was verified from the reproducibility of the results obtained between measurements

performed on different areas and the comparison of the results obtained from this method with those obtained by other direct methods or indirect size measurement methods such as NTA and TRPS as part of a metrological approach. The strategy proposed in this work could be proposed as part of best practice and metrological approach, including validation developed in the ISO standard 13322-1 in the absence of appropriate reference material. The spin coating method might be useful and would be worth trying when a segregation of particles according to their size is suspected on preparing samples for EM by easier methods.

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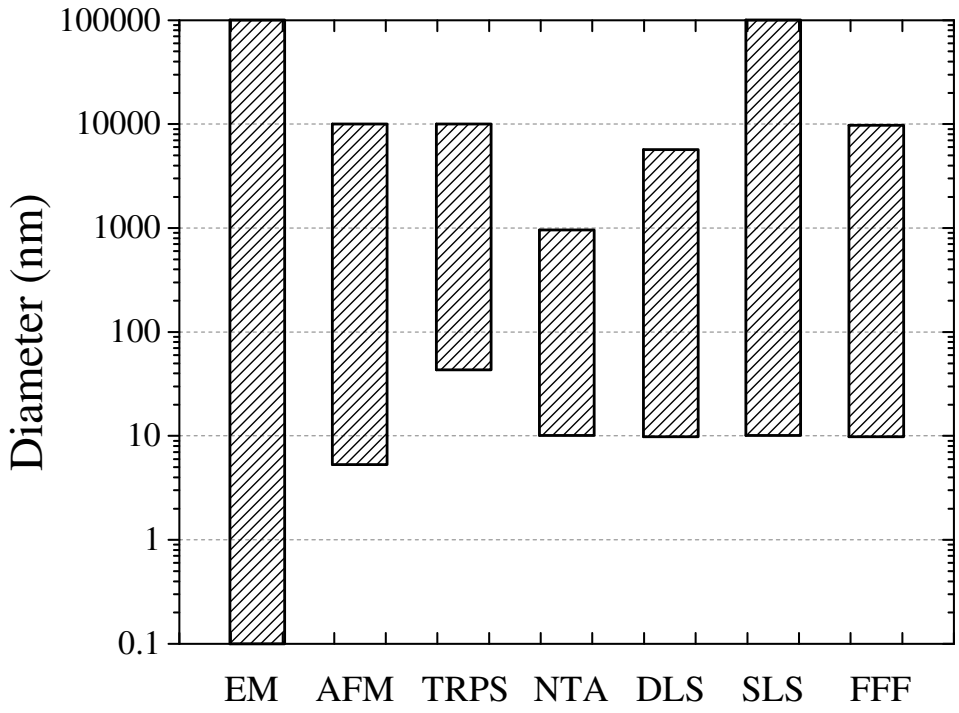
Figures

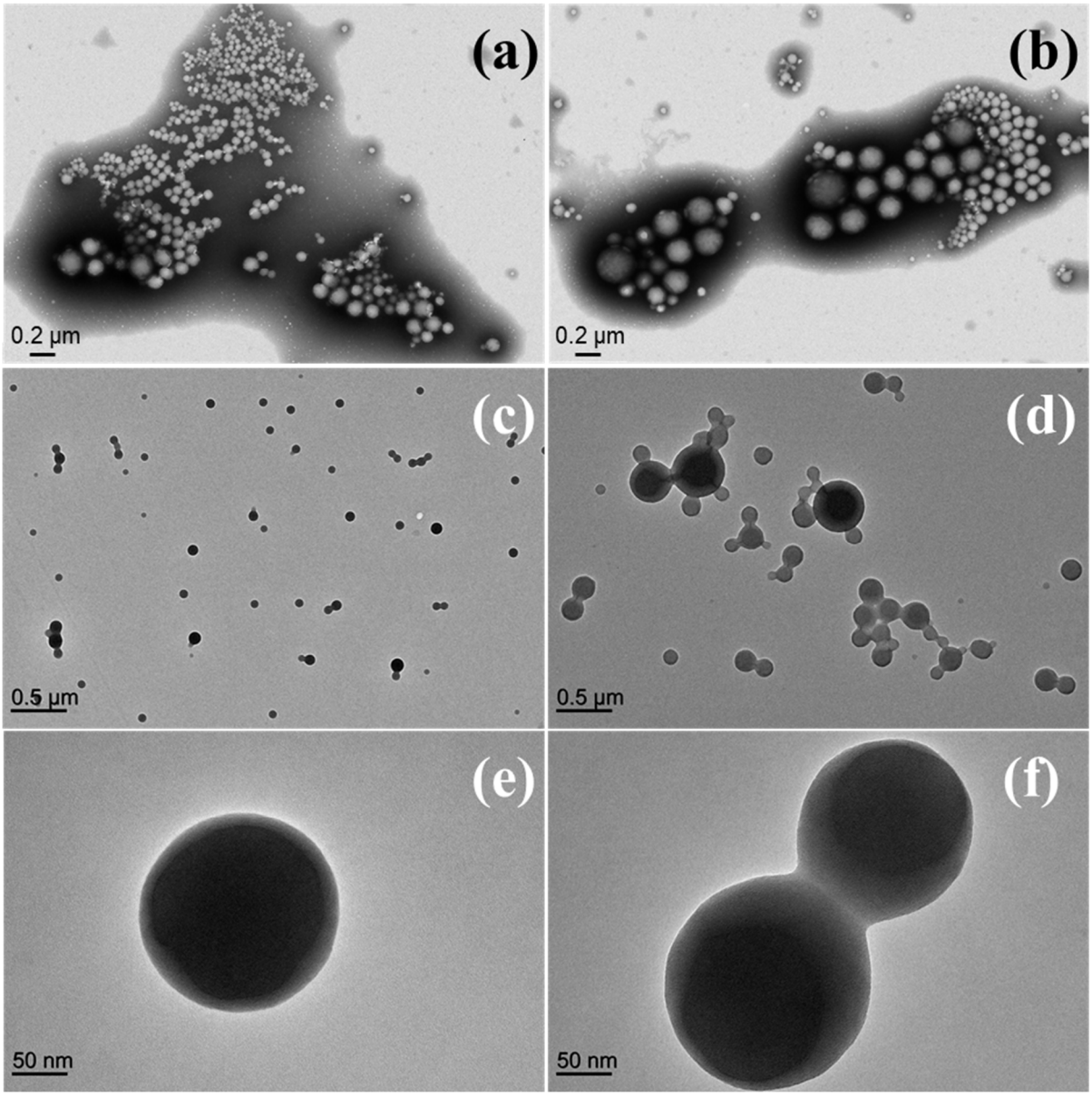
Fig.1. Size range limits for the most frequently used particle size measurement methods. AFM: Atomic force microscopy, DLS: Dynamic light scattering, EM: Electron microscopy, FFF: Field-flow fractionation, NTA: Nanoparticle tracking analysis, SLS: Static light scattering, TRPS: Tunable resistive pulse sensing.

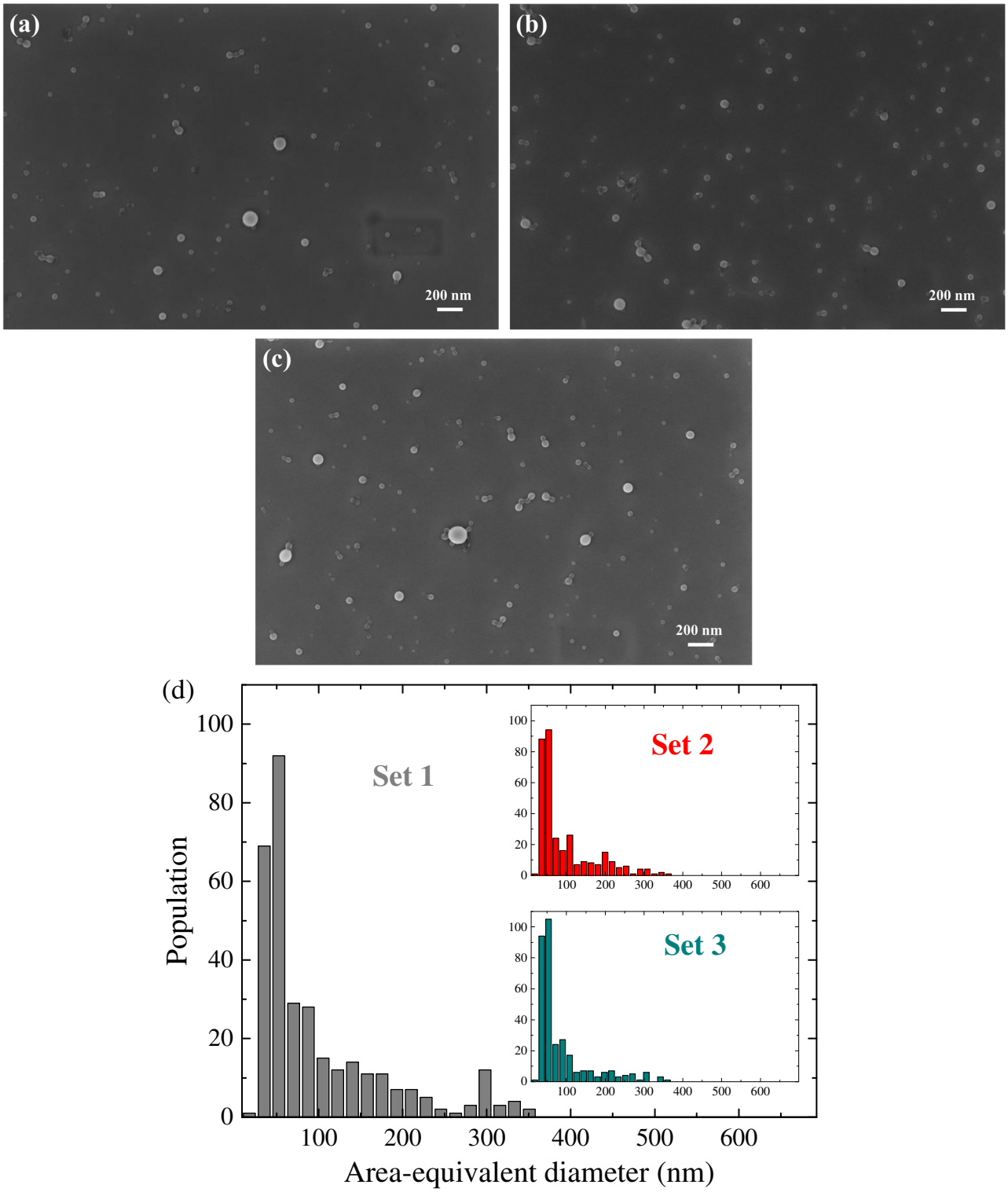
Fig 2. Images resulting from analysis of PIBCA nanoparticles coated with dextran with flotation for TEM investigation. (a) and (b): Flotation and drying with staining. Grid placed on top of a drop of the dispersion diluted at 400 µg.mL^{-1} with ultrapure water filtered on 0.22 µm. After 3 min the grid was removed from the drop of sample and the excess of liquid was left to dry at room temperature in a dust-free place. Then, the grid was placed on the top of a drop of solution of phosphotungstic acid (1%) for 30 sec. The excess of liquid was removed with a paper filter and the grid was left to dry in a dust-free space before introduction into the electron microscope. Electron microscope was a JEOL JEM 140 operating at 80 KeV equipped with a camera ORIUS SC1000 A2. (c) to (f): Flotation and drying with no staining. Grid placed on top of a drop of the dispersion diluted at 100 µg.mL^{-1} with filtered ultrapure water. After 3 min the grid was removed from the drop of sample and placed in a dust-free space until the liquid had dried off. Electron microscope was a JEOL JEM 140 operating at 120 KeV equipped with a camera ORIUS SC1000 A2.

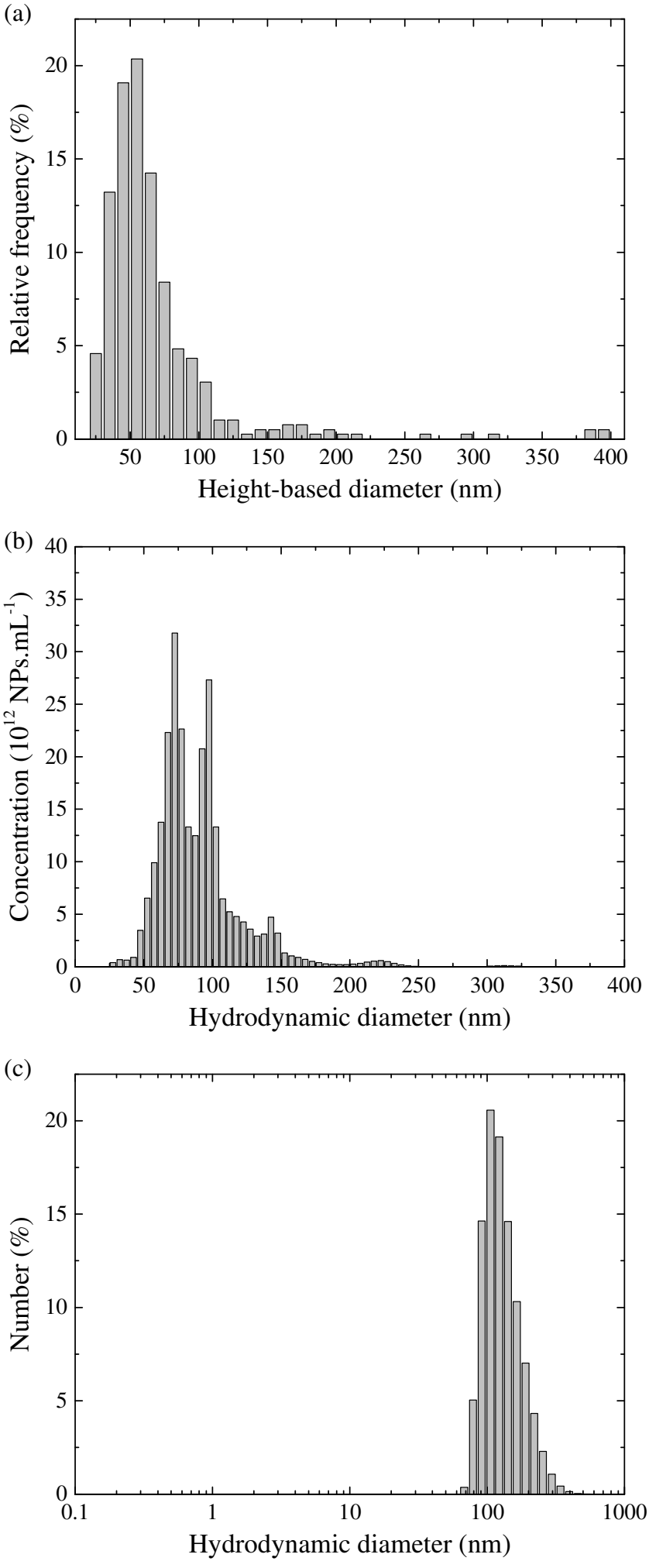
Fig 3. Evaluation of PSD of PIBCA nanoparticles coated with dextran. SEM investigation with spin-coating deposition: example of images used to measure size of nanoparticles and to establish the PSD (a) to (c) and number-based PSD (d) (Sets 1 to 3: 327, 327 and 326 counted nanoparticles). Dispersion diluted at 1 mg.mL^{-1}.

Fig. 4. Evaluation of PSD of PIBCA nanoparticles coated with dextran by (a) AFM (height, 393 counted nanoparticles, dispersion diluted at 100 µg.mL^{-1}), (b) NTA (dispersion diluted at 51 ng.mL^{-1}) and (c) DLS 173° (number-based PSD, dispersion diluted at 256 µg.mL^{-1}).

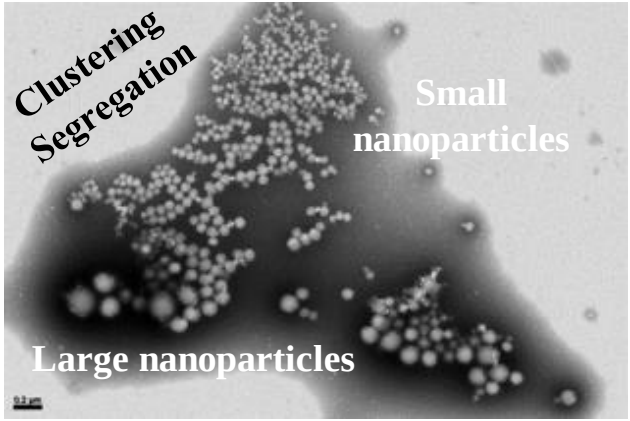

Diameter (nm)
100000
10000
1000
100
10
1
0.1
EM
AFM
TRPS
NTA
DLS
SLS
FFF


(a)
0.2 μm
(b)
0.2 μm
(c)
0.5 μm
(d)
0.5 μm
(e)
50 nm
(f)
50 nm


(a)
200 nm
(b)
200 nm
(c)
200 nm
(d)
Set 1
Set 2
Set 3
Population
Area-equivalent diameter (nm)
0
20
40
60
80
100
100
200
300
400
500
600


(a)
Relative frequency (%)
Height-based diameter (nm)
(b)
Concentration (10^{12} NPs.mL^{-1})
Hydrodynamic diameter (nm)
(c)
Number (%)
Hydrodynamic diameter (nm)

Deposition methods for grid preparation to evaluate particle size distribution (PSD) of multimodal dispersion of poly(isobutylcyanoacrylate) nanoparticles by direct methods based on microscopic techniques



Flotation

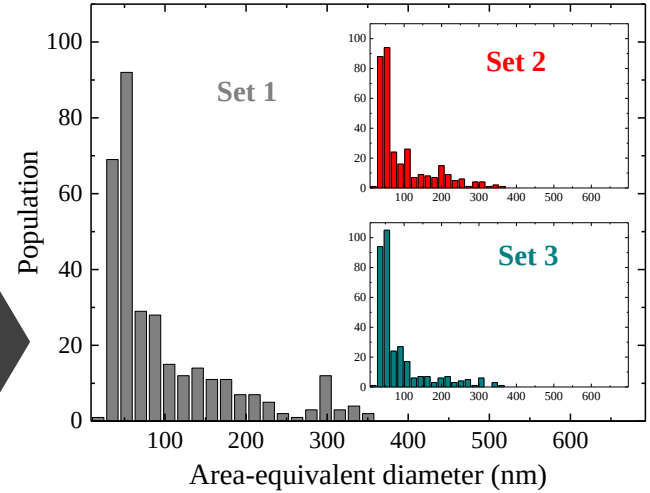
Transmission electron microscopy

Not suitable for relevant evaluation of PSD

Spin-coating

Scanning electron microscopy

Suitable for PSD evaluation



3 sets of 327, 327 and 326 nanoparticles providing similar PSD