



A direct comparison of experimental methods to measure dimensions of synthetic nanoparticles



P. Eaton^{a,*}, P. Quaresma^a, C. Soares^a, C. Neves^a, M.P. de Almeida^a, E. Pereira^a, P. West^b

^aREQUIMTE - LAQV, Departamento de Química e Bioquímica, Faculdade de Ciências, Universidade do Porto, 4169-007 Porto, Portugal

^bAFMWorkshop Inc, 1434 E 33rd St., Signal Hill, CA 90755, USA

ARTICLE INFO

Article history:

Received 26 September 2016

Accepted 2 July 2017

Available online 5 July 2017

Keywords:

Nanoparticles

Transmission electron microscopy

Atomic force microscopy

Scanning electron microscopy

Dynamic light scattering

ABSTRACT

Nanoparticles have properties that depend critically on their dimensions. There are a large number of methods that are commonly used to characterize these dimensions, but there is no clear consensus on which method is most appropriate for different types of nanoparticles.

In this work four different characterization methods that are commonly applied to characterize the dimensions of nanoparticles either in solution or dried from solution are critically compared. Namely, transmission electron microscopy (TEM), scanning electron microscopy (SEM), atomic force microscopy (AFM), and dynamic light scattering (DLS) are compared with one another. The accuracy and precision of the four methods applied nanoparticles of different sizes composed of three different core materials, namely gold, silica, and polystyrene are determined. The suitability of the techniques to discriminate different populations of these nanoparticles in mixtures are also studied.

The results indicate that in general, scanning electron microscopy is suitable for large nanoparticles (above 50 nm in diameter), while AFM and TEM can also give accurate results with smaller nanoparticles. DLS reveals details about the particles' solution dynamics, but is inappropriate for polydisperse samples, or mixtures of differently sized samples. SEM was also found to be more suitable to metallic particles, compared to oxide-based and polymeric nanoparticles. The conclusions drawn from the data in this paper can help nanoparticle researchers choose the most appropriate technique to characterize the dimensions of nanoparticle samples.

© 2017 Elsevier B.V. All rights reserved.

1. Introduction

Synthetic nanoparticles are being studied for potential use in many applications in diverse fields, such as medical diagnostics, therapy, structural materials, environmental applications, etc. Certainly, the main impetus for this research is that nanoparticles exhibit size-dependent properties that are very different to the constituent bulk material. Some examples include the optical properties of metallic nanoparticles, magnetic properties of iron oxide nanoparticles, optical properties of semiconductor nanocrystals (quantum dots), etc. Implicit in this, is that the actual dimensions of the nanoparticles are critical for their properties, and thus suitability for any application. Therefore, for any potential application, it is of extreme importance that suitable techniques are used to characterize the nanoparticles, in order to understand this structure–property relationship. If the common definition of a nanoparticle that it will have at least one dimension of less than

100 nm is accepted, there is still a large range of magnitudes that can be probed. For example, in the case of quantum dots, a change in diameter on the order of 0.3 nm can give a measurable change in optical properties [8]. In the case of optical absorption by metallic nanoparticles, a change of a few nanometres can make an appreciable difference [12]. It is not only physical properties that depend on size, since interactions with biological entities such as cells and biomolecules can depend greatly on the nanoparticle dimensions [22,25]. Although size control is important, some nanoparticle synthetic routes lead to populations with dispersion in size of many tens of nanometres.

Many different techniques have been applied to the study of nanoparticle dimensions during the past few decades of nanotechnology research [13,24]. Amongst these, microscopic techniques provide direct imaging of the dry particles. On the other hand, several methods can be applied to particle solutions, such as light scattering, which measures the mobility of particles in solution, methods based on sedimentation rate, chromatographic methods, and Coulter counting. Finally, crystallographic methods are also widely applied, although inapplicable to amorphous particles. These techniques are summarized elsewhere [4,13]. For ap-

* Corresponding author.

E-mail address: peter.eaton@fc.up.pt (P. Eaton).

Table 1
Principal characteristics of the methods used. Note that specifications and capabilities are quoted for best possible conditions, and depend strongly on sample type, conditions, and instrument. *Resolution is quoted for microscopic techniques, and typical detection limit for the light scattering technique.

Technique	Resolution/detection limit	Physical basis	Environment	Material sensitivity	Parameters measured
TEM	0.1 nm ^{a,b}	Scattering of electrons	Vacuum	Increases with atomic number	Size and shape
SEM	1 nm ^b	Emission of electrons	Vacuum	Somewhat increases with atomic number	Size and shape
AFM	1 nm ^a (XY), 0.1 nm ^a (Z)	Physical interaction with sample	Vacuum/air/liquid	Equal for all materials	Size and shape
DLS	3 nm ^b	Light scattering fluctuations due to diffusion	Liquid	Depends on refractive index	Size only

^a Eaton and West, [7]

^b Tiede et al., [24]

plications where nanoparticles are in solution, an ideal technique would seem to be high-resolution microscopy in liquid. Liquid cells are available for all high resolution microscopy techniques, however, current state of the art imaging in liquids is of considerably lower resolution than under dry or atmospheric conditions, and many other practical problems hold such methods back at present [5]. Ultimately, high-resolution microscopy in liquids of mobile nanoparticles is likely to remain challenging since the very motion of the particles reduces the time for image acquisition, greatly decreasing image quality. From the previous statements, it becomes apparent that a major difference between some of the methods applied is the question of nanoparticle environment. In some methods, the samples can be measured while freely moving in solution, while for others, they must be dried, or even placed in a high vacuum before measurement. In addition, the nature of the solution might prohibit the use of some methods. For example, light-scattering techniques can suffer from significant interference if the solution contains even small amounts of large contaminants (e.g. dust) [4]. Meanwhile, drying of solutions for microscopic analysis can suffer if the solutions contain large amounts of salt, or buffering molecules, due to formation of crystals or films covering small nanoparticles. When applicable in solution, some methods require that the concentration of the sample fall within a very narrow window (for example, nanoparticle tracking analysis [10]). The presence of other materials in solution may also alter results from some techniques. In addition, some methods are more or less suitable for certain types of nanoparticles. For example, electron microscopy techniques have varying sensitivities for different materials depending on their atomic number. Also, techniques that rely on drying the samples would be not suitable for materials whose form is altered by the vacuum required.

In the work described in this paper, we sought to assess the suitability of a number of very commonly applied methods to the precise characterization of the dimensions of several common types of nanoparticle. Specifically, the characterization methods tested were dynamic light scattering (DLS), and the microscopic techniques transmission electron microscopy (TEM), atomic force microscopy (AFM), and scanning electron microscopy (SEM). While all of these microscopic techniques are capable of imaging and measuring dried samples of nanoparticles of a variety of materials, they work in different ways, have different methods of contrast formation, and cannot all achieve the sample maximum resolution. Some properties of the different methods used are summarized in Table 1. For more details of these techniques and their applicability in nanoscience, the following references are recommended [7,23,27].

These methods are perhaps the most commonly used methods for characterization in use at this time. They were tested with only spherical particles, but for each nanoparticle material tested, two different sizes were produced and tested. In addition, the ability of the techniques to distinguish these two size populations in mixed samples was assessed. The precision of the techniques was tested by comparing the size dispersion of the results from each technique.

The nominal sizes ranged from around 15 nanometres to almost 100 nm. Three different materials, gold, polystyrene, and silica, were tested, and the results from each method on each sample compared.

It is important to emphasize here that direct comparison of the dimensions obtained from each technique is not the aim of this work. Indeed, such comparison is not feasible, because each technique actually measures different properties of the sample [26]. This can be particularly the case with nanoparticle dispersions due to deviations from true monodispersity in the population and other problems, even when comparing different instruments all calibrated in a manner that can be traced back to the international meter standard (traceable calibration) [9]. In particular, comparison of dimensions obtained from light scattering techniques such as DLS with direct microscopic measurements is highly complicated, and cannot be done directly. There are various reasons for this, including the fact that light scattering is actually measuring the hydrodynamic radii of the particles, which includes not just the particle itself, but the ionic and solvent layers associated with it in solution, under the particular measurement conditions. In addition, DLS is a dynamic measurement, extremely sensitive to the dispersion/aggregation behaviour of the particles in solution. This is highly difficult to infer from microscopic data on dried solutions [5]. Even amongst the microscopic methods, direct comparison of values is not feasible and even in cases where cross-calibration has been performed to ensure all instruments are comparable to each other, can be made only with caution, bearing in mind that each microscope measures a different property. For example, in TEM measurements of metallic nanoparticles, under the conditions used here, without shadowing or staining, almost all of the signal will come from the metallic core of the particles, ignoring any organic capping layer. On the other hand, the AFM finds it hard to distinguish between inorganic and organic materials, and measures the whole particles. SEM sensitivity is probably somewhere between the two, since sensitivity is greater for higher atomic number materials (this depends somewhat on the type of detector being used), but organic material may also be detected. All microscopic techniques were calibrated to standards, but the standards were non-traceable, and the instruments used here were not all cross-calibrated, so their actual calibrations would be somewhat different. Therefore, some variation in the actual measured dimensions is to be expected. Nevertheless, the aim of this paper was not to obtain accurate measurements of the particle sizes, but to assess the suitability of each technique for characterizing each type of sample, and specifically, the precision of the measurements, and if they were capable of distinguishing and separating the different size nanoparticle populations in mixed samples. Assuming a fixed population of sizes in the sample, the measured broadness of the distribution found by each technique will reflect the errors and other precision-reducing factors inherent in each technique. Broadening of distribution by such factors will also reduce the separation between populations in measurements of binomial distributions. Thus, in this paper, we use measured dispersity in the pop-

Table 2

Denominations (in bold) of the samples used in this work.

Gold	Polystyrene	Silica
Au1 —nominal diameter 15 nm	PS1 —nominal diameter 30 nm	Si1 —nominal diameter 50 nm
Au2 —nominal diameter 60 nm	PS2 —nominal diameter 90 nm	Si2 —nominal diameter 80 nm
AuMix —1:1 (vol:vol) mixture of Au1 and Au2	PSMix —1:1 (vol:vol) mixture of PS1 and PS2	SIMix —1:1 (vol:vol) mixture of Si1 and Si2

ulations, and binomial separations, to compare the performance of the techniques under study.

2. Materials and methods

Samples studied: 9 nanoparticle samples were characterised with 4 techniques. All nanoparticles are approximately spheres, differing in diameter. The sample names, and their expected content, are described in Table 2.

Preparation of gold nanoparticles: For gold nanoparticle (Au1 and Au2) synthesis, all the required glass material was previously washed with *aqua regia* and abundantly rinsed with deionised water and Milli-Q water. All the solutions were prepared with Milli-Q water.

Au1 were synthesised by the Turkevich method, with slight modifications [16]. Briefly, 125 ml of HAuCl₄ 1 mM solution was heated under reflux and vigorous stirring. When the solution started boiling, 13 mL of a pre-heated trisodium citrate 37 mM solution was added. The initial yellow solution turned dark red and after 15 minutes the flask was removed from the bath and cooled down to room temperature.

Au2 were obtained by a protocol with two distinct phases [3]: small gold nanoparticles (~15 nm) were synthesised and then successive growing steps on top of these initial small gold nanoparticles (seeds) were made, until the desired size was obtained. For the seed synthesis, 150 ml of a 2.2 mM trisodium citrate solution was heated under reflux and vigorous stirring. When the solution started boiling, 1 ml of HAuCl₄ (25 mM) was quickly injected. The colour of the solution changed from yellow to light red in 10 minutes. After this period, the flask with the seeds (S0 generation) was removed from the bath. For the seeded growth of gold nanoparticles, the oil bath temperature was set to 90 °C, and, when the temperature of the seed suspension was stable at 90 °C, 1 ml of a HAuCl₄ 25 mM solution was quickly injected. After 30 minutes, the reaction was finished (S1 generation obtained). This HAuCl₄ injection was repeated and after 30 minutes the S2 generation was ready. The sample was then diluted by extracting 55 ml of sample from the reaction flask and adding 55 ml of 2.2 mM trisodium citrate solution. After the temperature was again stable at 90 °C, the process of 1 ml HAuCl₄ 25 mM injection plus 30 minutes of reaction time was performed three times (S3, S4 and S5 were successively obtained). Small aliquots from each generation were analysed by UV–Vis spectroscopy in order to evaluate the size [12].

Preparation of silica nanoparticles: Fluorescent silica nanoparticles (Si1 and Si2) were prepared by a reverse microemulsion method adapted from that described elsewhere [6,20]. The fluorescence of the particles was characterized but was not relevant for the size studies described in this paper (results not described). The precursor dye was prepared using 1×10^{-4} M of rhodamine B isothiocyanate (RBITC) dissolved in 1 ml of water and an equimolar amount of (3-aminopropyl)triethoxysilane (APTES). The mixture was stirred overnight protected from the light at room temperature. A water in oil microemulsion was prepared with 1.77 ml of Triton X-100, 1.80 ml of 1-hexanol, 7.5 ml of cyclohexane, 400 µl of water and 100 µl of RBITC-APTES. The mixture was stirred for one hour protected from the light and after this period of time, 200 µl of tetraethyl orthosilicate (TEOS) and 100 µl of NH₄OH were added to the mixture and stirred. For Si1, this stirring process was al-

lowed to continue for 2 hours, and for Si2, the process was allowed to continue for 24 hours. To end the reaction and precipitate the nanoparticles, 20 ml of acetone was added to the solution which caused precipitation of the particles. The nanoparticles were separated from the reaction mixture by centrifugation and washed 3 times with ethanol.

Polystyrene nanoparticles: Polystyrene nanoparticles (P1 and P2), were purchased and used as received (with dilution using ultrapure water). The PS1 samples was sold as *Latex Spheres*, 0.03 µm, nominal diameter, and the PS2 samples were sold as *Latex Spheres*, 0.09 µm, nominal diameter. Both were purchased from Ted Pella, Inc.

Characterisation methods: three microscopy techniques were used on dried samples, and one light scattering technique used on the solution the dried samples came from.

Atomic force microscopy (AFM): A TT-AFM from AFM Workshop with a 15 µm scanner was used in vibrating mode. Probes were purchased from Applied Nanostructures, Inc., and had approximately 300 kHz resonant frequency, probe diameter <10 nm (App-Nano ACT). Samples were deposited onto freshly cleaved mica for this technique. AFM set-point was optimised for accurate scanning without inducing particle compression. Samples were air dried before scanning. As with other techniques, sample solutions were treated in an ultrasonic bath (30 seconds) before deposition to reduce aggregation. Images were analysed with a combination of the open-source program Gwyddion [19], and a custom particle-height measurement routine. In both cases, the analysis consisted of direct height measurement of individual nanoparticles. The maximum height from the substrate was taken as the particle diameter for this technique.

Scanning electron microscopy (SEM): FEI Quanta 400 FEG-SEM (located at CEMUP, Porto) was used. Imaging conditions varied somewhat between samples. For all samples, imaging was performed in high vacuum at 15 kV accelerating voltage. Gold nanoparticle samples were deposited on carbon tape, and for gold sample, backscattered electron images were analysed since in these samples, higher contrast was obtained from this technique compared to the secondary electron signal. For both silica and polystyrene samples, poor images were obtained from either secondary electron images or backscattered electron images of uncoated samples. For these samples, it was found to be necessary to coat the samples (with gold/palladium) in order to get high-contrast images. This of course can lead to some systematic error in the size measurements. This is discussed further in the results and discussion section. These samples were deposited on polished aluminium stubs. As with other techniques, all sample solutions were treated in an ultrasonic (30 seconds) bath before deposition to reduce aggregation. Images were analysed using the open source utility ImageJ [21]. ImageJ was used to calibrate the images according to the internal scale bar from the instrument, and diameter was measured directly from the images using the line tool. Each particle was measured in two axes and the result averaged to take account of any non-circularity in the profile.

Transmission electron microscopy (TEM): TEM specimens were prepared by placing one (10 µL) drop of the nanoparticle solution onto a carbon-coated copper grid and drying at room temperature. As with other techniques, all sample solutions were treated in an ultrasonic (30 seconds) bath before deposition to reduce aggrega-

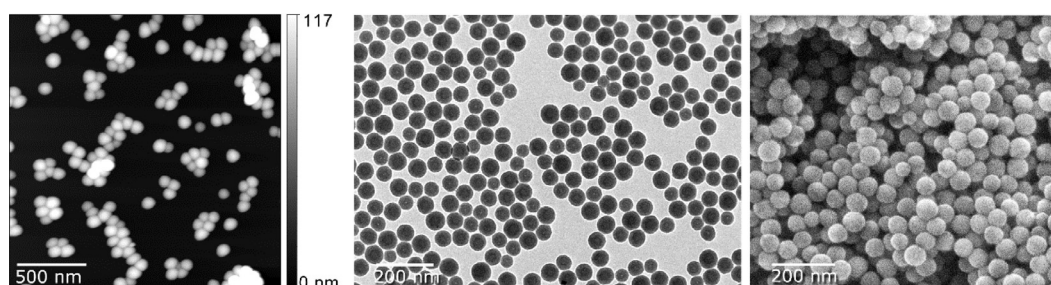


Fig. 1. Examples of silica (Si2) nanoparticle images from AFM (left), TEM (centre), and SEM (right).

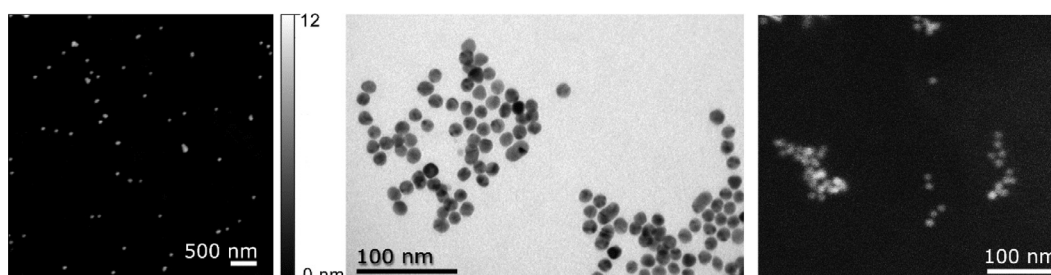


Fig. 2. Examples of gold (Au1) nanoparticle images from AFM (left), TEM (centre), and SEM (right).

tion. TEM was performed with a HITACHI H-8100 microscope operated at 200 kV. Images were analysed using the open source utility ImageJ (Rasband, 1997–2015), as described above for SEM.

Dynamic light scattering (DLS): DLS was carried out using a Malvern Zetasizer ZS instrument, using fresh disposable polystyrene cuvettes for each sample. Samples were subjected to ultrasonic re-dispersion as mentioned above before measurement. The Malvern Zetasizer v7.11 software, which uses the cumulants method of analysis was used to analyse the data. The software modelled each particle as a sphere, with appropriate refractive indices for the bulk material and the solvent. The same software was also used for analysis using the CONTIN algorithm. Gold and polystyrene particles were dispersed in water for measurement. Silica nanoparticles were dispersed in 100% ethanol as solubility was better in ethanol than in water. For these samples, which showed a tendency to aggregate, filtering with 0.2 μm syringe filters was tried, but made no significant difference to the results. Each sample was tested 3–5 times, and the results averaged.

Calculations: Signal to noise ratio, SNR was calculated as $\text{SNR} = \mu_{\text{sig}} / \text{RMSnoise}$, where μ_{sig} was the maximum intensity at the centre of the nanoparticle (with the average baseline adjusted to zero), and RMSnoise was the root mean square of the variation in signal in a region free of detectable particles, adjacent to the measured nanoparticle. To obtain the SNR in decibels (SNR_{dB}), the relation $\text{SNR}_{\text{dB}} = 20 \log_{10} \text{SNR}$ was used. SNR was calculated for at least 15 particles for each method, and results are expressed as mean $\pm$ standard deviation. To calculate the bimodal separation (S) of data from mixed samples, two Gaussian peaks were fit to the histograms, and the mean (μ) and standard deviation (σ) of each peak used in the following equation: $S = \mu_1 - \mu_2 / (2 \times (\sigma_1 + \sigma_2))$. Scattering cross-sections were calculated using the Mie Simulator v1.05R2, available at <http://www.virtualphotonics.org/software>.

3. Results and discussion

3.1. Microscopic techniques

Some illustrative images of each particle material are shown in Figs. 1, 2, and 3, for silica (Si2), gold (Au1), and polystyrene (PS2)

samples, respectively. Images of the other samples (Si1, Au2 and PS1) are included in the supporting information.

In general, scanning electron microscopy presented the most difficulties in obtaining images, and for every sample, several different imaging and sample preparation conditions were tested before optimal conditions were found. However, using TEM and AFM, standard deposition conditions worked simply and imaging conditions were those commonly used for nanoparticles imaging.

As mentioned in the material and methods section, difficulty was found in obtaining high quality images of uncoated polystyrene and silica nanoparticles by SEM. In particular, the smaller particles, for which low contrast was seen, were more affected by this. The images produced on the uncoated samples were not of sufficient quality to enable diameter measurements. However, after coating with a thin layer of gold/palladium, the image quality improved to the extent that it was possible to obtain reasonable images that enabled dimensional measurement. Prior to this, image quality was very low and imaging sufficient for diameter characterization was not possible (data not shown). The coating would have introduced an error into the characterization. From our data, this error could not be detected. However, based on the information from the manufacturer of the sputter coater, under the condition that were used, a film of 7 nm thickness could be produced. However, the thickness of this layer would not be the same all over the sample, and will depend on the angle between the target and the part of the sample to be imaged. In particular, in the case of spherical nanoparticles, where the diameter of the observed particles is measured, a thinner layer than would deposit on a horizontally oriented surface, would be expected. Fourteen nanometres would be the maximum dilation of a measured particle that could possibly occur, if the sample was completely covered even on vertical surfaces. In this case the diameter measurement would measure the thickness of the layer twice, once on each side of the particle. Thus there was likely an error of somewhere between 0 and 14 nm in these measurements due to coating.

The images of Si2 shown in Fig. 1 come from three very different techniques, and thus their overall appearance is quite different, even though all show the same sample. On the other hand, for this sample, all three techniques gave high quality images, which are suitable for measurements of sample dimensions. On very close

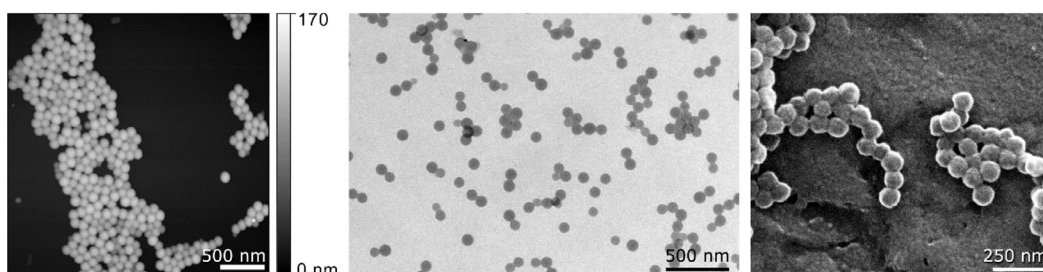


Fig. 3. Examples of polystyrene (PS2) images from AFM (left), TEM (centre), and SEM (right).

inspection, it can be seen from Fig. 1 that noise level in the SEM image is somewhat greater than in the other two techniques. The AFM data is considerably different to the other two techniques because it contains information in three spatial dimensions. When illustrating AFM height data, a colour scale is often included in the Figure (as shown in Fig. 1), to enable mapping of grey scale tones to height information, although accurate estimations of height data is more easily made with two dimensional profiles. In fact, in AFM images the height dimension (z axis data) is typically used to determine the diameter of spherical particles, and this method was used in this work. This is due to probe-sample convolution, measurements of samples laterally (along the x or y axes of the AFM dataset), is not normally used as it adds a systematic error to lateral dimension measurements [7]. Although this error is systematic, it is not trivial to remove it, since the shape of the probe, which influences the size of the error, is usually unknown. Nonetheless, various methods do exist to improve the accuracy of lateral dimensional measurements in AFM [1]. In the case of spherical particles, these methods are irrelevant, since vertical height data can be used, which is not prone to this error, and can be both very accurate and precise. In addition, z -axis data from AFM typically has lower noise and higher resolution than data collected in the x and y axes. In the cases of SEM and TEM, dimensions are measured directly from the XY distances in the images, thus the lateral magnification is important to optimize resolution in the dimensional data obtained. For AFM very high lateral magnification is not required, since dimensional data is exclusively measured in the z axis. For this reason, lateral magnification in AFM images is often low compared to the other two microscopies. Thus, observing Figs. 1, 2, and 3, it can be seen that the lateral dimensions of the AFM images are all greater than found in the TEM and SEM images.

From Fig. 2, it can be seen that for Au1, the smallest nanoparticle sample tested, the SEM images were very noisy. Au2 gave considerably better results (see Fig. S3, in the Supporting Information), indicating, that this is not only a problem of the material, rather, of the size. In principle, the SEM used had more than sufficient resolution to resolve these particles. In this case, the issue is more likely the size of the signal obtained for these small particles. In the conditions used, the depth of the volume probed by the SEM should be several hundred nanometres at least [18], thus the small nanoparticles actually contribute relatively little to the signal measured, and contrast is weak. For the polystyrene particles, similarly to the silica particles, poor images were obtained with uncoated samples in SEM. This was particularly the case with the smaller particles (PS1), which were almost impossible to image uncoated, and certainly did not produce images of sufficient quality to enable dimensional measurements. These particular particles were also highly challenging to image in the TEM, producing images with a kind of negative contrast, where the particles appear as “holes” in a darker, surrounding medium (see Figure in supporting information), in the AFM it was noted that although good contrast could be obtained on the particles themselves, a thin film was also

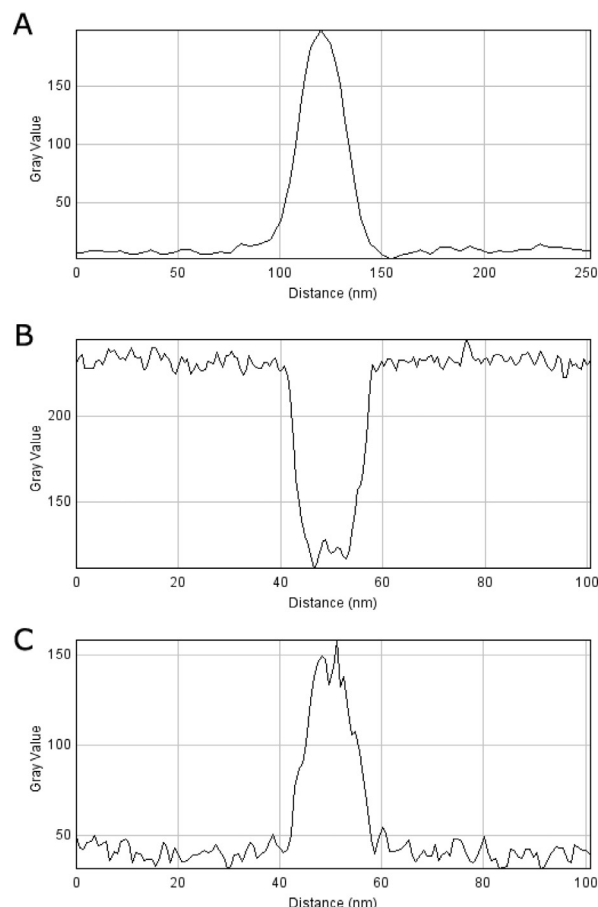


Fig. 4. Profiles measured through 3 nanoparticles from the Au1 sample, in data from the three microscopic methods used, A, AFM; B, TEM; and C, SEM (backscattered electron signal). The signal to noise ratio reported in the text was calculated from these profiles, comparing the maximum signal obtained at the position of the particle to the RMS noise in the background portion.

present after deposition. It seems likely that some low-molecular weight material was present in considerable concentration in this commercial sample. After coating, these samples could be imaged using the SEM, although in many cases it was difficult to separate the particles, possibly again due to a contaminating film.

In order to show more clearly the difference in image quality obtained from the three microscopic techniques, profiles were extracted from images of the Au1 sample, passing through isolated nanoparticles from all the three microscopic techniques. Example profiles are shown in Fig. 4. In the profiles in Fig. 4, the y axis values—“gray values”—represent the actual grayscale in the case of TEM and SEM. In the case of SEM, the backscattered electron signal was used. In the case of these two electron microscopies, the intensity reflects the electronic density of the materials under study,

Table 3

Summary of results from diameter measurements made with microscopic techniques.

	AFM Au1	AFM Au2	AFM PS1	AFM PS2	AFM Si1	AFM Si2	TEM Au1	TEM Au2	TEM PS1	TEM PS2	TEM Si1	TEM Si2	SEM Au1	SEM Au2	SEM PS1	SEM PS2	SEM Si1	SEM Si2
N	153	129	45	259	151	410	386	99	94	182	446	565	302	172	191	155	207	232
Median	11.2	51.0	16.6	82.8	52.8	68.8	13.5	58.5	30.4	87.1	45.0	59.1	11.7	52.3	32.0	90.3	43.6	63.4
Mean	11.2	50.5	16.7	80.4	53.5	68.4	13.7	59.1	31.2	85.8	43.8	57.7	12.1	52.0	32.4	88.7	45.0	63.1
5th Centile	8.9	41.8	9.8	61.9	44.8	53.9	11.8	48.1	21.3	67.0	31.7	45.4	8.6	33.2	23.2	72.2	34.2	53.1
95th Centile	13.4	58.7	23.1	92.2	64.3	80.8	16.6	70.0	41.0	97.4	53.6	66.4	18.3	66.5	42.8	100.0	57.8	72.3
Range as %*	37	33	80	38	36	39	34	37	63	35	50	36	81	64	60	31	53	30

* The range between the 5th and 95th centile expressed as a percent of the mean value.

but also depend greatly on the specific setup of the instrument, including such parameters as beam intensity, detector and camera sensitivity, for example. Therefore in this case, actual values of the grey value have no physical meaning. In the other, hand, in the AFM, the “z-axis” of the image, i.e. greyscale intensity, represents real height values. However, since it is not possible to compare the values from one technique with the others, in each case, the z scale was just converted to grey value.

These data clearly show a very much lower noise level compared to the size of the signal (i.e. better contrast) for AFM compared to either TEM or SEM, while the TEM image seemed to be marginally better than the SEM image.

In order to analyse further this data, the signal-to-noise ratio (SNR) of several of these profiles was calculated (see methods section). The SNR is a measure of how large the signal is compared to average background noise. The raw values are 113 ± 36 , 27 ± 10 , and 16 ± 5 for the AFM, TEM and SEM profiles, respectively (in dB these are 41 ± 3 , 27 ± 7 , and 24 ± 2 dB, respectively). These data confirm the impression from observing the profile – the AFM had considerably higher signal to noise ratio than either electron microscopy technique, while for our data, TEM had almost double the signal to noise ratio of SEM.

It is important to consider here, that the SNR in these cases do not directly correlate to a comparison of the performance of the techniques. These data show that under the conditions used, AFM was a far more sensitive technique in terms of detecting the nanoparticles compared to the background. However, this is not directly related to resolving power of the microscopes in question. For the AFM, the SNR in the z axis is hugely important, since this is the critical parameter used for most measurements of topography made in AFM. In the other techniques, the measurements are typically made laterally, and their most important parameter is lateral resolution. For AFM, lateral resolution is rather difficult to measure since it is dependent on the probe used, and probes are not only somewhat variable, even under optimal manufacturing conditions, they are also often altered during use (for example, by wear).

The diameter of the nanoparticles observed in each sample, including the mixtures of nanoparticles was measured from the images obtained. The ability to discriminate different sizes in a mixture is important for mixed samples, but can also be used as a method to judge whether the method used can really measure distributions of sizes, or is only a measure of an “average” size [14]. Histograms showing the results obtained from all microscopic methods are shown in Figs. 5, 6, and 7 for silica, gold and polystyrene samples, respectively. Table 3 also shows numerical analysis of the diameter measurements for each sample and method used.

Examining the histograms of the silica nanoparticle diameters in Fig. 6, several conclusions can be drawn. Firstly, one might notice that in general, the actual sizes measured between the three techniques are somewhat similar, although some small differences were found. As mentioned previously, all the instruments used here have not been fully cross-calibrated with each other. Exam-

Table 4

Calculated values of bimodal separation for each mixed sample as measured by all three microscopic techniques.

	AFM	TEM	SEM
SiMix	0.60	0.55	0.61
AuMix	3.16	2.45	0.89
PSMix	2.36	1.90	1.84

ining mean sizes of the samples in Table 3, it may be seen that although some samples did differ between the techniques, there was no significant difference in the sizes from the three techniques as a whole, i.e. no one technique consistently gave results larger or smaller than the other techniques. Therefore it seems the calibration of the three microscopes was probably similar. Without calibration to a standard traceable to the internal system of units, it's not possible to know for sure which of these are the most accurate. Finding significant differences in measured dimensions when comparing results from different laboratories, even when using calibration standards, is not uncommon [15]. However, actual comparison of the sizes obtained by the techniques is not the focus of this paper, rather the aim is to establish the suitability of the methods. Differences in results on individual samples by different methods can be due to differences in the properties measured by the three techniques (as discussed above), or sampling of different sub-populations by each method.

Returning to the silica samples, it can also be seen that all three microscopic techniques were able to distinguish the silica particles of different sizes, even though these two samples were relatively close to each other in size. Examining in particular the histograms of the mixed values, it's possible to see that the two peaks overlap, but can be distinguished in the histograms. In terms of separation, the AFM seems to have separated the two populations most clearly, and in the case of the SEM the two peaks are present, but very close to each other. To the casual observer, the SEM histogram might be mistaken for one broad peak, rather than two sharp peaks close to each other. In order to get some quantitative data on the separation of populations in mixed samples, the bimodal separation was calculated, as described in the methods section. This parameter is a measure of how far apart the peaks are located, compared to the standard deviations. Larger values indicate data with greater separation between the peaks, or smaller standard deviation of the data. The method used to calculate this parameter is given in the materials and methods section. The results are shown in Table 4.

According to this data, AFM achieved the greatest separation between peaks overall, with TEM generally larger than SEM, the exception being the SiMix data. In this case, actual bimodal separation values were very low for all techniques, SEM being the largest by a very small margin. These data also demonstrate that the SEM results separated very poorly the mixed gold sample. This could well reflect the lower signal to noise ratio of the SEM on this sample.

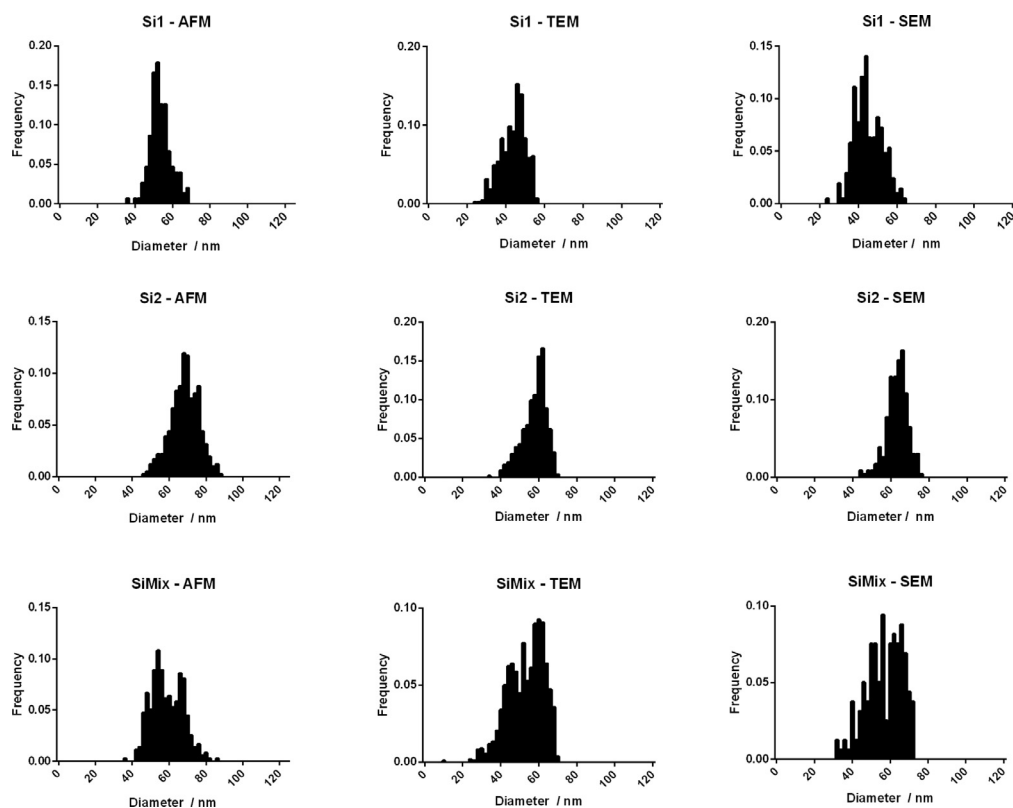


Fig. 5. Histograms resulting from measurements of particle diameter in dried samples of silica nanoparticle samples by three microscopic techniques. The left hand column shows results from AFM, the centre column from TEM, and the right hand column shows results from SEM. The upper row shows results from the Si1 samples, the centre row shows results from the Si2 samples, and the lower row shows results from the SiMix samples.

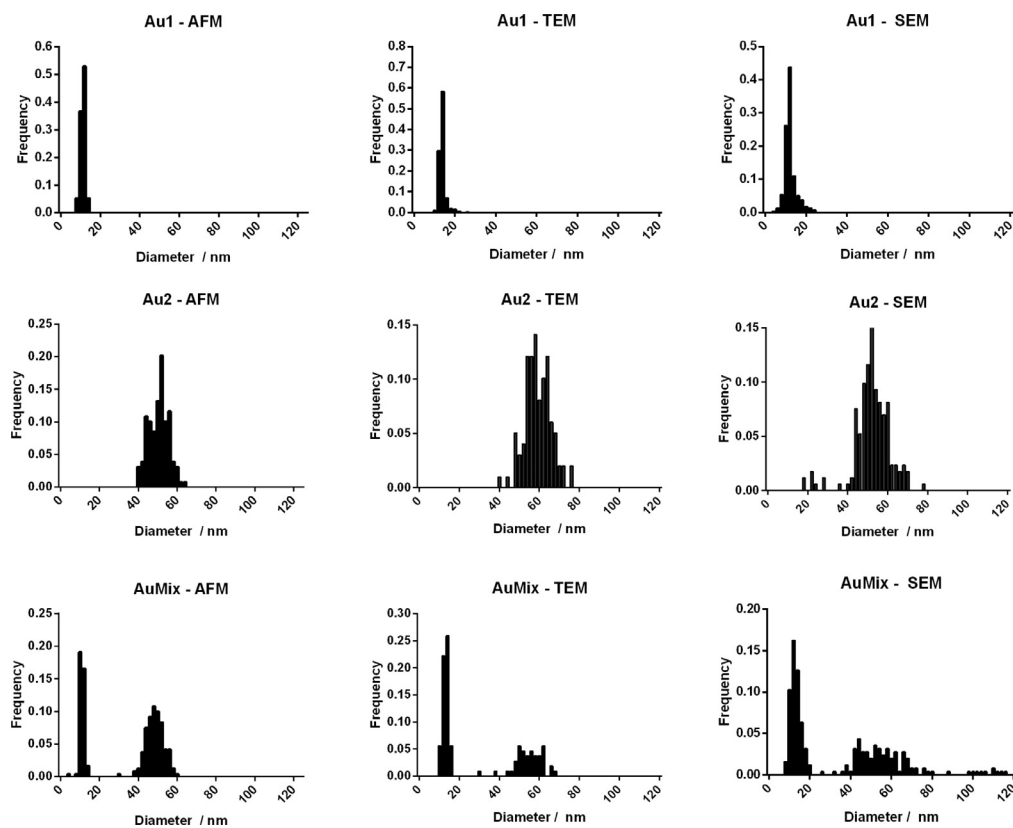


Fig. 6. Histograms resulting from measurements of particle diameter in dried samples of gold nanoparticle samples by three microscopic techniques. The left-hand column shows results from AFM, the centre column from TEM, and the right-hand column from SEM. The upper row shows results from the Au1 samples, the centre row shows results from the Au2 samples, and the lower row shows results from the AuMix samples.

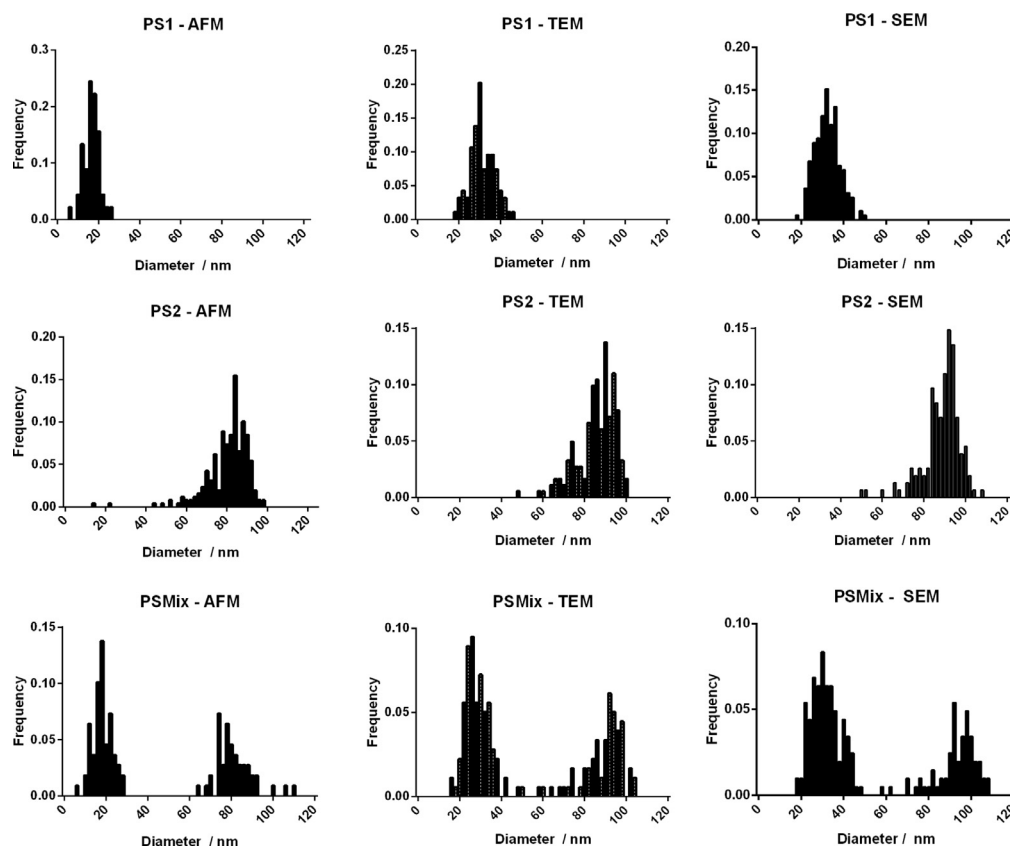


Fig. 7. Histograms resulting from measurements of particle diameter in dried samples of polystyrene samples by three microscopic techniques. The left-hand column shows results from AFM, the centre column from TEM, and the right-hand column shows results from SEM. The upper row shows results from the PS1 samples, the centre row shows results from the PS2 samples, and the lower row shows results from the PSMix samples.

The results on the gold particles (Fig. 6), show a much clearer separation of sizes overall, by all techniques. This is likely due to two factors. Firstly, the synthesis of near monodisperse gold particles is easier due to well established methods in the literature to produce this type of nanoparticle. The data for the Au1 sample showed very narrow size dispersions were registered for all the methods. Secondly, in the case of the gold samples, difference in size between the two populations was greater, in relative terms, than those in the other populations (the larger sample was around four times the size of small sample, versus three times in the case of polystyrene, and about one and a half times in the case of the silica). The data show that the population of the Au2 was considerably more disperse than the Au1, in this case, the synthesis was a multistep process, and there are more opportunities for population broadening during this process. Overall, in the case of the SEM, the results for all gold nanoparticles showed more disperse sizes than the other two techniques did. All methods were able to distinguish the two populations, but for the SEM, it was a less clear separation.

Looking at the histograms of the polystyrene nanoparticles, it is possible to see that the populations are somewhat more disperse than the gold nanoparticles, and the separation poorer, but it was possible to separate the populations in all methods, including in the mixed samples. Interestingly, the methods seem to show some skew in the populations, the larger particles apparently skewed to larger sizes, and the smaller particles skewed to small sizes. This effect was detected by all the microscopic techniques, which suggests it is a real characteristic of the samples, and also helps to validate the use of these techniques to characterize distributions of nanoparticle sizes. Of all the techniques, the AFM separated the populations best, with very few sizes detected between the two

main peaks (see Table 4). This perhaps reflects the fact that AFM has high contrast on these samples (there is practically no dependence of contrast in AFM height images on material type), while both electron microscopic techniques show poor contrast for organic materials, such as this polymer.

Table 3 shows some parameters extracted from the diameter measurement data (the same data from which the histograms were produced). Looking at the average values, either median or mean, overall, there is no clear trend in the data between the three techniques, suggested that all three microscopes were calibrated similarly. To measure the dispersion of the data, the 5th and 95th centile of each population was calculated. Expressing this range as a percentage of the mean value, gives a number that expresses dispersion of the population, ignoring outliers in the bottom or top 5% of the range, while also ignoring any systematic differences between the actual values given by the techniques.

Examining these range values, several trends are apparent. For most samples, at least as measured by AFM and TEM; the range as a percentage of the mean gave values between 30 and 40%. The exception to this is the PS1 sample. Since for this sample, every technique gives a much wider range of values (60 to 80% of the mean), we can conclude that this result is a feature of the sample, not the measurement. Indeed, observing the micrographs, it's possible to see something rather strange in this sample. By all techniques a large amount of amorphous material was observed in this sample, typically surrounding the nanoparticles. As mentioned in the discussion above, it seems likely that this sample contained some contaminant.

Another trend is that large values of the size range were obtained in SEM, in general, compared to the other two techniques.

Table 5

Size parameters obtained by the DLS for all samples. Each value reflects the average of 3–5 independent experiments.

	Au1	Au2	AuMix	PS1	PS2	PSMix	Si1	Si2	SiMix
Z-Average /nm	50.7	52.0	54.4	38.0	93.5	89.5	153.7	121.6	115.1
PDI	0.53	0.24	0.25	0.13	0.02	0.07	0.37	0.37	0.26

Size range was particularly large for the Au1 sample, which is in accordance with observations of the images. These nanoparticles were poorly resolved by this technique. The SEM only produced values of size range in the range of 30–40% of the mean value for the PS2 and Si2 samples; these are the largest samples examined. This suggests that only for relatively large particles (mean size >60 nm), SEM can compete with AFM and TEM. For these large particles, SEM gave quite similar size dispersions, compared to these two other techniques. In fact the dispersion of sizes in SEM was slightly smaller in SEM for these two large types of nanoparticles.

As stated previously, for monomodal distributions, assuming the populations of nanoparticles probed by each technique were the same, then if a technique shows smaller dispersion in the results, this technique would have greater precision in the measurements. Looking at the global results, if averaging the range as % of mean values across all samples for each technique, AFM had 44% dispersion on average, TEM 43% and SEM 54%. So, AFM and TEM were rather more precise, with SEM somewhat less so, overall. On the other hand, for large samples, SEM displayed precision which was as good as or better than the other microscopic techniques.

The TEM also gave a larger value than AFM for the Si1 sample; this may be due to poor contrast. The Si sample give fairly low contrast on the carbon grids, adding to the error in diameter determination, although the contrast was not as low as that found for the PS1 sample. Presumably this would be possible to overcome with some staining technique such as negative staining. In this case, it would be expected that TEM could determine the diameter as accurately as AFM. The AFM appeared to perform very badly for the PS1 sample, even though high contrast was seen on this sample (Fig. S1). The range of values was 80% of the mean value. In particular many small features were detected. From the EM methods, it was possible to determine that this Figure was contaminated with some non-particulate organic material; this might have affected the spread of values in the AFM data. While AFM has high sensitivity to almost any material, this can be a drawback for contaminated samples [2].

3.2. Light scattering

Dynamic light scattering was also used to characterize the solutions from which the samples for microscopy were made. This technique is very different to imaging of dried samples, and is sensitive to dynamic aggregation, aggregation, agglomeration, etc. Furthermore measurement of sizes from DLS data is an indirect method, based on determination of frequency of movement, and modelling of the size from this data. Thus, there are several reasons to expect different results from this technique compared to the microscopic techniques.

The first parameters that were extracted from DLS data were numerical parameters, namely the Z-average, and the polydispersity index, using the cumulants method. In general, these are the parameters reported for DLS data, although further characterization parameters can be extracted. Z-average is defined as the harmonic intensity averaged particle diameter. It is the primary parameter that comes from DLS data, and often described as the most stable parameter, and is commonly used in standard proto-

cols for description of nanoparticles in solution. This value is effectively a measure of the average hydrodynamic diameter of the objects detected in solution, weighted by volume squared. Although it is more directly obtained from the raw scattering data compared to some other methods of characterizing the size data from DLS, Z-average can only be compared to size as measured by other techniques under certain circumstances. Namely, these are that the sample is near-spherical in shape, has a low polydispersity, and that the size dispersion is monomodal [17]. The PDI is a dimensionless parameter that is a measure of the broadness of the dispersion of detected sizes. Thus, values below 0.1 can be defined as “Monodisperse”, i.e., they have a narrow dispersion of sizes. Values above 0.7 are considered too polydisperse for DLS analysis.

The values of z-average and PDI for all the samples examined are shown in Table 5.

Examination of this data reveals important implications for the use of DLS to characterize solution nanoparticle samples. The only samples that have results compatible with the nominal values were the monomodal polystyrene samples. In these samples, values of PDI compatible with monodispersity, i.e. close to or less than 0.1, were obtained. The PS2 sample, in particular, showed a very low PDI and a z-average very close to its nominal value. The PS1 sample also gave a value close to the nominal value, with a slightly larger PDI. The fact that these samples exhibit expected behaviour in this technique is not surprising in the context of the fact that polystyrene nanoparticle samples are the type of samples typically used to calibrate and validate the DLS technique. In fact, samples, of 100–300 nm diameter are typically used. Polystyrene nanoparticles are highly appropriate for DLS analysis since they are highly charged in solution, stable, and undergo relatively little dynamic aggregation in solution. In addition, it's highly likely that the nominal values reported by the vendor come from light scattering technique such as DLS; since it is a rapid, standardized technique, particularly for sample such as these. It is possible to see that the PS1 sample caused some small deviations from the nominal values, possibly due to low scattering cross-section and possibly due to presence of contaminants noticed in microscopy studies. Despite the low dispersity and close match to the nominal values of the DLS data on the simple polystyrene samples, the results on the mixed sample (PSMix) confirms that this technique is not useful for characterization of bimodal samples. The results of the PSMix sample were very close to those from the PS2 sample, with a very close z-average (89.5 versus 93.5), and a slightly larger PDI. Despite DLS not being able to separate bimodal distributions, based on first principles it could be expected to find an average result for the mixture value somewhere intermediate between the results for PS1 or PS2. However, the z-average value of PSMix is extremely close to that of PS2, and in fact it was within the variability of the results obtained on PS2 (data not shown). The reason for this is due to the difference in scattering cross section between 30 and 90 nm particles. Calculating the theoretical difference between the scattering cross sections, it is found that scaling up a model 30 nm nanoparticle to 90 nm results in an increase in scattering cross section of just over 1000 times. Thus, while the diameter of PS2 was nominally three times greater than that of PS1, the response from the DLS was likely three orders of magnitude greater. Considering

Z-average will exacerbate this situation further than only considering scattering cross section, since it is further weighted towards larger particles.

Turning to the other types of nanoparticles under study it can be seen that in general, the DLS results from silica and gold correlated poorly with microscopy results compared to the polystyrene nanoparticles in DLS studies. In the case of the gold particles, all of the PDI values were above 0.2, and in the case of the Au1 sample, the values were very high, at 0.53. In addition, the z-average for the Au1 and Au2 were almost identical. It is possible that in solution the Au1 particles dynamically aggregate and de-aggregate, given this large z-average result, which is considerably larger than any individual particles in the solution according to the microscopy data. The DLS raw data before averaging showed quite a large variability between one run and another, with no particular trend in the data, once again, suggesting that the DLS is detecting aggregation in solution. For the mixed sample, a z-average was obtained that was not intermediate between the large and small samples; in fact it was larger than the value for the large sample, clearly showing that DLS analysis using this parameter is not appropriate for mixed samples. By definition, Z-average is not appropriate for anything other than monomodal distributions. However, for the Au2 sample, the z-average was actually quite similar to results from microscopy, and while the PDI was large, this actually matched the microscopy results, where fairly broad dispersions were found for this sample. However, light scattering and microscopic techniques are measuring different properties of the sample, under different conditions. It is noteworthy that by all microscopic techniques, samples of Au1 showed what appeared to be small aggregates in the dried samples. While it is hard to correlate distribution of particles seen in dried samples to solution behaviour, it is tempting to infer that the microscopic results also indicated that in solution, these particles are likely very slightly aggregated. It is worth noting that this type of nanoparticle has been under synthesis for more than five years in our lab, and in our experience, the solutions are highly stable (no large-scale aggregation, or changes in UV-vis spectrum for months of shelf life), DLS results typically show larger sizes than found in microscopic studies.

For silica samples, the DLS results showed different behaviour again. In this case, the z-average for every sample was considerably larger than results from microscopy studies. In this case, the PDI values were also fairly large (although not as large as for Au1). In the silica samples, the sample that was the smallest according to microscopy studies gave the largest z-average, while the mixture gave the smallest z-average. These results clearly do not relate to the sizes of individual particles, but to some dynamic behaviour. In fact, the particles were not entirely stable in solution. If left for a matter of hours, the particles would aggregate and then sediment out of solution. However, this behaviour appeared to be reversible, the entirety of the samples being re-dispersed by sonication. Although no visible sedimentation occurred during the experiments, it is likely that this process was occurring and led the DLS to detect small aggregates in solution. During the DLS experiments, the samples were sonicated thoroughly and measured immediately afterwards. It was noted that if the results were repeated after a few minutes, different results could be found (data not shown). In this case the DLS produced results reflecting the solution behaviour of the silica particles, which could not be detected by the microscopy techniques.

The DLS also produces histograms of size in order to attempt to characterize dispersion of sizes. Results showing intensity averages are shown in Fig. S5. Note that to obtain such data, further assumptions are made about the sample. The more processing is applied to the raw data, the more likely that inaccurate results are obtained. Thus, number-average histograms, which should in principle be closer to microscopy data (which are, after all, plots of

number of nanoparticle versus diameter), can be considered to be less realistic than intensity-average histograms, because in order to change the data to number of nanoparticles, they involve more assumptions about the state of the sample. Nevertheless, number-average histograms are included for comparison, in the supporting information (Fig. S6).

Looking at the data in S5, it may be seen that, as with the z-average and PDI data, the results for the polystyrene particles most closely match the nominal sizes and microscopy data, with a lower peak for the PS1 sample than the PS2, and relatively narrow peaks. Of the two, the PS1 data is much broader than the PS2, as evidenced by the PDI values of these samples. However, the PS1 peak does not extend significantly about 100 nm, unlike the PS2 data. The PSMix peak is remarkably similar and there is no data seen in the region where the PS1 data occurs. Once again, this is likely due to the orders of magnitude lower scattering at these small sizes. The data confirms that bimodal populations cannot be characterized by this technique.

Moving to the gold nanoparticle data, the situation becomes more complicated. The histograms suggest at least two populations in each of the gold samples, Au1 and Au2. In the case of the Au1, there appears to be a broad main peak stretching from close to 10 nm up to several hundred nanometres, with what appears to be a small shoulder which reaches down to well below 10 nm. A second peak at very large diameters (from several hundred nanometres) passes beyond the actual axis of the plot in Fig. S5, and in fact the highest populated bin was around 5000 nm. Clearly this sample was very complex, and not according to the definition of DLS as being suitable for only monomodal distributions. Therefore it would seem these distributions of sizes are not to be trusted. Nevertheless, although in correct in details, they give further evidence of solution aggregation in this sample. The Au2 results are also somewhat unusual. The presence of a small peak around 10 nm would make sense if this was a mixed sample, but microscopy data suggested very few small nanoparticles in this sample. Surprisingly, the AuMix sample was actually the only one of these samples which showed a histogram suggesting a monomodal distribution. In the case of the silica particles, it was observed for all samples, that the distribution was somewhat skewed, with a pronounced tail at larger diameters. In every one of these cases, some data was detected at more than 1000 nm (although the numbers were very small for Si2 and SiMix). This actually ties in well with the idea that these samples contain very large aggregates, which eventually sediment out of solution due to their large size. Number average histograms (Fig. S6) also showed reasonable results for PS samples and multiple peaks for both Au1 and Au2. In the case of the silica sample, two populations were seen for Si60, and one for both Si90 and the mixed sample.

The CONTIN algorithm was also tested, since this method is reportedly better at analysis of multicomponent systems than the more commonly applied cumulants method. However, this method was also unable to distinguish individual peaks corresponding to the two size populations in the mixed samples. The method found only one peak for the AuMix sample (at 65.4 nm, on average), and only one peak for the PSMix sample (at 97.5 nm). For the SiMix samples, multiple peaks were found – the primary (most populated) peak was detected at 123.6 nm on average. A second peak, which was variable in position between runs, was detected at between 3000 and 4000 nm. In one run, a third peak was detected at 651 nm. Since the hydrodynamic diameters represented by these peaks are much greater than the sizes of the individual particles, they were almost certainly due to the presence of large aggregates in the solution. Further evidence comes from analysis of the DLS data on the individual silica samples, both the Si1 and Si2 samples showed at least two peaks in each run, the larger one variable in position, but always between 2500 and 4500 nm. These data show

how DLS cannot resolve individual populations of single particles for these samples, but is able to detect aggregation in solution, difficult or impossible for microscopy techniques.

4. Conclusions

All of the techniques used here have considerable value as methods to determine dimensions of nanoparticle samples. However, the most appropriate technique depends on sample type, as well as the type of information which is required. Considering the microscopic techniques, it was found that all the methods were able to characterize the samples, although metal coating to increase contrast was required for SEM to enable high-quality images. This introduced an error of up to 14 nm. These methods were also able to separate populations in heterogeneous samples. Of the three techniques, SEM was least appropriate for small nanoparticle types. AFM was rather independent of nanoparticle material, delivering very high contrast and signal to noise ratio on all samples. On the other hand, it can be sensitive to cleanliness of the sample. If rapid characterization of large number of nanoparticles is required, TEM offers the highest throughput, being capable of collecting images with thousands of nanoparticles at high magnification in a few minutes. The smallest nanoparticle discussed in this work had a diameter of 15 nm, while there are whole classes of nanoparticles smaller than this. For example, semiconductor quantum dots, which are typically nanocrystals with diameters frequently below 6 nm. Both AFM and TEM are capable of adequately characterizing these nanoparticles [11], while previous experience indicated that SEM and DLS would not be able to determine their dimensions.

The DLS was proved to be completely inappropriate for mixed samples, and it was confirmed that unstable samples lead to unreliable results with this technique. It also seemed to deliver poorer results with smaller nanoparticles. On the other, hand, the method does reveal details about the dynamics and stability of the nanoparticle solutions, which are impossible to obtain from microscopic methods. For example, from microscopic results, our silica samples appeared as well separated, un-aggregated particles, while DLS revealed the instability in these dispersions.

Which technique a researcher is likely to use most likely depends on the availability of, and familiarity with the methods. There are considerable differences in access to such techniques as these. Worldwide, more electron microscopes are installed than atomic force microscopes. DLS instruments are fairly widespread, but probably not used in as broad a range of fields as microscopes. On the other hand, the purchase cost of our TEM was approximately double that of the scanning electron microscope, and twenty times that of our AFM. The high cost of electron microscopes, as well as high maintenance costs typically means access to them can be difficult. The purchase price of the DLS was low, about double that of the AFM. In addition, these instruments are compact, have low maintenance costs, and simple to use, although data interpretation is more complex.

Overall, before choosing a technique to characterize a nanoparticle sample, it is recommended that researchers consider the type of information required and the appropriateness of the techniques to particular samples, in particular considerations such as the size of the nanoparticle, and the material of which it is composed. A combination of methods, with careful interpretation of the data is usually the best option.

Acknowledgements

Scanning electron microscopy was performed at Centro de Materiais da Universidade do Porto, CEMUP. Transmission electron microscopy was performed at the Electron Microscopy Lab-

oratory (Microlab) of the Instituto Superior Técnico, Universidade de Lisboa. Miguel Almeida thanks FCT for the fellowship SFRH/BD/95983/2013. Cristina Neves was supported by FCT grant SFRH/BD/61137/2009, and Cristina Soares by grant PTDC/CTM-NAN/109877/2009. UCIBIO thanks FCT for funding under Grant number UID/Multi/04378/2013.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ultramic.2017.07.001.

References

- [1] Y. Andrew, K. Ludger, Aspects of scanning force microscope probes and their effects on dimensional measurement, *J. Phys. D: Appl. Phys.* 41 (2008) 103001.
- [2] M. Baalousha, J.R. Lead, Characterization of natural and manufactured nanoparticles by atomic force microscopy: effect of analysis mode, environment and sample preparation, *Colloids Surf. A* 419 (2013) 238–247.
- [3] N.G. Bastús, J. Comenge, V. Puentes, Kinetically controlled seeded growth synthesis of citrate-stabilized gold nanoparticles of up to 200 nm: size focusing versus Ostwald ripening, *Langmuir* 27 (2011) 11098–11105.
- [4] S.K. Brar, M. Verma, Measurement of nanoparticles by light-scattering techniques, *TrAC Trends Anal. Chem.* 30 (2011) 4–17.
- [5] R. Brydson, A. Brown, C. Hodges, P. Abellan, N. Hondow, Microscopy of nanoparticulate dispersions, *J. Microsc.* 260 (2015) 238–247.
- [6] C.-L. Chang, H.S. Fogler, Kinetics of silica particle formation in nonionic W/O microemulsions from TEOS, *AIChE J.* 42 (1996) 3153–3163.
- [7] P. Eaton, P. West, *Atomic Force Microscopy*, OUP, Oxford, 2010.
- [8] A.I. Ekimov, A.L. Efros, A.A. Onushchenko, Quantum size effect in semiconductor microcrystals, *Solid State Commun.* 56 (1985) 921–924.
- [9] F. Meli, T. Klein, E. Buhr, C.G. Frase, G. Gleber, M. Krumrey, A. Duta, S. Duta, V. Korpelainen, R. Bellotti, G.B. Picotto, R.D. Boyd, A. Cuenat, Traceable size determination of nanoparticles, a comparison among European metrology institutes, *Meas. Sci. Technol.* 23 (2012) 125005.
- [10] V. Filipe, A. Hawe, W. Jiskoot, Critical evaluation of nanoparticle tracking analysis (NTA) by nanosight for the measurement of nanoparticles and protein aggregates, *Pharm. Res.* 27 (2010) 796–810.
- [11] C. Frigerio, J.L.M. Santos, J.A.C. Barbosa, P. Eaton, M.L.M.F.S. Saraiva, M.L.C. Passos, A soft strategy for covalent immobilization of glutathione and cysteine capped quantum dots onto amino functionalized surfaces, *Chem. Commun.* 49 (2013) 2518–2520.
- [12] W. Haiss, N.T.K. Thanh, J. Aveyard, D.G. Fernig, Determination of size and concentration of gold nanoparticles from UV–Vis spectra, *Anal. Chem.* 79 (2007) 4215–4221.
- [13] M. Hasselov, J.W. Readman, J.F. Ranville, K. Tiede, Nanoparticle analysis and characterization methodologies in environmental risk assessment of engineered nanoparticles, *Ecotoxicology* 17 (2008) 344–361.
- [14] C. Hoo, N. Starostin, P. West, M. McCartney, A comparison of atomic force microscopy (AFM) and dynamic light scattering (DLS) methods to characterize nanoparticle size distributions, *J. Nano Res.* 10 (2008) 89–96.
- [15] S. Jeremias, K. Virpi, B. Sten, K. Helge, L. Lauri, L. Antti, Intercomparison of lateral scales of scanning electron microscopes and atomic force microscopes in research institutes in Northern Europe, *Meas. Sci. Technol.* 25 (2014) 044013.
- [16] J. Kimling, M. Maier, B. Okenve, V. Kotaidis, H. Ballot, A. Plech, Turkevich method for gold nanoparticle synthesis revisited, *J. Phys. Chem. B* 110 (2006) 15700–15707.
- [17] J. Lim, S.P. Yeap, H.X. Che, S.C. Low, Characterization of magnetic nanoparticle by dynamic light scattering, *Nanoscale Res. Lett.* 8 (2013) 381.
- [18] J. Liu, High resolution scanning electron microscopy, in: *Handbook of Microscopy For Nanotechnology*, Kluwer Academic Publishers, Boston, 2005, pp. 325–360.
- [19] D. Necas, P. Klapetek, Gwyddion: an open-source software for SPM data analysis, *Cent. Eur. J. Phys.* 10 (2012) 181–188.
- [20] C.S.S. Neves, Development of fluorescent silica nanoparticles encapsulating organic and inorganic fluorophores; synthesis and characterization Ph.D. thesis, University of Porto, 2014.
- [21] Image J, Rasband, W.S. <http://imagej.nih.gov/ij/>, (accessed 19.07.16).
- [22] L. Shang, K. Nienhaus, G.U. Nienhaus, Engineered nanoparticles interacting with cells: size matters, *J. Nanobiotechnol.* 12 (2014) 11.
- [23] D.J. Smith, Characterisation of nanomaterials using transmission electron microscopy, in: A.I. Kirkland, J.L. Hutchison (Eds.), *Nanocharacterisation*, The Royal Society of Chemistry, 2007.
- [24] K. Tiede, A.B. Boxall, S.P. Tear, J. Lewis, H. David, M. Hasselov, Detection and characterization of engineered nanoparticles in food and the environment, *Food Addit. Contam. Part A, Chem. Anal. Control, Exposure Risk Assess.* 25 (2008) 795–821.
- [25] J. Wang, U.B. Jensen, G.V. Jensen, S. Shipovskov, V.S. Balakrishnan, D. Otzen, J.S. Pedersen, F. Besenbacher, D.S. Sutherland, Soft interactions at nanoparticles alter protein function and conformation in a size dependent manner, *Nano Lett.* 11 (2011) 4985–4991.

- [26] H.-G. Wolfgang, H. Dorothee, J. Klaus-Peter, F. Carl Georg, B. Harald, Current limitations of SEM and AFM metrology for the characterization of 3D nanostructures, *Meas. Sci. Technol.* 22 (2011) 094003.
- [27] W. Zhou, R. Apkarian, Z.L. Wang, D. Joy, Fundamentals of scanning electron microscopy (SEM), in: W. Zhou, Z.L. Wang (Eds.), *Scanning Microscopy for Nanotechnology: Techniques and Applications*, Springer, 2006.