Improved light scattering measurement with microscopy data for nanoparticle number-based size distribution

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Abstract. Size is the key feature of nanoparticle properties. Nanoparticle size distribution can be measured by microscopy technique and light scattering posterior to a separation technique. Discrepancies can be observed, especially in the case of polydisperse samples. Microscopy is more precise for size and shape measurement, while light scattering provides better information about concentration. An experiment is set up to investigate the feasibility of bringing comparability of Asymmetrical Flow Field-Flow Fractionation followed by Multi-Angle Light Scattering and AFM measurements. A sample made from a mixture of stock samples is built, after detailed characterisation of the stock samples. By obtaining comparability of MALS and AFM measurements, a new way to calibrate MALS instrument without the need for reference material is opened: using aliquots of the sample itself as a reference.

1. Introduction
Nanoparticles have grown as a huge market thanks to their new applications. Their size of the order of the nanometre, typically between 1 and 100 nm, provides them with unique properties. Their small size offers a larger specific surface area than the same substance at the macroscopic level enhancing surface reactivity. The small size of the nanoparticles goes together with potential risks on health and environment. For proper assessment of their properties, an accurate measurement of their size is thus of prime concern. Along that perspective, several EU regulations about cosmetics (1223/2009), labelling (1169/2011) and biocidal products (528/2012) [1,2,3] have been set up for the identification as “nanomaterial”. With some variations, these definitions all rely on assessing the number-based size distribution of the nanoparticles.

Numerous measurement techniques have been developed to measure the size of the nanoparticles. However, these techniques provide different average sizes, especially on polydisperse samples [4]. Based on different physical principles, different methods have different measurands, and provide different signal amplitudes – weights. Microscopy techniques are counting methods and provide a number-based size distribution. Light absorption and light scattering methods provide mean size weighted by either the volume of the particle (d³) or the volume squared (d⁶, called “intensity-weighted”).

Facing a lot of methods and discrepancies among them, reference materials were favoured for calibration of particle sizers. However, the selected reference material has to be as much as possible similar to the analyte and implicitly some prior knowledge is needed. Certified materials are not always available for all the instruments. In presence of polydispersity, different sensitivities relative to the size
give rise to different certified values \[5,6\]. One way out of this problem would be to characterise the sample using its size distribution instead of its average size and uncertainty. For ensemble techniques like Multi-Angle Light Scattering (MALS) and UV absorption, it is only possible after separation according to the size (or a size-related measurand). Asymmetrical Flow Field-Flow Fractionation (AF4) separates the nanoparticle sample according to the hydrodynamical size.

The purpose of the present paper is to set up a methodology to extract size distribution from MALS measurements, calibrated with traceable Atomic Force Microscopy (AFM) measurement, using the sample itself as a reference. This is achieved by collecting fractions of the sample from AF4, measuring them using AFM through a SI traceable method and correcting the MALS size measurement accordingly. The coupling of AFM to light scattering techniques has previously been applied for complementary characterisation of samples \[7, 8, 9, 10\] but their comparability has never been developed.

2. Experimental method

Size distribution measurement with MALS can be obtained when combined with a size-based separation technique. In the present experimental setup, MALS is combined with Asymmetrical Flow Field-Flow Fractionation, AF4. The same sample being measured by AF4-MALS and AFM, appropriate dilution condition should be found for both techniques.

Optimal AF4 separation requires well dispersed sample and low interaction of the dispersion with the separation membrane. AFM sample deposition on mica substrate as in \[11\], requires strong electrostatic binding interactions between the particles and the substrate. Added salt or surfactant necessary for the dispersion should be kept as low as possible.

In a first experiment (Experiment (1) in Figure 1), AFM and AF4-MALS are performed on two different monomodal samples obtained from the same manufacturer and prepared following a common method. In a second experiment, the measurements are performed on aliquots collected from AF4 fractionation of a mixture of the two previous samples. The sample undergoes a dual step separation: the first is preparative (producing aliquots) and the second is analytical (to obtain the MALS size distribution of the aliquots). In Experiment (2a) (see Figure 1), quality of the AF4 separation is assessed by reinjecting the aliquots and comparing the size distributions obtained by MALS after the first separation and after the second one. In Experiment (2b), size distribution measurements of the aliquots obtained by AF4-MALS and AFM are compared.

3. Material and method

3.1. Sample preparation

The samples are Polystyrene Polybead Microspheres presenting nominal size of 50 nm (52 nm; standard deviation 10 nm; 2.5% w/v) and 100 nm (88 nm; standard deviation 10 nm; 2.5% w/v). 0.5 mM of sodium dodecyl sulfate (SDS) in ultrapure water is used as eluent for the fractionation, after filtration.
through 0.1 µm regenerated cellulose filter. Samples are prepared by diluting stock samples 300x (v/v) into eluent for Experiment 1. For preparative separation (Experiment 2a), 5 µL of a mixture of the stock samples is injected, without prior dilution.

3.2. AF4-MALS fractionation and measurement

The AF4-UV-MALS setup is composed of an Agilent HPLC stack with an analytical scale fraction collector, a Wyatt Eclipse DualTec (AF4) and a Wyatt Heleos II (MALS).

The injection loop is made of PEEK tubing and has a volume of 20 µL. The separation channel is a short channel, with 350 µm spacer (wide model). The membranes were provided by Wyatt (regenerated cellulose, 10 kDa cutoff).

Size is measured by MALS and concentration for each time slice in the chromatogram is measured by the UV detector. UV wavelength is set to 220 nm for polystyrene.

The MALS and UV data are analysed using Particle module of Astra software. Data from the manufacturer show that the nanoparticles are spherical. The sphere model is thus used to fit the angular MALS intensities, and to determine the size of nanoparticles. The MALS calibration (referred to as “normalization” by the MALS community) is performed using Polystyrene Nanobead NIST traceable size standard (Disc Centrifuge Sedimentation certified mean diameter: 69.3 ± 0.9 nm; standard deviation 10.2 nm; 1% w/v), using the same setup, method and parameters as for the measurement.

The AF4 separation method development is performed on a mixture of 50 nm and 100 nm nominal size polystyrene nanoparticles samples. In view of further AFM measurement, the concentration of the collected aliquots should be maximal. This is achieved by a multifactor compromise: having the AF4 detector flow V_d (output to detectors) as small as possible, for a fixed separation quality (determined by V_x/V_d, as a first approximation); and collecting sample during a minimal amount of time around the maxima of concentration in the chromatogram. For a fixed detector flow, low cross-flow (V_x, flow responsible for separation according to size) also reduces the membrane-sample interaction. The recovery is thus expected to be higher, at the price of diminishing the separation power. The main features of the selected method are: detector flow V_d=0.5 mL/min and constant cross-flow V_x=0.75 mL/min. Sample is focused during 5 min (injected in the first 4 min) and the elution duration is 50 min. The two aliquots (later named aliquots 1 and 2) are collected during 1 min at instants 1 and 2, as illustrated on an example chromatogram in Figure 2, resulting in 0.5 mL aliquots.

![Figure 2](image)

Figure 2 UV and Light scattering at 90° (LS) chromatograms of the mixture of nominal size samples 50 nm and 100 nm. The two slices correspond to the collected fraction – aliquots 1 and 2 – for analysis after separation.
An Asylum Research (Oxford Instrument) MFP-3D Infinity AFM operating in AC mode is used in this work. All the measurements are performed in air at ambient laboratory conditions using PPP-NCHR silicon cantilevers.

Nanoparticles samples are prepared depositing a drop of sample dispersions (specifically prepared or aliquot collected after fractionation) on a poly-L-lysine-coated mica substrate. After 10 minutes, mica substrates are rinsed with deionized water and dried under filtered nitrogen flow.

AFM images are treated and analysed using SPIP software (Image Metrology), with Particle and Pores module. A first order plane fit is applied to the captured images before using SPIP Particle and Pores module to detect and determine the maximum height of the particle relative to the substrate surface, as measurand.

3.3. Data analysis
Data analysis for comparison of AFM and AF4-MALS measurement is performed in Mathematica. One is here interested in the size distribution itself and not the average size of the sample. The measurement result is thus summarized as a single number as average size ± standard deviation of the distribution. Average size and standard deviation are obtained by statistical analysis on weighted histograms, where one data point corresponds to one particle for AFM and one data point corresponds to one elution time slice for AF4-MALS.

4. Experiments and results

4.1. Experiment 1: Analysis of stock samples with common sample preparation
In a first step, two samples of nominal value of 50 nm and 100 nm are analysed separately using both AFM and AF4-MALS, with common sample preparation. Each sample dispersion was diluted 300 times (v/v) in 0.5 mM SDS.

The size distribution of the nominal 50 nm sample obtained by AF4-UV-MALS is presented in Figure 3. For each time slice, the size is measured by MALS and the relative concentration by UV. The average measured size with the corresponding standard deviation is 43 ± 6 nm.

![Figure 3 Chromatogram of the measured size with elution time (left) and size distribution, volume-based (right) for the 50 nm nominal sample.](image-url)

The size distribution of this sample obtained by AFM is presented in Figure 4 (left). The distribution is presented in its natural form: the number-based size distribution. The average measured size and the corresponding standard deviation is 29 ± 12 nm.

For microscopy, the signal processing consists in counting all the particles measured on the images, all particles having the same weight. For light absorption, as in UV, each atom of a nanoparticle absorbs the same amount of light as the other. As a consequence, the absorption is proportional to the number of atoms, that is to say the volume \((d_i)^3\) at a time-slice i. The measured size distribution by AF4-UV-MALS is thus volume-weighted. For light scattering based signal such as concentration measurement
with MALS, the light intensity in a given direction comes from interferences from light emitted by the atoms. It is thus proportional to \( d_i^3 \times d_i^3 = d_i^6 \), to first order.

A simple concentration reweighting formula can thus be written down to express a measurement of a given sample as it would be if it were performed on the same sample but with a different measurement technique:

\[
w_{\text{rew}}(d_i) = c \times w_{\text{meas}}(d_i) \times (d_i)^{n_{\text{rew}}-n_{\text{meas}}},
\]

where \( c \) is fixed afterwards for normalization as a probability density function:

\[
c = 1 / \sum d_i^{n_{\text{rew}}-n_{\text{meas}}}
\]

and \( n_{\text{rew}} \) (or \( n_{\text{meas}} \)) is 0, 3 or 6 if the output (or measurement) scheme is number-based, volume-based or intensity-based, respectively. It is applied on the more detailed level of information at hand, i.e., for every elution time slice for AF4-UV-MALS and for every particle for AFM.

The results of reweighting the previous measurement to number-, volume- or intensity-based schemes are presented in Table 1, and corresponding number-based distributions are shown in Figure 4.

| Weighting scheme   | AFM            | MALs           |
|--------------------|----------------|----------------|
| Number-based       | 29 ± 12 nm     | 40 ± 7 nm      |
| Volume-based       | 40 ± 8 nm      | 43 ± 6 nm      |
| Intensity-based    | 43 ± 6 nm      | 46 ± 5 nm      |

Table 1 Size comparison of 50 nm nominal size sample after conversion to common weighting scheme. Measurements (without reweighting) are in bold.

Figure 4 AFM (measured) and MALS (reweighted) size distributions, in number-based scheme. The red lines show the average size of the distributions.

In terms of average values, the results agree well for the reweighting in volume and intensity. There is a clear discrepancy for the number-based reweighting. This discrepancy can be explained by the low-size cut-off of the technique, as shown in the distribution of Figure 4: AFM measures particles as small as 10 nm while the cut-off of the MALS is 30 nm. This MALS cut-off is mainly due to small intensity leading to more difficult fitting of the signal.

For high sizes, potential discrepancies are enhanced by reweighting to intensity, though less pronounced. The lower statistics of AFM at high sizes lead to the more noisy aspect of the distribution, while the MALs results are smoother there, but similar high threshold (55 nm) is obtained. To summarize, AFM is more sensitive to smaller sizes and MALs to higher, comparatively, which leads to a discrepancy when reweighting distribution with nanoparticles smaller than 30 nm.

Figure 5 shows the results of AFM and MALs measurements, on the same scale, for the 100 nm nominal size sample. The observation is similar to the 50 nm nominal size sample, but to a lesser extent: AFM reveals some low size population between 50 nm and 75 nm, which MALs does not. As a consequence, the average size of the number-based size distribution for AFM is pulled towards smaller values (82 nm in Table 2) compared to its mode (about 90 nm, see Figure 5). However, the small particle size contribution is limited, such that there is a good agreement between the number-based reweighted average particle size.
Figure 5 AFM (left) and MALS (right) measurements of 100 nm sample.

Table 2 Size comparison of 100 nm nominal size sample after conversion to common weighting scheme. Measurements (without reweighting) are in bold. Results are comparable for all schemes.

| Weighting scheme | AFM   | MALS  |
|------------------|-------|-------|
| Number-based     | 82 ± 15 nm | 89 ± 8 nm |
| Volume-based     | 88 ± 9 nm  | 89 ± 7 nm  |
| Intensity-based  | 90 ± 6 nm  | 90 ± 6 nm  |

4.2. Experiment 2: Comparability of AFM and AF4-UV-MALS measurements on fractionated sample

4.2.1. Experiment (2a): Preparation of fractionated samples by the AF4-MALS and assessment of the separation quality.

The quality of the separation can be verified through a second separation of the aliquot, as illustrated in Figure 1, Experiment (2a). Good separation quality is necessary in order to reach comparability of AFM and MALS results.

5 µL of an equal mixture of undiluted 50 nm and 100 nm nominal size samples are injected in the 20 µL injection loop. Using only the few non-saturated photodetectors of the MALS, size measurement is still possible. The result is shown on light grey curve in Figure 6. Populations from both samples are separated. The chromatogram shows gaps in the size measurement of the 100 nm nominal size peak (see about 23, 25 and 30 min of elution time on Figure 6) when no size measurement is possible (all MALS photodetectors are saturated) or in transition regime (rapid changes with several photodetectors becoming saturated). Based on the knowledge from the 50 and 100 nm nominal samples, the chromatogram on Figure 6 shows that the separation is effective and initial populations from the stock samples (see Experiment 1) could be recovered, even though no baseline separation could be observed and peak tailing is present which is the sign of membrane overloading [12,13].

Aliquots 1 and 2 are collected from the apex of the two peaks (on light grey curve on Figure 6; illustrated on Figure 2). A careful cleaning of the AF4-UV-MALS is performed prior to the injection of the aliquots for a second fractionation (analytical separation). The same membrane is kept for the analysis of the aliquots. Aliquots 1 and 2 are separately injected resulting in the chromatograms black and dark grey, respectively. Size and concentration can be measured with no saturation of UV-MALS detectors, confirming the adequate concentration adjustment of the mother dispersion: their concentrations are similar to the samples used in Experiment 1.
Figure 6 Chromatogram of LS 90° (solid lines) and corresponding geometric diameter (dotted lines). Light grey is the mother dispersion (mixture of undiluted 50 nm and 100 nm samples). Black and dark grey curves are the chromatogram of aliquot 1 (associated with 50 nm sample peak) and aliquot 2 (associated with 100 nm sample peak).

With subsidiary injection of aliquots, a size distribution is available for each vial by AF4-MALS measurement, which makes possible a quantitative assessment of the improvement of monodispersity by comparing the AF4-MALS standard deviation from stock samples and after the second separation: for the most polydisperse sample (50 nm), the standard deviation is reduced from 6 nm to 4 nm in volume-based scheme (see Table 1 and Table 3).

4.2.2. Experiment (2b): AFM and MALS measurement of the same fractionated sample.

First AFM tests on aliquot 1 shows too small nanoparticle concentration. The aliquot is thus concentrated by evaporation in the vial under the fume hood at ambient temperature, and maintained in movement by an agitator. The sample is then deposited on a mica substrate, without poly-L-lysine coating. The re-concentration of sample obtained from AF4 separation for subsequent microscopy measurement should be optimized. Measured size distribution is presented on Figure 7.

Compared to the stock sample, AFM size distribution is narrower. Sizes below 20 nm are absent from the collected vial, showing that the separation is effective. Higher size limits are similar for aliquot 1 on 50 nm nominal size sample (from Experiment 1).

Figure 7 Measured size distribution by AFM of aliquot 1 (at the apex of the first peak).
The same reweighting methodology as in Experiment 1 is applied and mean size and associated standard deviation are obtained (Table 3). The MALS mean value fits well to the AFM mean value. Strangely enough, the size distribution is larger for the AFM than for the MALS (see Figure 8). This could be explained by insufficient concentration at peak edges for the UV-MALS detectors.

Compared to Experiment 1 on 50 nm nominal size stock sample, polydispersity is also reduced. This is reflected in two features: standard deviation are smaller for all reweighted measurement and the average values are all compatible and vary less depending on the reweighting scheme. It is important to note that the two separations (preparative and on aliquots; same AF4 method for both) were with low cross-flows, which is non-optimal separation for AF4.

For the number-based size distribution, the agreement is very good between both techniques: 36 ± 6 nm for AFM and 36 ± 4 nm for MALS. The two distributions nicely overlap, as shown on Figure 8.

Comparability of measurement can thus be achieved, already with slight separation (Vx/Vd ratio lower than 2) rigorously kept under control. Both instruments are comparable, if calibration is applied.

Table 3 Average sizes from the AFM and MALS size-distribution for aliquot 1. Measurements (without reweighting) are in bold.

| Weighting scheme | AFM       | MALS      |
|------------------|-----------|-----------|
| Number-based     | 36 ± 6 nm | 36 ± 4 nm |
| Volume-based     | 39 ± 5 nm | 37 ± 4 nm |
| Intensity-based  | 41 ± 5 nm | 38 ± 3 nm |

Figure 8 Superposed number-based size distribution by AFM (blue) and AF4-MALS (red) of the aliquot 1.

5. Development of a methodology for MALS calibration with AFM measurement

Previous experiment suggests that the proposed method could be used to build calibration sample in-house. The first step would be to perform AF4-MALS separation of a given sample (analogous to preparative separation in this work), keeping in mind to have high concentration while avoiding detectors saturation. An aliquot of the fractionation would then be measured by AFM in a traceable way. Special attention has to be brought to sample deposition on substrate and to AF4 separation in view of having monodisperse aliquot. The size distribution from AFM will be reweighted to intensity-scheme and the intensity-weighted average size computed. The uncertainty on the calibration directly depends on the monodispersity of the calibration aliquot. Analysis of the quality of the separation during validation is thus important. This average size will be used for the calibration (normalization) of the MALS. In a second step, the full AF4-MALS size distribution of the given sample is obtained from the full separation after application of the calibration.
6. Conclusions
In this work, comparability of MALS and AFM measurements is achieved through appropriate reweighting of the data. Considering the distribution as the final result instead of single numbers (average, standard deviation) is needed for proper characterisation of the polydisperse sample. The analysis of two stock samples shows that a common sample preparation can be found for AFM and AF4-UV-MALS measurement. If the sample is polydisperse, AFM measurement provides better information on the low size part of the distribution and MALS on the high-size part.

Separation by AF4 of a sample made from two separate polystyrene nanoparticle samples provides aliquots which are collected and measured as size distributions for both AF4-MALS and AFM. Separation quality has been assessed by these subsequent measurements, despite overloading and peak tailing from the injection of highly concentrated sample. The requirements in concentration for both techniques are not compatible and demand some engineering. The sample must be specifically reconcentrated for AFM measurement. Eventually, comparability between the instruments is achieved on aliquots obtained from AF4 separation, with similar distributions after proper reweighting. This is the first step of calibration by comparison. This opens the perspective of bringing SI traceability into MALS measurement via AFM measurements. This methodology avoids the drawbacks of introducing third-party reference materials and improves the calibration.

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