Quantification of nanoparticle concentration in colloidal suspensions by a non-destructive optical method

Sandhya Clement, Brint Gardner, Wan Aizuddin W Razali, Victoria A Coleman, Åsa K Jämting, Heather J Catchpole, Ewa M Goldys, Jan Herrmann and Andrei Zvyagin

1 ARC Centre of Excellence for Nanoscale Biophotonics, Macquarie University, Sydney, NSW, Australia
2 Nanometrology Section, National Measurement Institute Australia, Lindfield, NSW, Australia
3 Department of Physics, Faculty of Applied Sciences, Universiti Teknologi MARA Pahang, Jengka, Pahang, Malaysia
4 Institute of Molecular Medicine, Sechenov First Moscow State Medical University, Russia
5 Laboratory of Optical Theranostics, Nizhny Novgorod State University, Nizhny Novgorod, Russia

E-mail: andrei.zvyagin@mq.edu.au

Received 9 August 2017, revised 13 September 2017
Accepted for publication 19 September 2017
Published 26 October 2017

Abstract

The estimation of nanoparticle number concentration in colloidal suspensions is a prerequisite in many procedures, and in particular in multi-stage, low-yield reactions. Here, we describe a rapid, non-destructive method based on optical extinction and dynamic light scattering (DLS), which combines measurements using common bench-top instrumentation with a numerical algorithm to calculate the particle size distribution (PSD) and concentration. These quantities were derived from Mie theory applied to measurements of the optical extinction spectrum of homogeneous, non-absorbing nanoparticles, and the relative PSD of a colloidal suspension. The work presents an approach to account for PSDs achieved by DLS which, due to the underlying model, may not be representative of the true sample PSD. The presented approach estimates the absolute particle number concentration of samples with mono-, bi-modal and broad size distributions with <50% precision. This provides a convenient and practical solution for number concentration estimation required during many applications of colloidal nanomaterials.

Supplementary material for this article is available online

Keywords: nanoparticle concentration, extinction spectra, dynamic light scattering, Mie scattering, silica nanoparticles, nanoruby, particle size distribution

(Some figures may appear in colour only in the online journal)

Introduction

The number concentration of nanoparticles in colloidal dispersions is an important metric for a broad spectrum of studies in nanotechnology and nanomedicine [1–8]. For example, in in-vitro and in-vivo toxicity tests, the particle concentration is of critical importance to determine the maximum cellular dose [9–11]. Accurate measurement of the number concentration of nanoparticles—that is, particles in the size range from approximately 1 to 100 nm—is a particular challenge. In contrast to parameters such as particle size and size distribution, there are currently no certified reference materials, reference methods or dedicated techniques for measuring nanoparticle number concentration [8].

Historically, optical particle counting (OPC) has been applied to determine the particle number concentration by counting the number of particles contained in a known volume of suspension [12, 13]. However, OPC cannot be
used reliably to detect nanoparticles due to the diffraction-limited optical resolution in direct imaging OPC systems, and/or due to insufficient signal-to-noise ratios for individual particles, especially for particle sizes below ~300 nm in obscuration-based or ~50 nm in scattering-based OPC systems [14]. Transmission electron microscopy (TEM) is a powerful technique for the characterisation of nanoscale objects [15, 16], however, the determination of particle number concentration by this method is time-consuming and not suitable for rapid, routine measurement of samples in situ [16]. Gravimetric methods are regarded as the simplest technique to estimate the nanoparticle mass concentration [8]. In this method, the number of nanoparticles in colloidal suspension can be estimated by measuring the total mass of nanoparticles, and given knowledge about the average particle composition, size and geometry, to calculate the mass of a single particle. However, the mass balance detection limit determines the minimum detectable concentration and represents a challenge for practical concentrations used in many chemistry and biochemistry procedures.

Particle sizing techniques that rely on the interaction between particles and light, for example, dynamic light scattering (DLS) or differential centrifugal sedimentation, typically report particle size distribution (PSD) data, as a function of the measured intensity of the normalised scattered light for a particular size range [17–20]. By making assumptions about the particle geometry and applying Mie theory [21], it is possible to convert these measured intensity-based distributions into particle volume- or number-based distributions. This measurement can, in principle, be integrated to yield the particle concentration, provided the volume of the sample interacting with the laser beam, and the optical properties of the particles and suspension medium are known, although measurement artefacts such as stray light reflections from the cuvette walls make this difficult if achievable at all.

A range of emerging single-particle measurement techniques, such as particle tracking analysis [22, 23], tuneable resistive pulse sensing [24, 25], resonant mass measurement [26], and single particle inductively-coupled plasma mass spectrometry [27] also offer the possibility to measure particle number concentration based on the number of particles detected per unit volume of suspension. However, those techniques are not yet in common laboratory use, and, there are still challenges measuring particles below ~50 nm in diameter with these methods.

For a long time, it has been expected that the particle number concentration in colloidal suspension could be determined by measuring the optical extinction spectrum. In the simplest case of a monomodal PSD, and assuming the single-scattering regime which may be realised in dilute suspensions of non-absorbing colloidal particles, the absolute concentration can be theoretically determined by measuring the optical extinction coefficient, $\varepsilon_s$, defined by the following expression:

$$
\varepsilon_s = -\log(I_r/I_i),
$$

where $I_i$ and $I_r$ are the irradiances of incident, and transmitted light through the sample respectively. The number of photons incident on the sample is assumed to be reduced on passing through the sample by the scattering process alone. In this case, the number concentration, $C_s$ (number of particles per unit volume), is related to the optical extinction coefficient by

$$
\varepsilon_s \cong C_s \sigma_i L / \ln(10),
$$

where $L$ is the optical path length through the cuvette and $\sigma_i$ is the scattering cross-section. The measurement of $\varepsilon_s$ at a chosen wavelength, $[\varepsilon_s(\lambda)]$, is straightforward using a spectrophotometer. For homogeneous spherical particles, the scattering cross-section, $\sigma_i(\bar{d}, \lambda)$, can be obtained, for example, by using one of the online Mie scattering calculators (such as http://omlc.org/calc/mie_calc.html) and by specifying the mean particle diameter, $\bar{d}$, wavelength and relative refractive index. The relative refractive index is defined as $m = m(\lambda) = n_p(\lambda)/n_s(\lambda)$, where $n_p(\lambda)$ and $n_s(\lambda)$ denote the (wavelength-dependent) refractive indices of the particles and the suspension medium, respectively. Bulk refractive index values of a wide range of materials are available from internet resources (such as https://refractiveindex.info). Equation (2) is then applied to calculate the particle number concentration. If $\bar{d}$ is not known, the full optical extinction spectrum is used to infer $\bar{d}$ by fitting the simulated extinction coefficient as a function of $\lambda$ to the measured one $[\varepsilon_s(\bar{d}, \lambda)]$ by varying $\bar{d}$. More sophisticated inverse scattering algorithms have been developed to determine the PSD and $C_s$ for arbitrary PSDs [28]. These algorithms, however, require constraints based on educated guesses, and, in general, are sensitive to both measurement accuracy and measurement artefacts. It is commonly accepted that an additional measurement is needed to supplement the optical extinction spectrum to reduce the complexity and improve the accuracy of quantification of $C_s$.

In this work, we propose a simple, rapid and non-destructive method for which we coined the name EXDLS, to estimate the nanoparticle number concentration in a suspension of homogeneous, spherical nanoparticles based on the measurement of optical extinction using a standard spectrophotometer and the measurement of the PSD. These data are processed by a MATLAB program described below. In order to measure the PSD, we use DLS in this work, but, in principle, any method can be used. Spectrophotometry and DLS are widely available in many laboratories, and routinely used for the characterisation of nanoparticles. The concentrations deduced from these measurements using our theoretical modelling are compared to the nominal concentrations (based on manufacturer specifications) as well as the concentrations determined from TEM measurements and thermo-gravimetric analysis (TGA). We present the evaluation of systematic errors attributed to the DLS measurements and establish that absolute number concentration can be quantified within 50% error margins for monomodal, bimodal and broad PSD colloidal samples, whereas the relative $C_s$, useful for determining the dilution ratio, can be quantified with 5% accuracy. We believe that our results can guide planning of many nanotechnology and biochemistry procedures.

**Materials and methods**

Polystyrene latex (PSL) spheres of nominal size 20, 50, 90 and 200 nm (certified diameters: 20 ± 2 nm, 46 ± 2 nm,
92 ± 3 nm and 203 ± 5 nm; traceable to the SI unit of length as realised at the National Institute of Standards and Technology, USA) were purchased from Thermo Fisher Scientific (Australia). Silica nanoparticles (concentration, 1% by weight, certified intensity-weighted harmonic mean diameter 19 ± 0.6 nm) (ERM FD100, JRC IRMM) and nanoruby (synthesised in house according to the reported protocol [29]) were used for comparative studies. Low-volume (3.0 ml) polystyrene cuvettes were used for all measurements.

To validate the proposed theoretical model, reference PSL spheres with known concentrations were used. To begin with, we investigated PSDs and concentrations of monomodal samples. The as-received samples (stock concentration of 1% weight/weight) were diluted 60 times with deionised (DI) water (MilliQ, 0.22 μm final filter DI water, 18.2 MΩ cm). To quantify the number concentration of colloidal samples featuring bimodal distributions, several samples were prepared by mixing the monomodal PSL samples in different ratios, as well as unique initial dilution amounts, as specified in table 1 below.

The extinction spectrum for each sample was measured using an ultraviolet–visible–infrared spectrophotometer (Cary-5000, Agilent) for wavelengths ranging from 400 to 800 nm. The lower wavelength limit was chosen as 400 nm due to the use of disposable polystyrene cuvettes, which are known to absorb at shorter wavelengths. Zero and baseline corrections were carried out before each measurement to obtain the extinction spectra of the nanoparticles alone.

The DLS measurements of the relative distributions of the hydrodynamic diameter of the samples were carried out using a Zetasizer Nano ZS (Malvern Instruments, UK) equipped with a HeNe laser with λ = 633 nm. The instrument operates by detecting the backscattered laser light and recording its intensity fluctuations as a function of time. The autocorrelation analysis of the recorded signal yields the light intensity-based PSD which, by applying Mie theory, can be transformed into volume or number-based PSDs. In addition, we used a recently developed ‘protein analysis algorithm’ within the Zetasizer user interface to refine the PSDs to produce more accurate evaluations of modal diameters with a narrower PSD width, thus making it possible to resolve peaks in the bimodal distribution samples which could not be resolved by the default ‘general purpose’ algorithm. The PSDs derived from DLS analysis may not represent the true size distribution. This is because the scattering intensity increases rapidly with increasing particle size, causing the PSDs to be heavily weighted towards any larger particles that may be present in a sample [30]. This weighting [31] towards larger particles was also observed in our measurements where the smaller modes of the bimodal PSDs could not be resolved.

Both TGA and TEM were used to estimate the particle number concentrations in the cases of silica, whereas TEM alone was used for benchmarking the estimated number concentration of the colloidal ruby sample. A PerkinElmer STA6000 Thermogravimetric Analyser was employed for TGA measurements. The performance of the balance in the TGA instrument was verified using a Mettler Toledo XS204 analytical balance. The TGA was operated by separating the solid content of a colloidal sample from solvents and labile fractions associated with solids, which were vapourised during heating. The colloidal silica reference material ERM FD100 (JRC IRMM), well characterised in terms of its hydrodynamic diameter (Dh) [32], was chosen for the method validation. It was particularly suitable for TGA analysis because the high silica melting point (1710°C) allowed high temperature analysis, leading to more thorough separation of the solid fraction of the colloidal sample from volatile components. The temperature of the internal environment (including the crucible) was brought to room temperature (20°C) before inserting 15 μl of the colloidal silica. The heating program involved heating a crucible to 200°C before cooling back to 20°C. The mass of the sample was monitored throughout the heating and cooling cycle. The number of silica nanoparticles was determined using the following equation:

\[ N = \frac{M_{\text{total}}}{\sum p_n M_n} \]  

where \( N \) is the number of particles present, \( M_{\text{total}} \) is the total mass of particles, \( p_n \) is the fraction of particles in the sample of diameter bin \( n \) and \( M_n \) is the average mass of the diameter bin \( n \). The concentration is calculated by dividing the number of particles by the initial volume of the sample placed into the TGA crucible (15 μl in this case).

For cross-validation of our method for a broad size distribution we used a sample of nanoruby colloids and determined the effective concentration via particle counting from TEM images. The TEM images were taken using a PHILIPS CM10 system with an accelerating voltage of 100 kV. The sample was prepared by depositing a 0.3 μl droplet of the diluted nanoruby colloidal sample onto a 300-mesh copper grid, ensuring an entire droplet was contained on the grid, and left to dry. The dried nanoparticles were found predominantly localised in the droplet rim. By identifying and counting the number of nanoruby particles present in the rim fragment, the number of particles contained within the ring of the sample and hence the concentration of colloidal nanoruby particles was extrapolated.

| Sample | Diameter (nm) | Volume of PSL sample (μl) | Ratio of PSL suspension volumes | Volume of diluent water (μl) |
|--------|--------------|--------------------------|-------------------------------|-----------------------------|
| 1      | 20 ± 2       | 300                      | 5:1                           | 2640                        |
| 2      | 46 ± 2       | 60                       | 5:1                           | 2640                        |
| 3      | 46 ± 2       | 92 ± 3                   | 60                            | 2500                        |

Table 1. Bimodal PSD polystyrene samples prepared by mixing two monomodal samples.
Theoretical modelling and data processing

The developed numerical algorithm for quantification of the number concentration of particles in colloids, $C_s$, is illustrated in figure 1. Two measurements were carried out: the DLS measurement yielded the light intensity-based PSD, $f(d)$ of the particle diameter, $d$, sampled in bins (logarithmically spaced), while the $\varepsilon_r(\lambda)$ spectrum was acquired using a spectrophotometer. An example of such measurements is shown in figure 2. In the case of the monomodal PSD samples (figure 1(a)), the modal diameter $d_0 \approx \bar{d}$ was read out from the peak of PSD of scattered light intensity. The value of $\bar{d}$ was used to construct a new PSD, termed gamma-distribution, based on the Drude model as given in [33] (theoretical

Figure 1. Schematic diagrams of algorithms for processing optical extinction spectra, $\varepsilon_r(\lambda)$ and PSD by intensity, $f(d)$ to obtain the number concentration, $C_s$ in case of log-normal (a) monomodal and (b) bimodal PSD colloidal samples.

Figure 2. Concentration measurement of monomodal PSL 90 nm sample using EXDLS method. (a) Extinction coefficient as a function of wavelength; red line and dotted line graph show their corresponding fitting in extinction and particle size. Inset, the normalised value of the calculated concentration estimated using EXDLS method versus as-prepared number concentration and for different dilution of monomodal PSL 90 nm nanoparticles. (b) Intensity-weighted PSD obtained from DLS.
distribution, figure 1(a):
\[
f_{\mu}(d) = A \times \left( \frac{d}{d_0} \right)^\mu \exp \left[ -\mu \left( \frac{d}{d_0} \right) \right].
\] (4)

Here, \( \mu \) is related to the full-width of half maximum, \( \Delta d \) of the new PSD in the following way: \( \mu = 6.15 \left( \frac{d_0}{\Delta d} \right)^2 \). \( A \) is a normalisation coefficient obtained by integrating the PSD over all size bins:
\[
\sum_d f_{\mu}(d) = 1.
\] (5)

This model was introduced in seminal works on inverse scattering problems [34] and was/is broadly acceptable [35, 36]. It captures colloidal and aerosol PSDs more faithfully, commonly featuring an extended tail towards the large sizes, although there is always a possibility that particle distributions may have other shapes. The authors’ own experience also supports the choice of equation (4) as an adequate approximation of true PSDs [37].

Multiplying both sides of equation (5) with \( C_f \), we obtain the particle number concentration, expressed as a sum of size fractions \( C_f f \). A theoretical prediction of \( \varepsilon_{\text{th}}(\lambda) \) is given by:
\[
\varepsilon_{\text{th}}(\lambda) = C_f \sum_d \sigma(\lambda, d) f_{\mu}(d),
\] (6)

with \( \sigma(\lambda, d) \) calculated using Mie theory incorporated in Matzer’s Matlab subroutine (http://omlc.ogi.edu/software/mie/). The subroutine requires tabulated values of the refractive indices of the medium (water in our case) and particle bulk material. These are readily available, for example from the open internet databases [38]. Fitting of \( \varepsilon_{\text{th}}(\lambda) \) to the measured \( \varepsilon(\lambda) \) by varying \( \mu \) makes it possible to reconstruct \( f \) from the measured value of \( \mathcal{Z} \) and fitted value of \( \mu \) (fitted distribution, figure 1(a)) and to remove a constant background in \( \varepsilon(\lambda) \). Finally, the \( C_f \) is calculated using equation (6).

The calculation of \( C_f \) in the case of bimodal PSD is schematically explained in figure 1(b). This case is particularly important in many nanotechnology procedures, with most colloidal samples prone to aggregation manifested by the emergence of the second peak in PSD obtained by DLS and the appearance of larger sized peaks in the PSD. As in the case of monomodal PSDs, the algorithm relies on two measurements of \( f(d) \) and \( \varepsilon_d(\lambda) \), with the measurement examples presented in figure 3. In this case, two modal diameters \( d_1 \) and \( d_2 \) are determined from the modal value of the intensity peaks in the PSD and used as input parameters for the construction of a new PSD, \( f_{\mu}(d) \):
\[
f_{d}(d) = A_1 \times \left( \frac{d}{d_0} \right)^\mu \exp \left[ -\mu \left( \frac{d}{d_0} \right) \right]
+ A_2 \times \left( \frac{d}{d_1} \right)^\mu \exp \left[ -\mu \left( \frac{d}{d_1} \right) \right]
\] (7)

with the same normalisation condition as given in equation (5). In order to determine the weighting coefficients for each term in equation (7), we recalculated the PSD of scattered light intensity to PSD of nanoparticle numbers, by reading out the values of \( f(d) \) at \( d_1 \) and \( d_2 \). The number-weighted PSD was calculated by using Mie theory at the fixed wavelength at which the DLS system operated. In the case of the Zetasizer Nano ZS, the calculation of PSD by number is built into the proprietary software. An additional complication arose from the logarithmically spaced hardware correlator. This led to the size bin growing proportionally to the size values, and hence required an additional correction. For example, the second term in equation (7) was multiplied by a factor of \( d_1/d_2 \). The fitting procedure was carried out using an algorithm, as described in the case of monomodal PSDs.

**Results**

**Monomodal PSD samples**

This method was first applied to estimate the number concentration of PSL particles with a nominal size of 90 nm and nominal stock concentration of 1% weight/weight. This stock was diluted 60 times (final concentration: \( 3.5 \times 10^{11} \) particles ml\(^{-1} \)). The acquired PSD and extinction coefficient of this sample are shown in figures 2(a) and (b). The estimated particle number concentration was determined to be \( 2.5 \times 10^{11} \) particles ml\(^{-1} \), as compared with the value of \( 3.5 \times 10^{11} \) particles ml\(^{-1} \) calculated from the manufacturer’s nominal mass fraction. The same sample was used to test the EXDLS method of the quantification of absolute \( C_f \) and relative accuracy in case of several sample dilution ratios. The acquired extinction spectra and PSD by intensity and their fitting are presented in figure 2; the resultant \( C_f \), normalised to the as-prepared \( C_f \) and benchmarked against the normalised as-prepared \( C_f \), is presented in figure 4, together with other sample values. An inset of figure 2(a) shows a plot of the normalised values of the EXDLS number concentrations versus as-prepared concentrations of a monomodal sample for three different dilutions (diluted sequentially in 2:1 ratio). From the parameters of the linear fit (goodness \( R^2 = 0.99 \), slope fit of 0.95), we estimated the error of the dilution measurement to be less than 5%. Note that this systematic error was due to the large value of \( \varepsilon_d \) reaching 1, where the onset of the multiple-scattering regime is expected. This measurement set an empirical boundary between the multiple and single-scattering regimes, leading to the requirement that the tested sample dilutions must have \( \varepsilon_d < 1 \).

**Bimodal PSD samples**

In order to test the applicability of the developed EXDLS method to samples with more complex PSDs, we prepared bimodal PSD samples by mixing two monomodal PSL samples in ratios presented in table 1. The dilutions were verified by checking the count rate during the DLS measurement set-up sequence. The results of the DLS measurements are shown in figure 3 (right panel; data, histogram and black squares; fitting, black solid line). The extinction measurements were carried out and recorded as shown in figure 3 (left panel; data, black dashed line; fitting, red solid line).

We compared our results for both monomodal and bimodal samples to the nominal concentrations determined
from the mass fraction specified by the manufacturer. This nominal value is represented by the horizontal dashed line in figure 4. The calculated number concentrations were normalised against the nominal concentrations for all samples for ease of comparison. Black circles at the base of figure 4 represent the relative size difference between polystyrene spheres presented in each sample.

The concentrations obtained by our EXDLS method as compared with the nominal concentrations gave a good estimate of the particle number concentration within an error margin of 50%. This error was systematic producing an underestimation of the nominal concentration. We believe that the main source of this error can be attributed to the DLS measurements, which yielded the PSD in terms of the

Figure 3. Concentration measurement of bimodal samples using EXDLS method. Extinction coefficient as a function of wavelength (left) and intensity weighted particle size distribution obtained from DLS (right) for (a) sample containing 20 and 50 nm particles at a ratio of 5:1. (b) Sample containing 50 and 90 nm particles at a ratio of 5:1. (c) Sample containing 20 and 90 nm particles at a ratio of 10:1. Note that the red line and dotted lines in the left and right panels show the corresponding fit to extinction spectra and particle size distribution.
Comparison of EXDLS and TGA methods

TGA is a destructive method of determining the total mass of solids in a sample. In our case, the total mass of silica was measured in ERM FD100 by heating the sample to evaporate the solvent and other labile components associated with the solid silica. The mass of the silica sample placed in TGA system was monitored as a function of temperature and plotted, as shown in figure 5. The onset of water evaporation was noticeable at 80 °C followed by the stabilisation of mass loss with further temperature increase. The TGA measurements were conducted in triplicate, as represented in figure 5. TEM measurements of ERM FD100 shown in the inset of figure 5. The PSD analysis by TEM for this sample was carried out on an ensemble of 38 157 particles. The number concentration was calculated using the density of silica specified in the ERM FD100 certificate of analysis.

In order to calculate the number concentration using our EXDLS technique, the silica sample PSD and extinction spectra were also measured by DLS and spectrophotometry (see supporting information section S1 available online at stacks.iop.org/NANO/28/475702/mmedia). The $C_s$ determined by EXDLS, TGA with PSD determined by TEM, and the nominal 1%-by-weight concentration stated by the manufacturer are listed in table 2.

The TGA measurements, supplemented with the PSD obtained from the TEM imaging and analysis (denoted TGA-TEM) agreed to within 3.3% with the manufacturer’s nominal concentration. $C_s$ determined by EXDLS agreed with that of TGA-TEM and the nominal $C_s$ within 27% error.

Comparison of EXDLS and TEM particle counting methods

We further tested our EXDLS method using a colloidal sample of ruby nanoparticles prepared in-house by femtosecond laser ablation [29] characterised by a broad PSD. $C_s$ of this sample was also determined by performing TEM imaging of a drop of suspension allowed to dry on a grid, and counting the number of all particles in selected field-of-views. The nanoruby particles exhibited a broad PSD, as shown in the TEM image in figure 6(a) and the corresponding PSD in figure 6(b). The concentration of the nanoruby sample was too low to measure by TGA.

This sample was challenging for DLS because its smaller size fractions were underestimated due to the overshadowing of their scattering signals by the strong signals of larger scatterers. In order to recover the true PSD of the nanoruby sample, TEM imaging and analysis was used. In this case, we used the measurement of the optical extinction spectrum and the PSD as obtained by TEM. We diluted the as-produced nanoruby sample with water by a factor of 100 before depositing a 0.3 μl ($3 \times 10^{-4}$ cm$^3$) droplet onto a TEM grid. Figure 6(c) shows a ring of nanorubies obtained on the TEM grid after the droplet dried. The rim of particles was seen to be...
present in 33 squares of the grid. We determined the total number of particles present on the slide by counting particles present in one of the grid squares and multiplying the obtained value by 33. We repeated the same procedure for 3 different TEM images. By assuming that the distribution of particles is uniform about the rim, and observing that negligible particles were present in out-of-the-rim areas, we were able to calculate the sample \( C_s \).

The number concentration of nanorubies in the colloidal sample obtained by using TEM together with the value obtained using EXDLS method (see supporting section S2) are plotted in figure 7.

Our EXDLS method reported a value of \( C_s \) that was within 22% of the error margin of the TEM counting method.

**Discussion**

As noted above, the reported method produces a consistent underestimate of the colloidal concentration, where the main source of the systematic error is due to the disparity of DLS and extinction spectrum measurements. DLS yields the distribution of the hydrodynamic diameter of the particles [39, 40], whereas the distribution of the diameters of particulate scatterers is required as an input to the optical extinction spectrum to determine the absolute colloidal concentration. Considering monomodal PSDs, the absolute concentration is determined by using equation (2):

\[
C_s \approx 2.3\varepsilon s / \sigma L.
\]

The overestimated mean hydrodynamic diameter obtained from DLS, \( \bar{d} \), results in the underestimated \( \sigma_s \) and hence underestimated \( C_s \). \( \bar{d}_h \) is greater than \( \bar{d} \) by at minimum the hydrodynamic double layer thickness. This layer may be formed by charged moieties of the colloidal solution, and is often referred as Stern layer. The thickness of Stern layer depends on the colloidal particle surface charge, surface moieties and solution composition, and is generally, poorly controllable. As a rule of thumb, increasing the ionic strength of a solution reduces the Stern layer thickness due to a screening effect, thus reducing \( \bar{d}_h \). At the same time, an increase in ionic strength is often accompanied with particle aggregation, and the net effect is thus complex.

One can think of applying a correction factor to convert \( \bar{d}_h \) to \( \bar{d} \) aiming to reduce the error. By applying a correction factor of 10% so that \( \bar{d}_h = 1.1\bar{d} \), \( C_s \) was recalculated and compared with the base values of \( C_s \), and presented in figure S3. The application of such a correction factor shows a tendency to overestimate \( C_s \). This is not surprising considering its variability versus nanoparticle surface charge, surface moieties and ionic strength of the solution. The 10% correction seems to be reasonable for a limited range of \( \bar{d}_h \).

Another source of the method systematic error common to all optical scattering methods is due to the critical dependence of the particle \( \sigma_s \) on its size, \( d \), reaching \( d^6 \) in the Raleigh scattering regime. The contribution of smaller diameter particles to \( \varepsilon_s \) is underestimated due to the eclipsing...
effect of the larger diameter particles, which also results in underestimation of \( C_r \). In order to illustrate this source of error, figure S4 shows two extinction plots of two ‘90 nm’ and ‘90:20 nm’ polystyrene bead samples, where the concentration of 20 nm sample fraction is 5-fold greater than that of 90 nm sample fraction. As one can see, although the concentration of 20 nm sample greatly exceeds that of 90 nm sample, its contribution to the overall extinction spectrum is minute and can be easily overlooked. This source of error is especially prominent in case of broad PSD samples, where size modes are not resolved.

A further complication arises from the need to convert the intensity-based PSD measured by DLS into a number-based PSD. This strongly non-linear process will emphasise systematic deviations of the measured PSD from the true PSD, especially for the case of samples with PSDs that are not monomodal and narrow.

In order to ameliorate the dominant systematic source of error for quantification of \( C_r \), the following method improvements are envisaged. Extension of the extinction spectrum towards the shorter wavelength range improves recovery of the smaller PSD fraction, as can be appreciated by observing the increasing separation between the extinction curves in figure S3. Improvement of DLS methodology towards more accurate estimation of PSD width is helpful. Systematic studies of generic hydrodynamic diameter dependencies will enable corrections to the physical diameters to be made, hence improving the accuracy of quantification of \( C_r \). It is worthwhile to discuss the upper size limit of the EXDLS method. We tested the method with a largest particle size of 500 nm (data not shown), with no noticeable degradation of the accuracy. The data processing algorithm is based on Mie scattering theory, which has no size limitations provided that the particles are spherical. As such, the algorithm is robust, with the only limitation being the onset of the single-scattering regime, i.e. \( \mu_s = C_r \ll 1 \), which has implication on the upper particle size limit due to the large scattering cross-section, \( \sigma_r \) of larger size particles, although it can be compensated by reducing \( C_r \). The other limitations to the maximum applicable particle size is the issue of particles sedimenting during the data acquisition process, which becomes an issue for particles with diameters in the micrometre range.

We believe that our method may also be suitable for quantification of the concentration of non-spherical particles. In the Rayleigh limit, i.e. for particles with diameters much smaller than the wavelength, the scattering cross-section can be calculated as

\[
\sigma_s = \frac{3m^2V^2(2m^2 - 1)^2}{2\pi(m^2 + 2)^2},
\]

where \( m \) is the effective refractive index expressed as a ratio of the refractive indices of a scatterer \( n_s \) and suspension medium \( n_{ss} \); \( m = n_s / n_{ss} \); \( k \) is the wavenumber in vacuum and \( V \) is the volume of a non-spherical particle whose principal dimensions are comparable. A correction factor for this expression was obtained by Min et al [41] for the case of continuous distribution of ellipsoids (CDE), as defined by Bohren and Huffman [42], where equal-V randomly oriented CDE-distributed ellipsoids were assumed:

\[
\frac{\sigma_r}{\langle \sigma_r^{\text{CDE}} \rangle} = \frac{9}{2(m^2 + 2r(m^2 - 1) - 2 \ln m)},
\]

where \( \langle \sigma_r^{\text{CDE}} \rangle \) is the averaged scattering cross-section of the ellipsoid ensemble. \( \langle \sigma_r^{\text{CDE}} \rangle \) appears greater than \( \sigma_r \), although only marginally (3%) for most organic particles (\( m = 1.2 \)), reaching 30% for the highest refractive index crystalline nanomaterials (\( m = 2.0 \)). CDE portrays simple and realistic particle distribution with a negligible probability of occurrence of extremely elongated needles.

Using the \( T \)-matrix method in combination with geometrical optics and anomalous diffraction theory, the optical cross-sections of size–shape distributions of prolate and oblate spheroids with aspect ratios up to 5 can be computed within an accuracy of about 8% for all values of the size parameter and a wide range of refractive indices [43]. Reference [43] reports that for randomly oriented spheroids with the aspect ratio ranging from \( \nu/2 \) to 2 (representing prolate and oblate spheroids), the averaged scattering cross-sections can be approximated by an equal-volume sphere cross-section to within 20%. This was examined for the broad range of the size parameter expressed as \( 0 < \frac{2\pi^{2}(m - 1)}{\lambda} < 30 \) and was well beyond the Rayleigh range.

We believe the method may also be suitable for the quantification of the number concentration of heterogeneous particles. A multi-layered spherical structure is a very common type of heterogeneous particles represented by quantum dots, upconversion nanoparticles, etc [44]. The layered structure is oftentimes required to seclude particles from the environment and/or to endow them with additional functionalities. Assuming non-absorbing particles (which are beyond the scope of this paper), \( \sigma_r \) of multi-layered spherical particles can be calculated in the framework of Mie theory and lends itself to straightforward extension of the presented method. Porous colloidal particles represent another practically important class of heterogeneous particles, especially considering nano-drug delivery applications in the life sciences. A strong similarity between porous spheres with multiple, randomly located medium inclusions and hollow spherical shells has been found [41]. In the Rayleigh domain, a hollow spherical shell is a good approximation to a porous sphere for the absorption and scattering cross sections. It is much easier to carry out calculations for hollow spheres than for heterogeneous porous particles. DLS techniques are immune to particle composition, provided colloidal particles are non-absorbing in the laser illumination spectral range (in case of Zeatsizer Nano, the laser wavelength 633 nm). The particle composition is employed only when the intensity-weighted PSD is converted to a number-based PSD. The software we have developed can carry out these calculations, and commercially available DLS instrument are provided with data processing software that routinely performs this calculation.

In an analytical case of a colloidal nanomaterial with an unknown refractive index, the problem of quantification of the colloidal concentration is exacerbated and \( C_r \)
quantification precision is likely to deteriorate. Introducing additional constraints on the refractive index to be determined appears useful for recovering \( n_p(\lambda) \) of the tested nanomaterial. For example, Griffin et al [REF] have reported on a true estimate of the refractive indices of PSL, silica particles and casein micelles of bovine milk by applying Rayleigh–Gans–Debye theory to fit \( \varepsilon_2 \). The following constraint was applied for the method to hold: \( \frac{2mL}{\lambda} |1 - m| < 0.40 \), where \( d \) falls in the nanometre range for the reported \( n_p \) [45]. An additional measurement improves the robustness and accuracy of \( C_s \) quantification. For example, an additional set of data of \( \varepsilon_2 \) acquired by varying \( n_p \) enabled the accurate determination of \( n_p \) of silica particles in colloidal solution and estimation of the ensemble \( \Gamma \) [9]. If the variation range of \( n_m \) encompasses \( n_p \), the measurement of \( n_p \) is straightforward, and its value is read out at the minimum value of \( \varepsilon_2 \) plotted versus \( n_m \) as it has been demonstrated for the measurement of refractive index and its dispersion of up-conversion nanoparticles [46]. Measurement of the composite refractive index of a colloidal sample by using an Abbe refractometer represents another useful approach.

Having noted the above, the strength of the proposed EXDLS method is in providing a rapid easy-to-apply method for the estimation of particle number concentration. Although it is likely that with complex improvements the accuracy of the method could be improved, these are somewhat not in the spirit of the intention of the work, which is to provide an easily adoptable solution to scientists working at the bench.

**Conclusion**

In this work, we demonstrated a simple, non-destructive method suitable for rapid evaluation of the number concentrations of composition-homogeneous, non-absorbing nanoparticles in colloidal suspension. Only two measurements, routinely performed by common commercially available instruments are required to calculate the concentration of nanoparticles. These are the optical extinction spectrum and the normalised PSD. The method performance was evaluated by using several samples with monomodal, and more challenging bimodal and broad size distributions, and comparing them to nominal concentrations, and concentrations determined by orthogonal methods. We were able to measure, within 50% accuracy, the number concentrations of these different colloidal nanoparticle samples. The errors in our calculations were largely attributed to inherent inaccuracies in the PSD determination by DLS for bi-, multi-modal or broadband distributions. This reported EXDLS method of determination of the number concentration of nanoparticle colloids is likely to be suitable for the requirements of many nanotechnology applications. This may be the case, in particular, where only minute amounts of sample are available, such as in low-yield reactions, or for the measurements of doses in toxicity studies.

**Acknowledgments**

This work was partially supported by Australian Research Council (ARC) through its Centre of Excellence scheme (CE140100003); and Russian Science Foundation (7-19-01416).

**ORCID IDs**

Sandhya Clement @ https://orcid.org/0000-0001-9556-3508
Andrei Zvyagin @ https://orcid.org/0000-0001-8799-2257

**References**

[1] Luo X, Morrin A, Killard A J and Smyth M R 2006 Application of nanoparticles in electrochemical sensors and biosensors Electroanalysis 18 319–26
[2] Orringer D A, Koo Y, Chen T, Kopelman R, Sagher O and Philbert M 2009 Small solutions for big problems: the application of nanoparticles to brain tumor diagnosis and therapy Clin. Pharmacol. Ther. 85 531–4
[3] Sametband M, Shwekey I, Banin U, Mandler D and Almog J 2007 Application of nanoparticles for the enhancement of latent fingerprints Chem. Commun. 1142–4
[4] Tiquia-Arashiro S and Rodrigues D 2016 Extremophiles: Applications in Nanotechnology (Berlin: Springer) pp 163–93
[5] Tiwari D K, Behari J and Sen P 2008 Application of nanoparticles in waste water treatment World Appl. Sci. J. 3 417–33
[6] Elsabahi M and Wooley K L 2012 Design of polymeric nanoparticles for biomedical delivery applications Chem. Soc. Rev. 41 2545–61
[7] Prasad G 2009 Biomedical applications of nanoparticles Safety of Nanoparticles (Berlin: Springer) pp 89–109
[8] Shang J and Gao X 2014 Nanoparticle counting: towards accurate determination of the molar concentration Chem. Soc. Rev. 43 7267–78
[9] Khlebtsov B N, Khanaideev V A and Khlebtsov N G 2008 Determination of the size, concentration, and refractive index of silica nanoparticles from turbidity spectra Langmuir 24 8964–70
[10] Jiang W, Kim B Y, Rutka J T and Chan W C 2008 Nanoparticle-mediated cellular response is size-dependent Nat. Nanotechnol. 3 145–50
[11] De Jong W H, Hagens W I, Krystek P, Burger M C, Sips A J and Geertsma R E 2008 Particle size-dependent organ distribution of gold nanoparticles after intravenous administration Biomaterials 29 1912–9
[12] Schmoll L H, Peters T M and O’Shaughnessy P T 2010 Use of a condensation particle counter and an optical particle counter to assess the number concentration of engineered nanoparticles J. Occup. Environ. Hygiene 7 535–45
[13] Heim M, Mullins B J, Umhauer H and Kasper G 2008 Performance evaluation of three optical particle counters with an efficient ‘multimodal’ calibration method J. Aerosol Sci. 39 1019–31
[14] David Y 1996 Direct-reading instrumentation for workplace aerosol measurements. A review Analyst 121 1215–34
[15] Borchert H et al 2005 Determination of nanocrystal sizes: a comparison of TEM, SAXS, and XRD studies of highly monodisperse CoPt3 nanoparticles Langmuir 21 1931–6
[16] Pyrz W D and Buttery D J 2008 Particle size determination using TEM: a discussion of image acquisition and analysis for the novice microscopist Langmuir 24 11350–60

[17] Hoo C M, Starostin N, West P and Mecartney M L 2008 A comparison of atomic force microscopy (AFM) and dynamic light scattering (DLS) methods to characterize nanoparticle size distributions Nanoscale 10 89–96

[18] Kato H et al 2009 Reliable size determination of nanoparticles using dynamic light scattering method for in vitro toxicology assessment Toxicol. Vitro 23 927–34

[19] Laidlaw I and Steinmetz M 2005 Analytical Ultracentrifugation, Techniques and Methods (Cambridge: The Royal Society of Chemistry) pp 270–90

[20] Steinbrück A, Csáki A, Ritter K, Leich M, Köhler J and Kato H 2010 Critical evaluation of nanoparticle tracking analysis (NTA) by NanoSight for the measurement of nanoparticles and protein aggregates Anal. Chem. 10 104–13

[21] Hergert W and Wriedt T 2012 The Mie Theory: Basics and Applications (Berlin: Springer) pp 1–151

[22] Gallego-Urrea J A, Tuoriniemi J and Hassellöv M 2011 Applications of particle-tracking analysis to the determination of size distributions and concentrations of nanoparticles in environmental, biological and food samples TRAC Trends Anal. Chem. 30 473–83

[23] Filipe V, Hawe A and Jiskoot W 2010 Critical evaluation of nanoparticle tracking analysis (NTA) by NanoSight for the measurement of nanoparticles and protein aggregates Pharm. Res. 27 796–810

[24] Vogel R et al 2016 A standardized method to determine the concentration of extracellular vesicles using tunable resistive pulse sensing J. Extracell. Vesicles 5 11242

[25] Coumans F A et al 2014 Reproducible extracellular vesicle size and concentration determination with tunable resistive pulse sensing J. Extracell. Vesicles 3 25922

[26] Weinbuch D et al 2013 Micro–flow imaging and resonant mass measurement (archimetdes)–complementary methods to quantitatively differentiate protein particles and silicone oil droplets J. Pharm. Sci. 102 2152–65

[27] Pace H E, Rogers N J, Jarolimek C, Coleman V A, Higgins C P and Ranville J F 2011 Determining transport efficiency for the purpose of counting and sizing nanoparticles via single particle inductively coupled plasma mass spectrometry Anal. Chem. 83 9361–9

[28] Crawley G, Counil M and Di Benedetto D 1997 Size analysis of fine particle suspensions by spectral turbidimetry: potential and limits Powder Technol. 91 197–208

[29] Edmonds A M et al 2013 Nano-ruby: a promising fluorescent probe for background-free cellular imaging Part. Part. Syst. Charact. 30 506–13

[30] Instruments M 2012 A basic guide to particle characterization. Malvern Instrument Limited, UK http://atascientific.com.au/publications/wp-content/uploads/2012/07/ MRK1806-01-basic-guide-to-particle-characterisation.pdf

[31] Razali W A, Sreenivasa V K, Bradac C, Connor M, Goldys E M and Zvyagin A V 2016 Wide-field time-gated photoluminescence microscopy for fast ultrahigh-sensitivity imaging of photoluminescent probes J. Biophotonics 9 848–58

[32] Braun A, Franks K, Kestens V, Roebben G, Lamberty A and Linsinger T 2011 Certification of Equivalent Spherical Diameters of Silica Nanoparticles in Water, Certified Reference Material ERM-FD100 (EUR 24620) (Luxembourg: Publications Office of the European Union) (http://publications.jrc.ec.europa.eu/repository/handle/JRC61819)

[33] Khlebtsov N G 2008 Determination of size and concentration of gold nanoparticles from extinction spectra Anal. Chem. 80 6620–5

[34] Shifrin K S 1998 Physical Optics of Ocean Water (New York: American Inst. of Physics)

[35] Mishchenko M I, Travis L D and Lacis A A 2002 Scattering, Absorption, and Emission of Light by Small Particles (Cambridge: Cambridge University Press)

[36] Khlebtsov N G and Mel’nikov A G 1992 Spectroturbidimetry of polydisperse systems with spectral dispersion of optical constants taken into account J. Appl. Spectrosc. 56 268–73

[37] Colpin Y, Swan A, Zvyagin A V and Plakhonk T 2006 Imaging and sizing of diamond nanoparticles Opt. Lett. 31 625–7

[38] Beveratos A, Broui R, Gacoin T, Poizat J-P and Grangier P 2001 Nonclassical radiation from diamond nanocrystals Phys. Rev. A 64 061802

[39] Khlebtsov B and Khlebtsov N 2011 On the measurement of gold nanoparticle sizes by the dynamic light scattering method Colloid J. 73 118–27

[40] Sikora A, Shard A G and Minelli C 2016 Size and ω-potential measurement of silica nanoparticles in serum using tunable resistive pulse sensing Langmuir 32 2216–24

[41] Min M, Hovenier J W and de Koter A 2003 Shape effects in scattering and absorption by randomly oriented particles small compared to the wavelength Astron. Astrophys. 404 35–46

[42] Bohren C F and Huffman D R 2008 Absorption and Scattering of Light by Small Particles (New York: Wiley)

[43] Min M, Hovenier J W and de Koter A 2003 Scattering and absorption cross sections for randomly oriented spheroids of arbitrary size J. Quant. Spectrosc. Radiat. Transfer 79 939–51

[44] Sreenivasa V K A, Zvyagin A V and Goldys E M 2013 Luminescent nanoparticles and their applications in the life sciences J. Phys.: Condens. Matter 25 194101

[45] Griffin M C A and Griffin W G 1985 A simple turbidimetric method for the determination of the refractive index of large colloidal particles applied to casein micelles J. Colloid Interface Sci. 104 409–15

[46] Sokolov V I et al 2015 Determination of the refractive index of beta-NaYF4/Yb3+/Er3+/Tm3+ nanocrystals using spectroscopic refractometry Opt. Spectrosc. 118 609–13