Toward a standardization of physico-chemical protocols for nanomedicine characterization: I. Size measurements

Fanny, Varenne[1,2], Jérémie, Botton[3,4], Claire, Merlet[1,2], Moritz, Beck-Broichsitter[1,2], François-Xavier, Legrand[1,2], Christine, Vauthier[1,2,a].

[1]*Univ Paris-Sud, Institut Galien Paris-Sud, Faculty of Pharmacy, Châtenay-Malabry, France*

[2]*CNRS UMR 8612, Institut Galien Paris Sud, Châtenay-Malabry, France*

[3]*Univ Paris-Sud, Faculty of Pharmacy, Châtenay-Malabry, France*

[4]*INSERM UMR1153, Epidemiology and Biostatistics Sorbonne Paris Cité Center (CRESS), Team « Early Origin of the Child's Health and Development » (ORCHAD), University Paris Descartes, Paris, France*

Résumé. Des nanomatériaux sont proposés pour améliorer le diagnostic, la prévention et le traitement de maladies sévères. Ils constituent un arsenal de technologies qui ont ouvert la voie aux nanomédecines. Parmi les différents paramètres utilisés pour décrire les propriétés de ces nanomatériaux qui se présentent souvent sous la forme de dispersions, la taille et la distribution de taille sont essentielles pour appréhender leur comportement in vivo. Quelques méthodes de mesure peuvent être appliquées pour réaliser des mesures de taille et de la distribution de taille des dispersions. La diffusion dynamique de la lumière (DLS) est une méthode bien établie et citée dans la norme ISO 22412:2008(E). Elle est retenue par les agences de santé. Toutefois, on peut relever une absence de critères qualité des données générées par cette méthode dans la littérature. Pourtant, la qualité des données est primordiale pour s'assurer de la fiabilité des résultats d'une mesure. L'objectif de ce travail a été de définir des précautions opératoires et des critères qualité pour les mesures de taille réalisées par DLS. En tenant compte de ces précautions et de ces critères, un protocole de mesure de taille a été validé et appliqué à la caractérisation de différents nanomatériaux.

Nowadays nanomaterials have a clear impact on our life. Their application as nanomedicine offers promising possibilities to improve medical diagnosis and therapy of severe diseases. Due to their small size, nanomaterials can find their way into medical products by improving drug delivery in treatment of severe diseases for which no treatment based on conventional formulations are yet available [1-7]. In contrast, nanomaterials developed for other applications or occurring as pollutants may cause toxicities in organs in which they accumulate. Among the different characteristics of nanomaterials that are influencing the in vivo fate, nanomaterial sizes and size distribution are crucial parameters to warranty efficacy and safety of treatments of nanomedicines and safety of nanomaterials produced for other in industrials applications. Accurate size measurements are needed to ensure reliable results for nanomaterial characterization [8, 9]. To this aim, validation of size measurement protocol must be performed to prove that it is sufficiently acceptable, reliable and adequate for all element of it scope [10-14]. Thus, Shekunov *et al.* recommended to evaluate precision and trueness of size measurements protocols [9]. Among methods that are suitable to evaluate size and size distribution of nanomaterials, DLS is described in the standard ISO [10] and well-established in company and academic laboratories. It is also recognized by health agencies i.e. Food and Drug Administration and European Medicines Agency. The reliability of measurements is pending to a series of precautions and quality criteria that were not yet all described in a single document of the literature.

In addition, due to the increasing number of nanomaterials with various compositions, there is a need for establishing standardized and validated protocols for size and size distribution measurement by DLS [15]. One standardized protocol was proposed by the Nanomedicine Characterization Laboratory, Frederick, MD, USA [11] but there was no mention that is was validated while the validation is aimed to demonstrate the robustness, the precision and the trueness of the protocol to be apply to the characterization of size of nanomaterials with different specificities and compositions.

The aim of this work was to establish a standardized protocol for size measurement performed by DLS and to define quality criteria to be applied for the validation of the protocol. The validation was then achieved with appropriate certified reference materials with assigned SI traceable values including a standard dispersion of 60 nm and applied for characterization of various nanomaterials used in nanomedicine as polymer nanoparticles and liposomes.

DLS is used to determine diameter of nanomaterials by measuring diffusion velocity nanomaterials due to Brownian motion [10]. It is random movement of

---

[a] christine.vauthier@u-psud.fr

nanomaterials due to bombardment of solvent molecules at nanomaterial surface defined by the translational diffusion coefficient, D. The translational diffusion coefficient is converted into hydrodynamic diameter of nanomaterial, d_H, using the Stokes-Einstein Equation given by Eq. 1.

$$d_H = \frac{k_B \cdot T}{3\,\pi \cdot \eta \cdot D} \tag{1}$$

where k_B is the Boltzmann's constant, T is the absolute temperature and η is the dynamic viscosity of the dispersing medium at the temperature of size measurement.

Dispersed nanomaterials are illuminated by a monochromatic and coherent laser light beam and nanomaterials scattered light is measured by a fast photon counter at a given scattering angle. The scattered light intensity from the moving dispersed nanomaterials fluctuate as a function of time and is converted into correlation function using a correlator (Figure 1). The correlation function could be analyzed either by fitting a single exponential according to Cumulants method described in the standard ISO [10] for determining the z-average diameter and the polydispersity index or by fitting a multiple exponential for evaluating nanomaterial intensity size distribution using Non-Negative Least Squares (NNLS) or CONTIN methods.
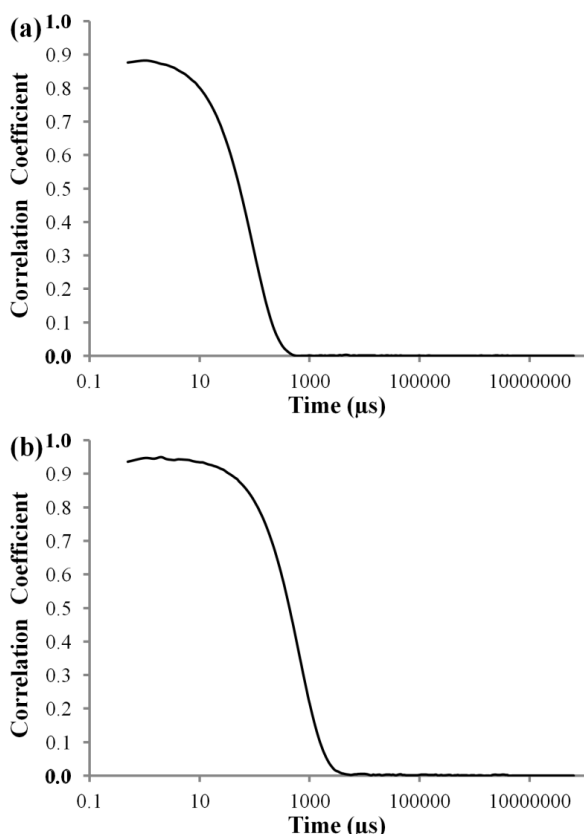

Figure 1. Correlation functions. (a) Small particles of 60 nm. (b) Large particles of 400 nm.

As quality criteria, stringent selection of measurement cells and respect of strict precautions of sample preparation were found as essential to perform reliable size measurements. Disposable cells were used to avoid cross-contamination. The optical quality of measurement cells was carefully inspected and measurement cells presenting scratches or impurity in polystyrene were discarded. Depending of suppliers, this lead to a high rejection rate (up to 85 %) due to the poor quality of the cells. Cells that passed this selection were pre-rinsed three times with filtered ultrapure water and stored in dust free environment before use. To fill up cells, no bubble or dust should be introduced in the measurement cells, the protocol recommended by the supplier of the DLS instrument was followed and samples were diluted with freshly filtered solvents stored in flasks also pre-rinsed with filtered solvents.

As quality of data obtained from DLS are essential for reliability of size results, quality criteria were defined to ensure successful measurements. During measurement, the following points were controlled:

Correlation function
It was checked that the correlation function reflecting the probability to find the nanomaterial at the same place showed a smooth decay exponential with a flat baseline for each run. The Figure 2 (a) showed the screen print displaying the correlation function during measurement of the standard of 60 nm prepared following all of the above mentioned precautions. In Figure 2 (b), the correlation function showed typical signed of dust contamination obtained here with a sample spiked with dust.

Count Rate curve
Another criteria of quality of measurements was assessed from the stability of the count rate during measurement. This represents the number of photons collected by the detector (given in kilocounts per second (kcps)) expressing the intensity of the signal received by the detector. Although the principle of the measure is based on an analysis of fluctuations of signal intensity with time, these fluctuations are expected to remain in a narrow range of intensity without spikes and/or drift during measurements. A curve that fulfilled these characteristics was shown in Figure 3 (a) acknowledging a good quality of the measure. Curves shown in Figures 3 (b) and 3 (c) showed artifacts due to the presence of aggregates in the sample and to containing dust particles respectively. Measurements for which such curves were obtained were discarded according to the quality measurement used the developed protocol.

Another series of quality criteria was set from examining data provided after measurements and calculations:

Correlogram
Correlograms provided in the results after measurements should be consistent with the curves of the correlation function observed during measurements. For quality measurements, it was set that the correlogram given by the three measurements of the protocol showed the smooth typical shape of exponential function presented in
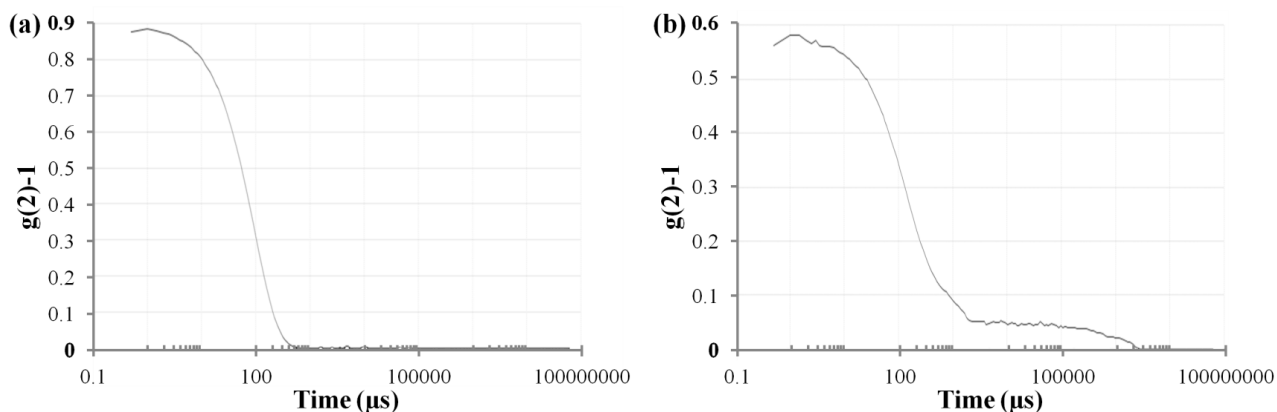
Figure 2. (a) Correlation function obtained from screen print taken up during the measurement of dilute standard 60 nm (Thermo Scientific, LTX3060A, Production batch: 3060-033, Certified batch: 3060-007, Packaging: 42430, Expiration date: August 2016, Analysis date: April 2014), dilution at 1/178 in NaCl 10 mM prior filtered with a 0.22 µm filter. The sample was prepared with all precautions defined to obtain satisfactory quality of measurements. (b) Correlation function obtained from screen print taken up during the measurement of dilute standard 60 nm (Thermo Scientific, LTX3060A, Production batch: 3060-033, Certified batch: 3060-007, Packaging: 42430, Expiration date: August 2016, Analysis date: January 2015), dilution at 1/170 in NaCl 10 mM prior filtered with a 0.22 µm filter, spiked with dust to create an artifact due to dust contamination.
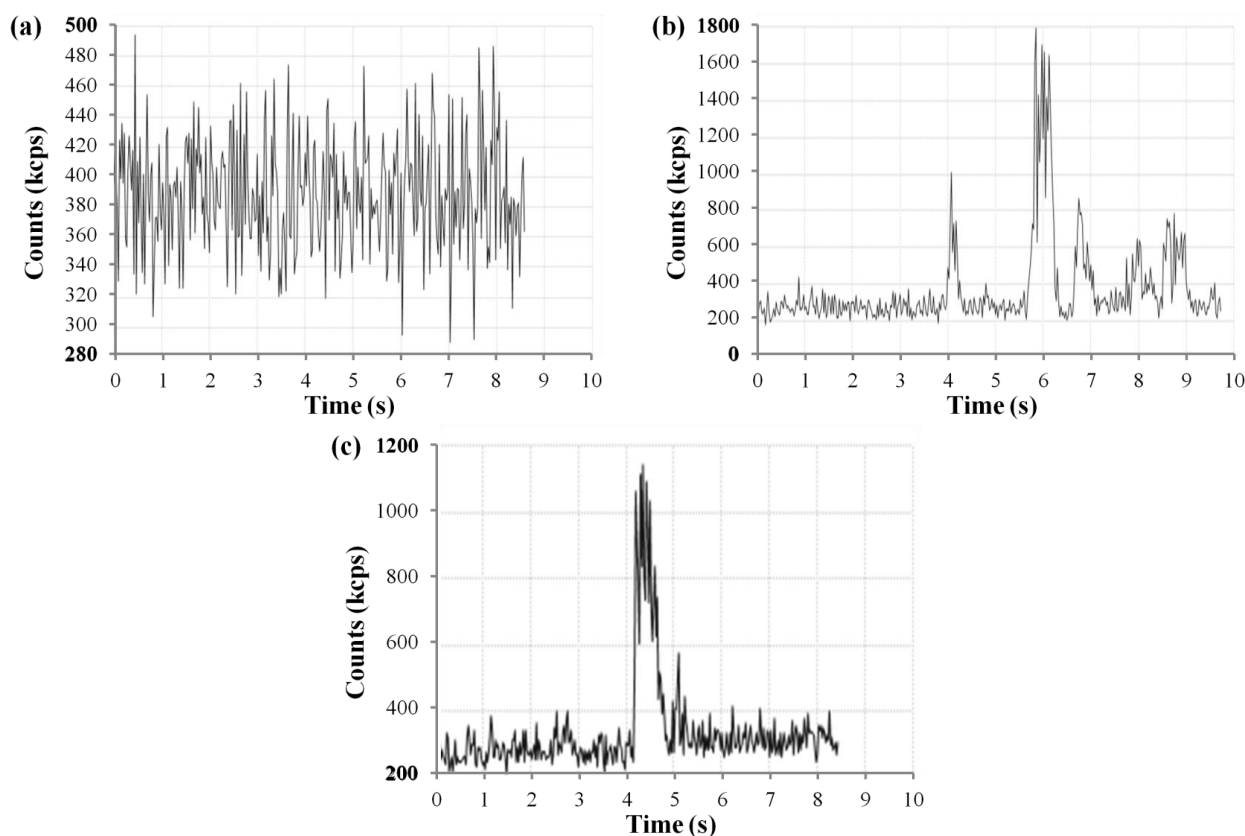

Figure 3. (a) Count rate curve obtained from screen print taken up during the measurement of standard dilute 60 nm (Thermo Scientific, LTX3060A, Production batch: 3060-033, Certified batch: 3060-007, Packaging: 42430, Expiration date: August 2016, Analysis date: April 2014), dilution at 1/178 in NaCl 10 mM prior filtered with a 0.22 µm filter. The sample was prepared with all precautions defined to obtain satisfactory quality of measurements. (b) Count rate curve obtained from screen print taken up during the measurement of aggregated nanoparticles. (c) Count rate curve obtained from screen print taken up during the measurement of dilute standard 60 nm (Thermo Scientific, LTX3060A, Production batch: 3060-033, Certified batch: 3060-007, Packaging: 42430, Expiration Date: August 2016, Analysis date: January 2015), dilution at 1/170 in NaCl 10 mM prior filtered with a 0.22 µm filter, spiked with dust to create an artifact due to dust contamination.

Figure 4 (a). The baseline was requested to reach zero. A baseline that do not return to zero could indicate non random movement of nanomaterials including sedimentation, presence of aggregates (Figure 4 (b)) or large particles including dust (Figure 4 (c)), these measures were considered as not having met our quality criteria. From our quality criteria, all three measurements needed to fulfill quality criteria to consider further measurements done on one sample. If one of the series of three measurements did not satisfied this criteria,
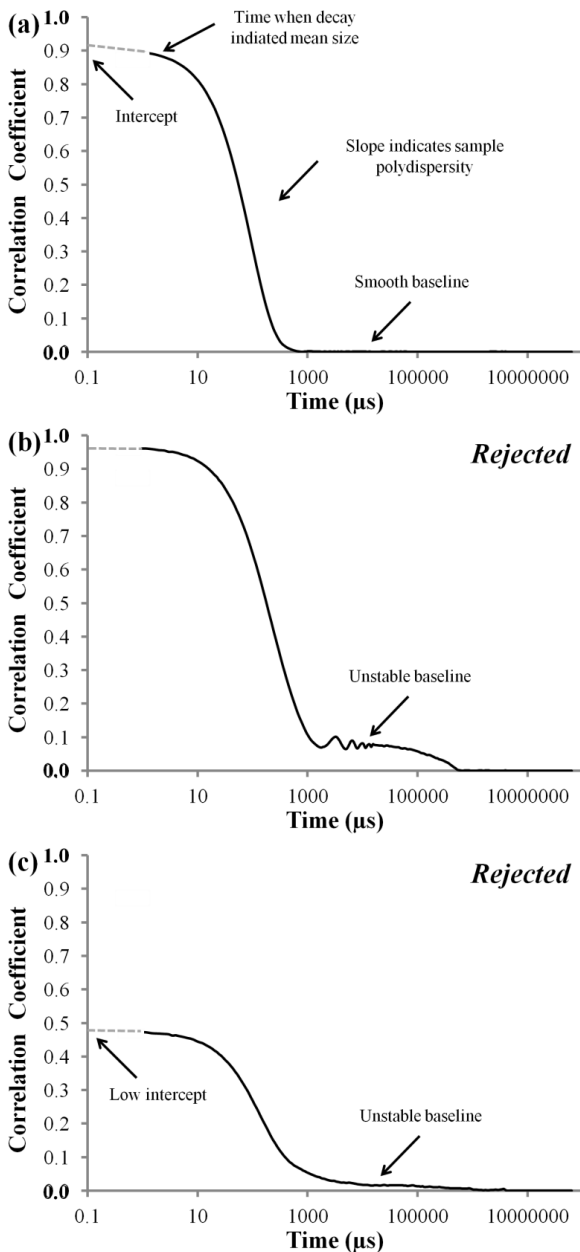
correlogram using the cumulant method described in the standard ISO [10]. It should be less than 0.005. For example, two cumulant fits are presented in Figure 5, one resulting from the analysis of dilute standard of 60 nm (Figure 5 (a)) and the other from the analysis of dilute standard of 60 nm spiked with dust to create an artifact within the measure (Figure 5 (b)). Cumulant fit error was equal to $4.90.10^{-4}$ for the standard of 60 nm prepared taking all precautions to avoid dust and bubbles in the measurement cells. This value fulfilled the recommendations. In contrast, for the sample contaminated with dust, the cumulant fit error was much above the thresholds with 0.0201.

Figure 4. (a) Correlogram obtained for dilute standard 60 nm (Thermo Scientific, LTX3060A, Production batch: 3060-033, Certified batch: 3060-007, Packaging: 42430, Expiration date: August 2016, Analysis date: April 2014), dilution at 1/178 in NaCl 10 mM prior filtered with a 0.22 µm filter. The sample was prepared with all precautions defined to obtain satisfactory quality of measurements. (b) Correlogram obtained for aggregated nanoparticles. (c) Correlogram obtained for dilute standard 60 nm (Thermo Scientific, LTX3060A, Production batch: 3060-033, Certified batch: 3060-007, Packaging: 42430, Expiration date: August 2016, Analysis date: January 2015), dilution at 1/170 in NaCl 10 mM prior filtered with a 0.22 µm filter, spiked with dust to create an artifact due to dust contamination.
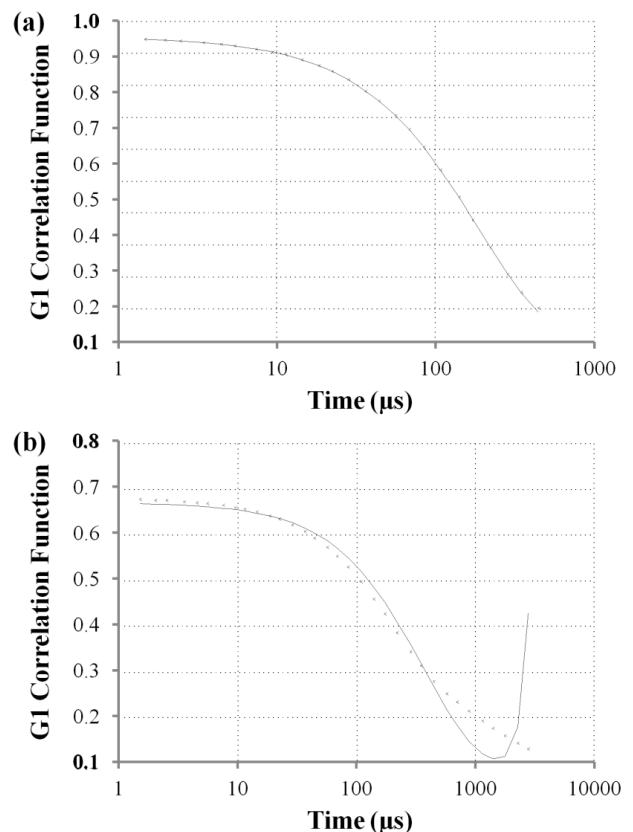
Figure 5. (a) Cumulant fit obtained for dilute standard 60 nm (Thermo Scientific, LTX3060A, Production batch: 3060-033, Certified batch: 3060-007, Packaging: 42430, Expiration date: August 2016, Analysis date: April 2014), dilution at 1/178 in NaCl 10 mM prior filtered with a 0.22 µm filter. The sample was prepared with all precautions defined to obtain satisfactory quality of measurements. (b) Cumulant fit obtained for dilute standard 60 nm (Thermo Scientific, LTX3060A, Production batch: 3060-033, Certified batch: 3060-007, Packaging: 42430, Expiration date: August 2016, Analysis date: January 2015), dilution at 1/170 in NaCl 10 mM prior filtered with a 0.22 µm filter, spiked with dust to create an artifact due to dust contamination. Values of the cumulant fit error were $4.90.10^{-4}$ agreeing with a good quality of measurement and 0.0201 indicating a poor quality respectively.

measurements were performed on newly prepared samples.

Cumulant fit error

Cumulant fit error is a measure of the quality of the cumulant fit that represents the closeness of agreement between experimental correlogram and calculated

Intercept

The intercept refers to the amplitude of the correlogram at time zero that is to say the intersection of the correlation curve and the y-axis of correlogram as shown in Figure 4 (a). It is used to evaluate the signal to noise ratio for measured sample. Nanomaterials with satisfactory

characteristics will provide intercepts greater than 0.6. Nanomaterials with best characteristics will give intercepts in excess of 0.9. In the protocol used in this work, value of the intercept was set at a minimum of 0.9 for standards and at a minimum of 0.6 for unknown samples. All measurements that showed a value below these thresholds were rejected (Figure 4 (c)). The three measures given from characterization performed with the protocol needed to fulfill this quality criteria requirement. If at least one measure failed, another completely independent measurement was undertaken with a newly prepared dilution of the sample.

Mean Count Rate (Intensity of signal)

The mean count rate corresponds to the measurement of the intensity of the signal monitored by the detector. It is expressed in kilocounts per second (kcps). Dilutions of nanomaterials were selected to adjust their scattered intensity within the range recommended by the supplier of the measurement instrument between 50 and 500 kcps finding the best compromise between the concentration in nanomaterials of the dispersion and the attenuation of the scattered signal. As this parameter depends on nanomaterial concentration in the dispersion, it should not vary between three consecutive measurements. It is also expected to remain almost constant measuring same samples diluted at the same concentration in nanomaterials. It was verified that the mean count rate was stable over multiple measurements on the same sample and comprised within a range of the mean value $\pm$ 5 % for all measurements that were performed on same samples. Several factors can explain variations of the mean count rates. A decrease may indicate the loss of nanomaterials due to sedimentation or a change of temperature of the sample between measurements. An increase of the mean count rate may indicate the occurrence of aggregation or change of the temperature of the sample. A random variation of the mean count rate between successive measurements may indicate that the dispersion is unstable.

Attenuation and measurement position

Attenuation and measurement position are parameters chosen by the instrument depending on the optical characteristics of a sample including its concentration. In fact, the signal received by the detector must be more or less attenuated for being in the range of 50 and 500 kcps by application of « filter » while the position of measurements depends of the transparency of the sample to avoid eventual multiple diffusion phenomena in the scattering signal. For low concentrated samples that are quite translucent, minimization of reflection by increasing scattering volume is necessary. The more efficient measurement position is also closed to the centre of the measurement cell. For the more concentrated samples that are more turbid and scatter much more light, measurement positions are taken up near the cell wall reducing multiple scattering by minimizing path length of scattered light. Consistently, the values of attenuation and measurement position are expected to be the same while measuring same samples with the same instrument.

Number of runs and run duration

Choice for the number of runs and run duration are related to size and light scattering properties of nanomaterials and to the optical characteristics of the instrument. These parameters are expected to be set at the same value while performing measurements on identical samples with the same apparatus. It was controlled that this was the case in all measurements performed on the same sample.

Result Quality Report

The supplier of the instrument has developed a size quality report [16]. Twelve tests are carried out on raw data for a given sample for easier interpretation of data from a DLS measurement. A description of the tests is given in Table 1. Results were considered to meet quality criteria when all tests fell within the specifications defined in these tests. In contrast, if any test failed, a warning message indicated that the results did not satisfy quality criteria giving possible reason for failure. In the framework of the quality criteria determined in the protocol, it was requested that all three measurements performed within the frame work of the protocol satisfy this quality control tests to accept the results provided by the sample. If one of the three measurements failed, size determination was repeated on a new sample.

Table 1. Summary of the tests included into the Result Quality Report [16].

Test number	Description
1-4	Control of the Z-average diameter (Cumulant method)
5	Control of the t value of polydispersity index
6	Control of the intercept value
7	Control of multimodal analysis
8	Control of the in range value
9	Control of the mean count rate (if it is within the defined range (20 - 500 kcps))
10	Control of the total number photons collected by the detector (< 10000)
11	Control of the cumulant fit error
12	Control of the distribution fit error

Taking into account of all defined criteria and operating precautions, validation of a protocol of size measurement by DLS was performed by investigating its robustness, precision (repeatability and intermediate precision) and trueness using appropriate certified reference materials with assigned SI traceable values. To assess the robustness, measurements were carried out by varying experimental parameters that could influence results of size measurements such as the temperature of the sample, the volume introduced into measurement cells and the analyst. The repeatability and the intermediate precision were respectively evaluated from measurements performed within day and over several

days. Contribution of each factor to the total variance were estimated using nested design and analysis of variance ANOVA. The repeatability standard uncertainty was less than the acceptability threshold given in the standard ISO [10]. It was shown that the validated protocol proposed in this work could be applied to quality control measure size of a series of polymer nanoparticles of various compositions and liposomes.

To conclude, this paper describes handling precautions and quality criteria that were followed ensuring reliability of size measurements. Taking into account of all, this work has provided a standardized and validated protocol for characterizing size of nanomaterials by DLS including polymer nanoparticles and liposomes that were used to develop nanomedicines. This protocol was demonstrated to be suitable to measure size of nanomaterials of various compositions including polymers and lipids.

References
[1] Organisation for Economic Co-operation and Development (OCDE), Regulatory frameworks for nanotechnology in foods and medical products: Summary results of a survey activity, DSTI/STP/NANO(2012)22/FINAL, 21 March 2013. http://search.oecd.org/officialdocuments/publicdisplaydo cumentpdf/?cote=DSTI/STP/NANO%282012%2922/FIN AL&docLanguage=En (consulted on May 2015).
[2] Draft guidance from FDA, Considering Whether an FDA-Regulated Product Involves the Application of Nanotechnology, 14 June 2011. http://www.fda.gov/RegulatoryInformation/Guidances/uc m257698.htm (consulted on May 2015).
[3] Reflection paper on the data requirements for intravenous liposomal products developed with reference to an innovator liposomal product, EMA/CHMP/806058/2009/Rev 02, 21 February 2013. http://www.ema.europa.eu/docs/en_GB/document_library /Scientific_guideline/2013/03/WC500140351.pdf (consulted on May 2015).
[4] Joint MHLW/EMA reflection paper on the development of block copolymer micelle medicinal products, EMA/CHMP/13099/2013, 17 January 2013. http://www.ema.europa.eu/docs/en_GB/document_library /Scientific_guideline/2013/02/WC500138390.pdf (consulted on May 2015).
[5] Report of the Joint Regulator -Industry Ad Hoc Working Group: Currently Available Methods for Characterization of Nanomaterials, 17 June 2011. http://ec.europa.eu/consumers/sectors/cosmetics/files/pdf/ iccr5_char_nano_en.pdf (consulted on May 2015).
[6] Organization for Economic Co-operation and Development (OCDE), Guidance manual for the testing of manufactured nanomaterials: OECD's sponsorship programme; First revision ENV/JM/MONO(2009)20/REV, 2 June 2010. http://search.oecd.org/officialdocuments/displaydocumen tpdf/?cote=env/jm/mono%282009%2920/rev&doclangua ge=en (consulted on May 2015).
[7] FDA advisory committee for pharmaceutical science and clinical pharmacology meeting Topic 2 Nanotechnology - Update on FDA Activities, 9 August 2012. http://www.fda.gov/downloads/AdvisoryCommittees/Co mmitteesMeetingMaterials/Drugs/AdvisoryCommitteefor PharmaceuticalScienceandClinicalPharmacology/UCM31 4585.pdf (consulted on May 2015).
[8] M. Gaumet, A. Vargas, R. Gurny, F. Delie, Eur. J. Pharm. Biopharm. **69** 1 (2008).
[9] B.Y. Shekunov, P. Chattopadhyay, H.H.Y. Tong, A.H.L. Chow, Pharmaceut. Res. **24(2)** 203 (2006).
[10] ISO 22412:2008(E): Particle size analysis - Dynamic light scattering (DLS).
[11] NIST-NCL Joint Assay Protocol, PCC-1 Version 1.1, Measuring the Size of Nanoparticles in Aqueous Media Using Batch-Mode Dynamic Light Scattering, November 2007, revised February 2010. http://ncl.cancer.gov/NCL_Method_PCC-1.pdf (consulted on May 2015).
[12] ISO 5725-1:1994: Accuracy (trueness and precision) of measurement methods and results - Part 1: General principles and definitions.
[13] ICH Harmonized Tripartite Guideline, Validation of analytical procedures: Text and Methodology Q2(R1), Current Step 4 version, Parent Guideline dated 27 October 1994, (Complementary Guideline on Methodology dated 6 November 1996 incorporated in November 2005). http://www.ich.org/fileadmin/Public_Web_Site/ICH_Pro ducts/Guidelines/Quality/Q2_R1/Step4/Q2_R1__Guideli ne.pdf (consulted on May 2015).
[14] Guide to the expression of uncertainty in measurement, JCGM 100:2008 GUM 1995 with minor corrections. http://www.bipm.org/utils/common/documents/jcgm/JCG M_100_2008_E.pdf (consulted on May 2015).
[15] P.-C. Lin, S. Lin, P.C. Wang, R. Sridhar, Biotechnol. Adv. **32(4)** 711 (2014).
[16] Malvern, Size quality report for the Zetasizer Nano, Version 9, 2014 (available on Malvern's site, required registration to access to this note, consulted on May 2015).