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Toward a standardization of physico-chemical protocols for nanomedicine characterization: II. Zeta potential measurements

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Résumé. La caractérisation des nanomédecines est une étape importante pour garantir la qualité des produits. Actuellement les paramètres les plus étudiés sont la taille et la distribution de taille, la charge de surface par la mesure du potentiel zêta. Parmi les différentes approches utilisées pour caractériser la charge de surface des nanomatériaux, la mesure de potentiel zêta est la plus accessible. Une méthode de mesure décrite par la norme ISO 13099-2:2012(E) est basée sur la mesure de la mobilité électrophorétique des nanomatériaux placés dans un champ électrique par analyse de phase de la lumière diffusée. Reconnue par les autorités de santé, cette méthode est bien implantée dans les laboratoires de recherche et dans l'industrie. Cependant, il peut être noté qu'aucun critère de qualité destiné à juger de la fiabilité des données produites par cette méthode ne sont donnés dans la littérature. Dans cette étude, des critères qualité ont ainsi été définis et des précautions expérimentales ont été identifiées. En suivant l'ensemble de ces précautions et des critères qualité identifiés, un protocole de mesure de potentiel zêta a été validé. Ce papier présente les critères qualité utilisés pour réaliser la validation du protocole de mesure de potentiel zêta défini.

At present, nanotechnologies have been introduced in many applications including building, transportation, cosmetics, food, healthcare and medicine. Surface properties of nanomaterials designed to be used in nanomedicine are greatly influencing their in vivo fate hence the success of the delivery. Among different approaches that can be used to characterize properties of nanomaterial surfaces, measurement of the zeta potential giving information on the surface charge is the most accessible. Zeta potential belongs to key physico-chemical properties of nanomaterials that have to be evaluated as described in guidances given by health authorities [1-3]. So, the reliability of zeta potential measurements is crucial. A method for zeta potential measurement is described in the standard ISO [4] and recognized by health agencies (Food and Drug Administration and European Medicines Agency). The principle of this method is based on the measurement of the electrophoretic mobility of dispersed nanomaterials placed in an electrical field by electrophoresis light scattering (ELS) using phase analysis light scattering (PALS). The zeta potential of nanomaterials can then be calculated from the electrophoretic mobility of the nanomaterials from well-established models. This method is well implanted in laboratories because commercial measurement instruments are available. However, it can be identified that there is a need for standardized and robust protocols to perform reliable measurement of this parameter that may apply to a wide range of nanomaterials [5]. So far, one protocol describes measurements of zeta potential of nanomaterials giving handling precautions for sample preparation and measurement cell selection. It was proposed by the Nanomedicine Characterization Laboratory, Frederick, MD, USA [6]. However, there was no mention on quality criteria of zeta potential measurements by ELS given within this protocol and published in the literature. Quality criteria are fundamental to ensure reliability of measurements including zeta potential measurement.

Thus, the aim of the work was to set up operating precautions and to define quality criteria for measurements of zeta potential by ELS using PALS. Taking into account the precaution raised and the quality criteria established, a standardized protocol of measurement of zeta potential was validated using one certified reference material with electrophoretic mobility at 2.53 ± 0.12 $\mu m.cm.V^{-1}.s^{-1}$ and one reference material with zeta potential at - 42 ± 4.2 mV. This protocol was

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applied to polymer nanoparticles developed to be used as nanomedicine.

Zeta potential can be determined from electrophoretic mobility measurement. In practice, the dispersion of nanomaterials is placed into a measurement cell equipped with two plated-gold electrodes. For zeta potential measurements to be used with Zetasizer instruments developed by Malvern is described in Figures 1 (a) and 1 (b). When an electrical field is applied, charged nanomaterials are subjected to two phenomena namely electrophoresis characterized by electrophoretic mobility, mainly based on their charge density, and electroosmosis induced by charges stranded at the cell surface.

Zeta potential, ζ, of dispersed of nanomaterials is related to the electrophoretic mobility, μ_{ep}, by the Henry Equation (Eq. (1)).

$$\mu_{ep} = \frac{2\ \varepsilon\ .\ \zeta\ .\ f(\kappa\, a)}{3\ \eta} \quad (1)$$

where ε is the dielectric constant, $f(\kappa\, a)$ is the Henry's function and η is the viscosity of medium.

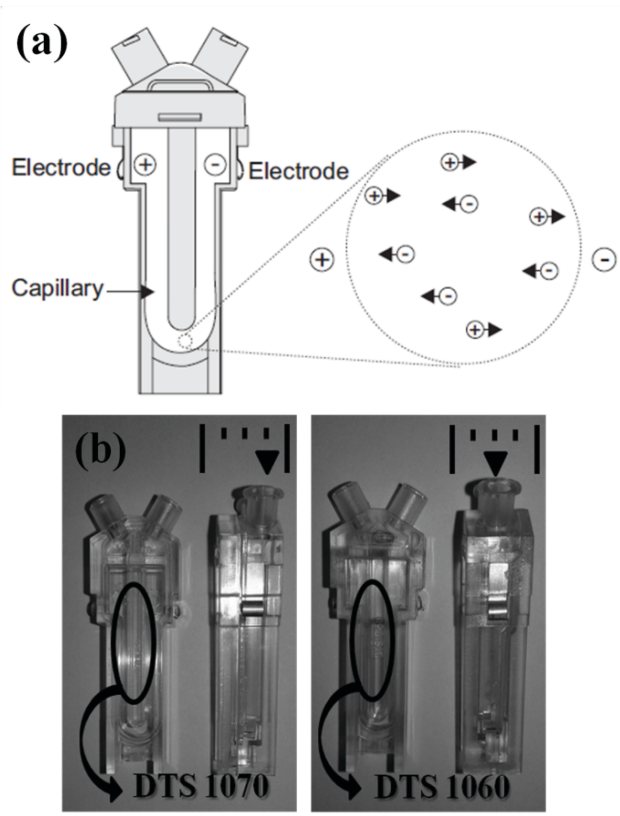

Figure 1. Electrophoresis measurement cells designed to be used in Zetasizer instruments of Malvern. (a) Scheme of the cells. (b) Type of cells: note the slight difference that must be identified for correct placement of cells in the instrument and selection while programming parameters of measurements. Adapted from [7] and with permission.

Electrophoretic mobility of nanomaterials can be evaluated by ELS by measurements of the Doppler shifts in scattered light. In practice, this is done by determining phase difference between the beat frequency and a reference frequency of two laser beams one passing through sample subjected to electrophoresis and one arriving directly at the detector. There are several ways that instruments can analyze the signal produced in PALS. It is important to understand how the analysis is performed to achieve a critical review on the quality of measurements and identify occurrence of artifacts. In the instruments developed by Malvern on the Zetasizer Nano ZS series, the technique called M3-PALS is used [8]. The technique M3 consists in achieving two mobility measurements. A Fast Field Reversal (FFR) measurement is carried out at the centre of the measurement cell giving a mean evaluation of electrophoretic mobility. A Slow Field Reversal (SFR) measurement is then performed to improve resolution of electrophoretic mobility distribution. In this mode, the electroosmosis effect that is created by the used conditions to establish the electric field affects the mobility of nanomaterials. Difference between mean electrophoretic mobility obtained from the FFR and SFR measurements is calculated to evaluate the electroosmotic flow and normalize the distribution. The M3-PALS technique was developed to provide with accurate measurements on nanomaterials having low electrophoretic mobility and measurements of high conductivity samples. Difference phase plots are obtained from the two modes of measurements. They are converted into frequency plot using a Fourier transform analysis. The electrophoretic mobility distribution hence zeta potential distribution are deduced from frequency plots.

Although providers of instruments try to simplify operations needed to perform measurements, the quality of measurements also greatly depends on efforts from analysts to prepare good quality samples and on care of the measurement cell preparation. Measuring electrophoretic mobility of nanomaterials, all factors that may affect the formation of an homogeneous electric field in the measurement cell may create artifacts that can introduce a bias in the measurements. Several artifacts can be avoided taking stringent precautions in preparing the sample and filling out the measurement cells. Several artifacts occurring during measurements can be detected through a carefully review of all measurements parameters provided over the measurement and after measurements. In this paper, we suggest a protocol for preparing samples and measurement cells to perform electrophoretic mobility hence zeta potential measurement by ELS. Advises on artifacts and on their detection during measurements were suggested. This describes in an extensive way method followed to perform electrophoretic mobility and zeta potential measurements during the validation of a measurement protocol. A procedure for quality control measurement on unknown nanomaterials was then suggested.

As described below, care was taken to select and to prepare measurement cells. Cells were preselected based on several criteria: absence of scratches or apparent impurities in polycarbonate that can interfere with optical measurements; cleanliness appearance and homogeneity

of the electrodes both inside and outside of the cells to obtain an homogeneous electric field; tight attachment of the electrodes in the measurement cells that is also required to obtain the establishment of an homogeneous electric field during measurement. Prior use, cells and caps were rinsed sequentially thoroughly with filtered ultrapure water, filtered ethanol and filtered ultrapure water again. Disposable syringes were used and washing liquids were flushed by each port of the cells to rinse the cell walls and the electrodes of each side of the cells. Cells were then stored capped in a dust free environment before use. Before filling with samples, they were checked again for default on cell walls and electrodes. Cells that did not meet all defined criteria were discarded.

Disposable syringes were used to fill the cells with the sample following the instruction of the supplier. Specific care was taken to avoid introducing bubbles and dust in the measurement cell and to respect the maximum filling level which depended on the type of cells (DTS 1070 versus DTS 1060 cells). During removal of the syringe, it was checked that no bubbles were introduced and that the electrodes remained fully immersed in the samples. Caps were inserted as indicated by the supplier. One cap was inserted in one port and then the second cap was placed on the second port of the cells DTS 1070 without pushing too much to avoid cell pressure. For cells DTS 1060, the two caps were placed simultaneously on the two ports. Cells were inspected once more to ensure that no bubbles were present and that the outside electrodes were dry. The cells were inserted in the instrument for temperature equilibration and measurement according to the orientation mentioned by the supplier [9]. Finding the right orientation of the cells may be not obvious as it related to subtle geometric characteristics of the cell. Nevertheless, this is an important point to consider as a significant difference in count rate may be generated if the cell is positioned in the wrong orientation. In extreme cases, measurements may even be not possible due to a too low intensity of the scattered light.

To prepare samples, all dispersants used to prepare dilutions of samples were freshly filtered with 0.22 µm filter. All flasks including caps used for preparation of dispersant or samples were carefully cleaned with filtered ultrapure water and stored in a dust free environment. Suitable minimum and maximum concentrations depend from optical properties of nanomaterials, nanomaterial size and polydispersity. However, dispersions need to be optically clear to let the laser beam penetrate sample while the scattered light will be detected at a forward angle. If sample concentration is too high, the laser is attenuated by nanomaterials reducing the intensity of detected scattered light. The optimized concentration of the dispersion was evaluated as described in the technical note [10].

To perform the validation, quality of data obtained from ELS were considered as essential for the reliability of the results. Quality of data was appreciated using different indicators during measurements i.e. trace of the phase plot and count rate for each run curve and on the measurements data i.e. trace of the final phase plot, trace of the frequency plot, mean count rate, attenuation, conductivity and result quality report.

Control achieved during measurement

Phase plot

The Figure 2 gives a good quality phase plot i.e. well defined without noise obtained for the analysis of Zeta Potential Transfer Standard using General Purpose (GP). It was checked for each run and results considered showed a phase plot as illustrated in Figure 2.

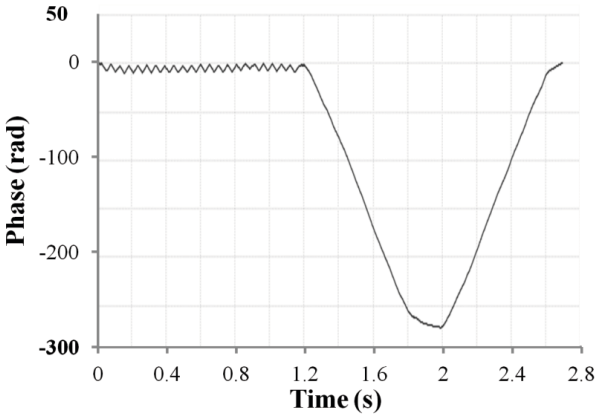

Figure 2. Phase plot obtained from screen print taken up during the measurement of Zeta Potential Transfer Standard (Malvern, DTS1235, Batch: 091408, - 42 ± 4.2 mV, Expiration date: February 2015, Analysis date: May 2014), measurement value: -41.1 ± 0.7 mV using GP analysis model.

Count Rate curve

The intensity of the signal is given by the count rate curve that appears on the screen during measurement. It gives the number of photons detected per time unit expressed in kilocounts per second (kcps). A stable count rate over time indicated that measurements were performed in good conditions. So, curves displayed as shown in Figure 3 (a) were taken to acknowledge measured fulfilling our quality criteria. In contrast, curves of count rates shown in Figure 3 (b) indicated that the count rate was unstable during measurement and were considered to not comply with the quality of measurements that was expected. Such curves were observed when bubbles formed on electrodes during measurements.

Control achieved on the measurements data

Final phase plot

The final phase plot given for one measurement represents the difference in phase between measured frequency and reference frequency as a function of time in the course of measurement. The phase plot using the GP analysis model showed two parts (Figure 4 (a)). The first part occurs as well defined alternating lines with positive and negative slopes. It gives the phase difference with time while applying the FFR. These slopes are

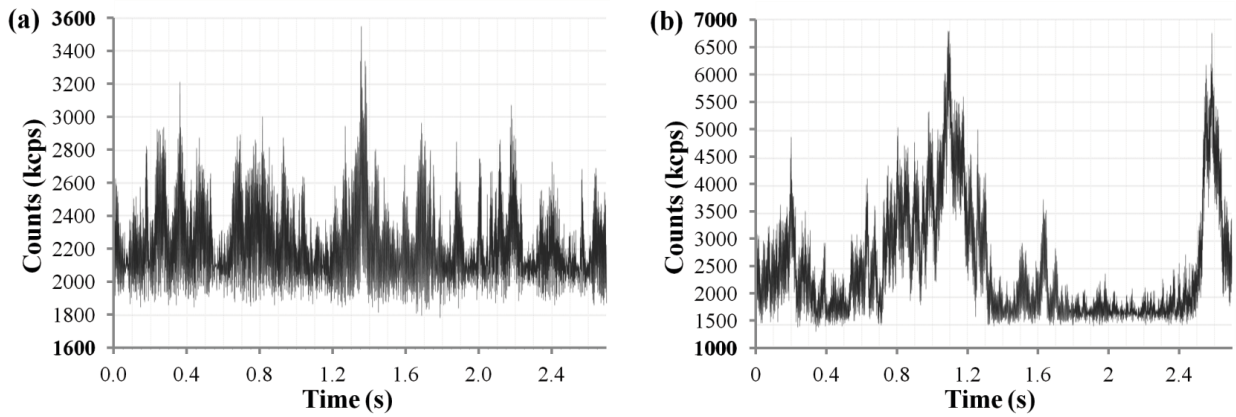

Figure 3. (a) Count rate curve obtained from screen print taken up during the measurement of Zeta Potential Transfer Standard (Malvern, DTS1235, Batch: 091408, - 42 ± 4.2 mV, Expiration date: February 2015, Analysis date: May 2014), measurement value: - 41.1 ± 0.7 mV using GP analysis model. (b) Count rate curve obtained from screen print taken up during the measurement of dispersion 113 ± 10 nm (Polysciences Inc, 00876, Batch: 610905) diluted with NaCl 15.4 mM using GP analysis model. Presence of bubbles on the electrodes.

averaged for determining the mean phase difference and hence mean electrophoretic mobility. The second part represents a smooth negative or positive peak corresponding to the application of SFR used for the determination of the distribution of the electrophoretic mobility of the moving material. Following the quality control approach developed in the present work, it was checked that the slopes of the difference of phase with time were well defined and smooth as shown in Figure 4 (a). Figures 4 (b) and 4 (c) show typical plots that were considered to correspond to poor quality phase plot. It could be determined that these poor quality data were generated respectively by sedimenting nanomaterials and formation of bubbles on the electrodes during the analysis. Both slopes of the FFR and SFR parts of the measurement were not well-defined and appeared quite noisy. Besides sedimentation of nanomaterials, artifacts that can produce poor quality of phase plot were observed with samples having an inappropriate concentration in nanomaterials and/or high conductivity. It is noteworthy that samples with high conductivity may cause heating by Joule effect degrading sample and/or the electrodes. For those samples, the supplier of the instrument recommend to perform measurement using the Monomodal analysis model but no distribution will be provided in that case and only mean zeta potential would be determined.

Frequency plot

The frequency plot corresponds to the frequency spectrum provided a Fourier Transform analysis of the SFR part of the measurement. It is used to determine the electrophoretic mobility distribution and hence zeta potential distribution. On good quality frequency plot, the baseline should be smooth and not noisy as shown in Figure 5 (a). An example of noisy and poor quality frequency plot resulting from the analysis of dispersion 113 ± 10 nm diluted in NaCl 15.4 mM with bubbles on the electrodes is presented in Figure 5 (b). Others factors can explain the obtaining of poor quality frequency plots. A low concentration of samples produces insufficient scattering signal resulting in a poor signal to noise ratio. On the contrary, if the concentration of the sample is too high, attenuation of scattering is applied resulting also in poor signal quality. This explained that sample concentration must be optimized as described in the technical note [10]. For samples having a high conductivity, application of the electric field during the SFR part of the GP analysis model may induce degradation of sample and/or electrodes that may be detected reviewing the quality of the frequency plot.

Mean count rate

The mean count rate represents the mean intensity of the signal expressed as the average number of photons collected by the detector during measurement. It is expressed in kilocounts per second (kcps). The range that was taken to assess good measurement quality was comprised between 20 - 500 kcps. This range was recommended by the supplier of the instrument. It was expected to provide sufficient signal and remain in linear range of the detector.

Attenuation

Attenuation is a parameter that is related to the concentration of the sample. The intensity of signal received by the detector may be more or less attenuated to fit in the range of 20 and 500 kcps. The apparatus is choosing a filter to fulfill this conditions. The value of attenuation should be the same measuring samples at same concentration with the same apparatus. It was verified that this condition was fulfilled for measurements achieved during the validation of the protocol of zeta potential measurement.

Conductivity

The electrical field generated in the measurement cell and hence the value of conductivity of the sample must be stable considering same sample for measurements carried out under precision conditions with the same apparatus. Thus, this parameter was also checked to comply with the requirements in the quality criteria defined in the present work.

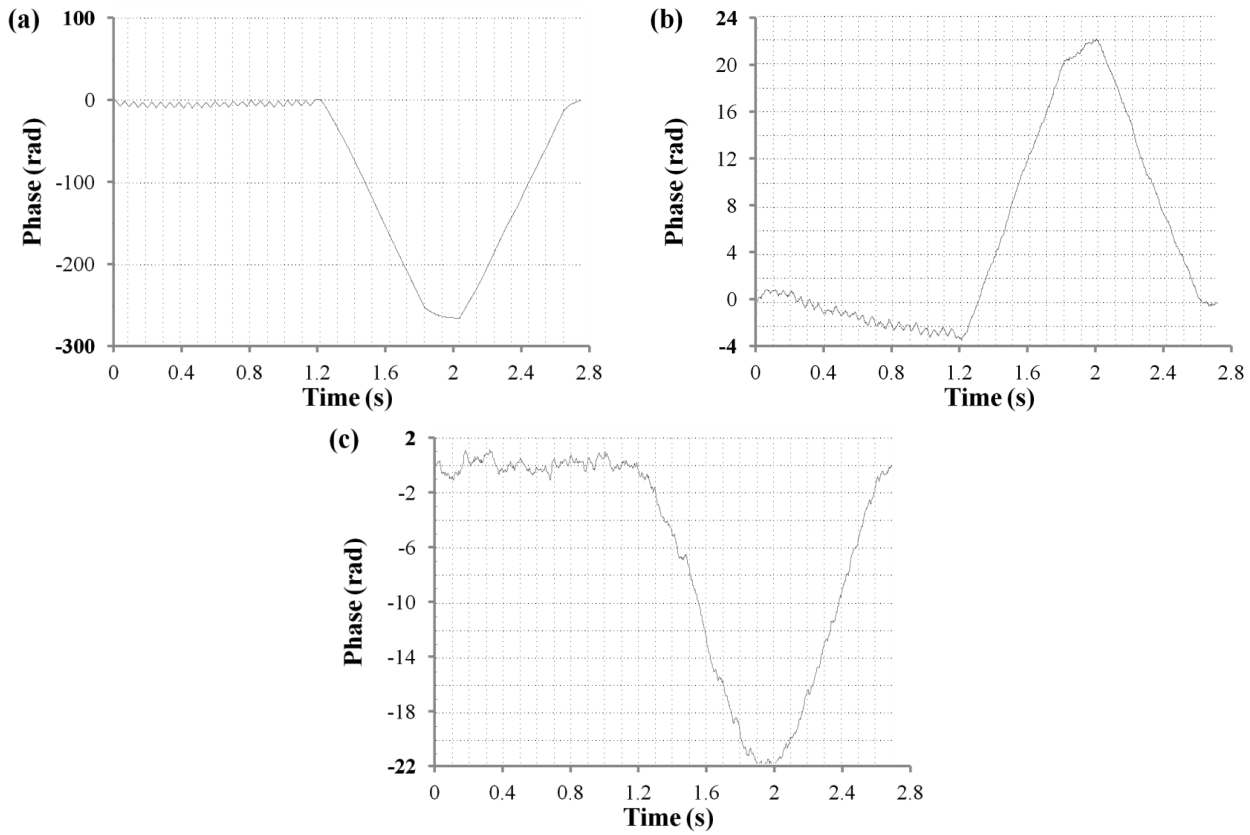

Figure 4. (a) Example of good quality phase plot obtained for measurement of Zeta Potential Transfer Standard (Malvern, DTS1235, Batch: 091408, - 42 ± 4.2 mV, Expiration date: February 2015, Analysis date: May 2014), measurement value: - 41.1 ± 0.7 mV using GP analysis model. (b) Example of phase plot of poor quality obtained with sedimenting particles using GP analysis model. (c) Example of phase plot of poor quality obtained for the measurement of dispersion 113 ± 10 nm (Polysciences Inc, 00876, Batch: 610905) diluted with NaCl 15.4 mM using GP analysis model while bubbles could be seen on the electrodes while checking after measurements.

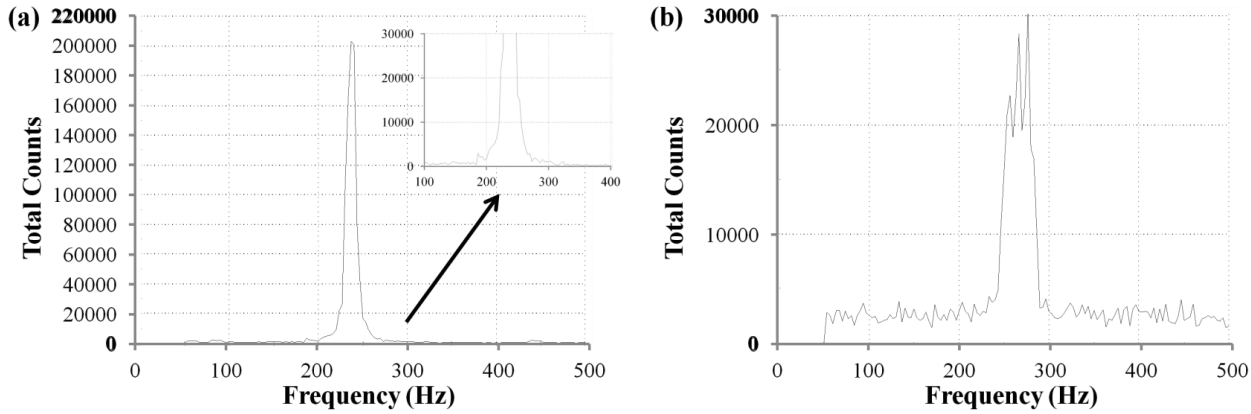

Figure 5. (a) Frequency plot obtained for the measurement of Zeta Potential Transfer Standard (Malvern, DTS1235, Batch: 091408, - 42 ± 4.2 mV, Expiration date: February 2015, Analysis date: May 2014), measurement value: - 41.1 ± 0.7 mV using GP analysis model. (b) Frequency plot obtained for the measurement of dispersion 113 ± 10 nm (Polysciences Inc, 00876, Batch: 610905) diluted with NaCl 15.4 mM using GP analysis model. Presence of bubbles on the electrodes was observed after measurement.

Result quality report

A quality report was developed by the supplier of the instrument for helping interpretation of data obtained from ELS measurements. Six tests are performed on raw data collected on given sample to establish this Quality Report [11]. Description of tests is given in Table 1. If only one test is out of specification, a warning message is displayed giving advice to improve results. If none tests fail, the results meet quality criteria. Only results of measurements that meet the quality criteria given by this quality report were taken into account for the validation of the protocol and for quality control measurements performed on unknown samples.

Taking into account all handling precautions and the above defined quality criteria, a protocol of zeta potential measurement by ELS was validated by investigating its robustness, precision (repeatability and intermediate precision) and trueness. Two standards with zeta potential

at - 42 ± 4.2 mV and with electrophoretic mobility at 2.53 ± 0.12 µm.cm.V^{-1}.s^{-1} were used to perform the validation of this protocol. To assess the robustness, measurements were performed by varying experimental parameters that could influence measurements hence final results. These included the temperature of the sample, the batch of measurement cells, the type of measurement cells and the analyst. The repeatability was determined under conditions stipulating that measurements were performed within day. Measurements to evaluate intermediate precision were performed over several days. The trueness was determined from measurements performed under intermediate precision conditions. Relative standard uncertainties of repeatability, intermediate precision and trueness were found lower than acceptability thresholds given in the standard [4]. The protocol was then applied on different types of nanomaterials to establish its range of applications.

Table 1. Summary of the tests included into the Zeta Potential Quality Report [11].

Test number	Description
1	Control of the quality of phase plot
2	Control of the quality of distribution plot data
3	Control of the limits of zeta potential distribution (if those are within the analyzed range)
4	Control of the conductivity of the sample
5	Flare originating from cell wall
6	Control of the position of the attenuator

In conclusion, this work describes operating precautions and quality criteria that are essential for the reliability of results obtained by ELS using PALS. From quality control criteria defined to accept or reject measurements, it explained how to detect the occurrence of possible artifacts during measurement. Taking into account of all, this work has provided a validated protocol to characterize nanomaterials with positive and negative values of electrophoretic mobility and zeta potential respectively. This work has also established that the validated protocol can be applied for characterizing zeta potential of various types of polymer nanoparticles applying a quality control approach.

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References

[1] 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).

[2] 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/displaydocumentpdf/?cote=env/jm/mono%282009%2920/rev&doclanguage=en (consulted on May 2015).

[3] 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/CommitteesMeetingMaterials/Drugs/AdvisoryCommitteeforPharmaceuticalScienceandClinicalPharmacology/UCM314585.pdf (consulted on May 2015).

[4] ISO 13099-2:2012(E): Colloidal systems - Methods for zeta-potential determination - Part 2: Optical methods.

[5] P.-C. Lin, S. Lin, P.C. Wang, R. Sridhar, Biotechnol. Adv. **32(4)** 711 (2014).

[6] NCL Method PCC-2, Measuring Zeta Potential of Nanoparticles, April 2008, revised November 2009. http://ncl.cancer.gov/NCL_Method_PCC-2.pdf (consulted on May 2015).

[7] Malvern, Zetasizer Nano Series User Manual, Issue 2.1, July 2004.

[8] Malvern, Technical Note, Measuring zeta potential using phase analysis light scattering (PALS), 2014 available on Malvern's site, required registration to access to this note, consulted on May 2015)

[9] Malvern, Zetasizer Nanoseries Accessories Guide, Issue 1.1, April 2013.

[10] Malvern, Technical Note, Concentration Limits for Zeta Potential Measurements in the Zetasizer Nano.

[11] Malvern, Technical Note, Zeta potential quality report for the Zetasizer Nano, 2014 (available on Malvern's site, required registration to access to this note, consulted on May 2015).