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F. Varenne, J.-B. Coty, J Botton, F.-X. Legrand, H. Hillaireau, et al.. Evaluation of zeta potential of nanomaterials by electrophoretic light scattering: Fast field reversal versus Slow field reversal modes. *Talanta*, 2019, 205, pp.120062. <10.1016/j.talanta.2019.06.062>. <hal-02983998>

HAL Id: hal-02983998

<https://hal.science/hal-02983998v1>

Submitted on 30 Oct 2020

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Evaluation of Zeta Potential of Nanomaterials by Electrophoretic Light Scattering: Fast Field Reversal *versus* Slow Field Reversal Mode

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Published in : Talanta: Volume 205, 1 December 2019, 120062,

<https://doi.org/10.1016/j.talanta.2019.06.062>

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Abstract

Zeta potential of nanomaterials designed to be used in nanomedicine is an important parameter to evaluate as it influences *in vivo* behaviour hence biological activity, efficacy and safety. As mentioned by the International Organization for Standardization (ISO), electrophoretic light scattering is a relevant method for evaluating zeta potential. The present work aimed to validate a new protocol based on the application of Fast Field Reversal mode and to explore its scope with nanomaterials investigated as nanomedicines. Its scope was then compared with that of an already validated protocol which uses both Fast Field Reversal and Slow Field Reversal modes. The new protocol was validated within the framework of the application of the Smoluchowski approximation. Its performances complied with the ISO standard. The protocol could be applied to evaluate mean zeta potential of soft nanomaterials including polymer-based nanomedicines and liposomes. However, it appeared unsuitable to evaluate zeta potential of dense nanomaterial including rutile titanium dioxide nanoparticles. Compared with the previously validated protocol which only applied to the determination of zeta potential of polymer nanoparticles, this new validated protocol gives access to the determination of zeta potential to a wider range of nanomedicines under conditions complying with quality control assessments.

Key words: Nanomaterials, Zeta potential, Electrophoretic light scattering, Slow field reversal, Fast field reversal, Scope.

Abbreviations

ANOVA: Analyse of variance

CRM: Certified reference material

DLS: Dynamic light scattering

ELS: Electrophoretic light scattering

FFR: Fast Field Reversal

IBCA: Isobutylcyanoacrylate

ISO: International Organization for Standardization

PALS: Phase analysis light scattering

PIBCA: Polyisobutylcyanoacrylate

RM: Reference material

SFR: Slow Field Reversal

1. Introduction

Nanomedicines have tremendous promises for designing better treatments for diseases and as tools for diagnosis [1-17]. They include materials of various nature occurring as particles at the nanometer size scale (1-1000 nm) [6,18]. Although nanomedicines have the potential to revolutionize medicines, several challenges remain to be addressed to accelerate their translation to the clinic [19,20]. One of these challenges is the assessment of their quality and safety, which is closely linked to their physicochemical properties [21,22]. Indeed, many aspects of the biological activity of nanomedicines including their pharmacokinetic, their biodistribution and interactions with cells are controlled by physicochemical properties of the particles composing the nanomedicine [23]. Paramount parameters include the size, the shape, the surface properties and the mechanical stiffness, designated as the 4S [24,25]. Among these, surface properties have recently been pointed out as having a major influence on the activity and safety of nanomedicines [26,27]. The characterization of surface properties of nanomaterials composing nanomedicines is not easy to perform. An electric potential of nanomaterial surface at the slipping plane between the layer of solvent molecules and ions strongly associated with the nanomaterial surface and solvent molecules and ions of the bulk, called zeta potential, can be determined. Because of its definition, the zeta potential greatly depends on the nature and salt concentration of the dispersing medium in which the nanomaterials are dispersed to perform measurements. It defines a property of the system including the nanomaterials and the dispersing media [28,29]. Nevertheless, providing that sample preparation for measurement including the composition of the dispersing medium is fully described, zeta potential can be used in quality control assessment to provide information on the stability of dispersions. It is also considered as a means of predicting the *in vivo* behavior hence biological performances of nanomedicines [30]. Health agencies have included the evaluation of the zeta potential in the list of characteristics needed to define a nanomedicine [23].

The different techniques that can be used to evaluate the zeta potential are summarized in the table 1. In practice, the methods measure the electrophoretic mobility of the particles dispersed in the dispersing media and this parameter is converted into a value of zeta potential according to models based on assumptions that depend on the characteristics of the nanomaterial-dispersing medium system including the size of the nanomaterials and the ion concentrations and the conductivity of the dispersing medium [28,29]. The value of zeta potential generally provided by marketed instruments implemented the different techniques generally corresponds to an apparent zeta potential as no correction for the surface conductivity is applied in the calculations. This correction is generally not applied in the calculations as it needs difficult to assess additional parameters [31,32]. Achievement of quality control assessment required standardized procedures and validated protocols.

The International Organization for Standardization (ISO) establishes rules and guidelines or characteristics for activities or for their results for which the aim is to achieve optimum degree of order in a given context. So, these guidelines are used to perform quality control analysis which aim to provide with as much as comparable results wherever the analysis is performed. It is noteworthy that the ISO standards are developed by academic or industrial experts from sector covered in ISO standards. This means that the ISO standards reflect a wealth of international experience and knowledge. A few ISO standards for the analysis of nanomaterials are available. However, the number of standards being developed is increasing. There are three ISO standards describing the

evaluation of the zeta potential of nanomaterials of the nanometer size [33-35]. It is one of the very few parameters defining nanomaterials of this size range that have been described in ISO standard. Besides, health organizations have established a list of methods that they found suitable to evaluate the zeta potential of nanomedicines. ISO standards are available for optical electrophoresis as electrophoretic light scattering (ELS) [34] or acoustic electrophoresis [35] while no ISO standard is devoted to apply tunable resistive pulse sensing and nanoparticle tracking analysis methods. Quality and safety of nanomedicines must be assessed in a quality control process that requires the application of validated measurement protocols [36]. ELS is rather simple to implement and the evaluated zeta potential greatly depends on the experimental conditions and models applied to convert the measured electrophoretic mobility into a value of zeta potential which is an apparent zeta potential. It is noteworthy that evaluation of zeta potential may be affected by aggregation and sedimentation, inability to perform evaluation with high ionic strengths as electrode blackening may occurred. These issues should be controlled to carry out reliable zeta potential evaluation [37]. Thus, size of nanomaterials should be measured before and after zeta potential evaluation to ensure that no aggregate is formed with application of electric field. Few validated protocols were published over the last few years [38-40]. One of these measures the electrophoretic mobility of the dispersed nanoparticles based on ELS coupled to Doppler shifts and M3-Phase Analysis Light Scattering (PALS) [38]. Its performances were evaluated with a certified reference material (CRM) with a positive value of electrophoretic mobility and a reference material (RM) with a negative value of zeta potential. This protocol could be transfer to several laboratories demonstrating same performances as those defined in the donor laboratory [39]. It can be applied to evaluate the zeta potential of several types of polymer-based nanomedicines dispersed in sodium chloride 1 mM [38]. However, the application of this protocol to the evaluation of zeta potential of nanomedicines of high density is problematic because measurement quality criteria were not met [40]. It was assumed that the poor-quality criteria obtained from these measurements was due to a sedimentation of the particles during measurement. In the validated protocol, two modes are applied to produce the electric field in the measurement cell the slow field reversal (SFR) and the fast field reversal (FFR) modes¹. The FFR mode consists in reversing quickly the applied electric field. In this mode, the electroosmosis corresponding to the flow of the dispersant under the electric field applied is assumed to be insignificant and the dispersed nanomaterials reach apparent velocity depending only on electrophoresis. The evaluation of velocity is carried out at the centre of the measurement cell and is used to calculated a mean value of zeta potential. In the SFR mode, the applied electric field is reversing slowly avoiding electrode polarization. This second mode can be used to evaluate the distribution of zeta potential which is defined by the different populations of zeta potential for a given sample of dispersed nanomaterials. The zeta potential distribution is provided by the frequency spectrum obtained from the SFR part of the measurement using a Fourier transform analysis. Combining the two modes of production of the electric field in the measurement cell allows the determination of the average value and an evaluation of the distribution of zeta potential of the system during the same measurement. However, as for size measurement by dynamic light scattering (DLS), the distribution of zeta potential assessed by ELS is subject to caution and is relative to the light scattering signal intensity. The determination of zeta potential distribution by ELS suffers from the same types of limitations then determining size distribution by DLS as both are determined

¹Malvern, Zetasizer Nano Series User Manual, Issue 1.1 (2013). <https://www.malvernpanalytical.com/en/learn/knowledge-center/user-manuals/MAN0485EN.html> (consulted on March 2019).

by methods based on light scattering which suffer from a generally low resolving power. In the case of the zeta potential, the quality of zeta potential distribution depends on the aggregation or sedimentation of nanomaterials, the sample concentration and conductivity and the measurement duration. Thus, the use of the SFR mode can be problematic in case of measurements performed on particles of high density dispersed in a medium of low density since the light scattering signal monitored during measurements can be biased by the sedimentation of the particles. Due to the rapid reversing field, it was assumed that the FFR mode would be less sensitive to this bias. So, the purpose of the present work was to validate a new protocol using only the FFR mode and to investigate its scope to evaluate zeta potential of nanomedicines composed of nanomaterials of various nature. Very few reference materials for zeta potential are available that is a difficulty to validate a measurement protocol. The validation of the new protocol was achieved using the available reference material having a negative zeta potential. This material is provided as a dispersion of particles for which the determination of the zeta potential from the electrophoretic mobility complies with the application of the approximation of Smoluchowski. Thus, the validation of the protocol was achieved within the framework of the Smoluchowski approximation.

After validation, it was then applied to determine the zeta potential of a series of pristine nanomaterials obtained after manufacture consistently with guidance's given in a recent paper considering zeta potential measurements in nanomaterial environment - health and safety that can apply to the development and quality control assessment of nanomedicines [41]. The nanomaterials were chosen among different types of materials of interest being evaluated as nanomedicines including one type of polymer nanoparticles, two types of liposomes and one type of titanium dioxide nanoparticles. Samples of these nanoparticles were prepared as they are obtained to be evaluated in biological systems according to the applications they were designed for. Here the purpose being to develop analytical methods to ensure quality control assessment of the nanomedicines, the analyzed dispersion corresponded to the formulation obtained after synthesis. As the protocol was to be validated applying the Smoluchowski approximation, the nanomedicines selected to investigate the scope complied with the application of this model considering their range of size (> 100 nm). Preparation of samples for zeta potential evaluation were diluted in a solution of sodium chloride at a low concentration (1 mM) to also comply with the application of this model. Finally, a comparison of data obtained from the evaluation of zeta potential with the previously validated protocol was achieved with nanomaterials included in the scope of this latter protocol.

Table 1. Main characteristics of methods used to evaluate zeta potential of dispersions of nanoparticles.

Method	Principle	Advantages	Drawbacks
Global methods			
ELS with PALS [38-40]	Frequency shift between light scattered by nanomaterials in movement and reference beam	<ul style="list-style-type: none"> - Quick analysis - Easy to use - Low sample consumption 	<ul style="list-style-type: none"> - Accuracy evaluation of zeta potential distribution depending on the aggregation or sedimentation of nanomaterials, the sample concentration and conductivity and the measurement duration²
Electroacoustic spectroscopy [42,43]	Nanomaterial motion relative to a given dispersant induced by ultrasound	<ul style="list-style-type: none"> - Accuracy evaluation of zeta potential of non-aqueous dispersions of nanomaterials - Accuracy evaluation of zeta potential of concentrated dispersions of nanomaterials 	<ul style="list-style-type: none"> - Needed to know density contrast, viscosity and dielectric permittivity of dispersant and weight fraction of nanomaterials for calculation of zeta potential - Diameter of nanomaterials should be above than 100 nm for dilute samples and above 300 nm for volume fraction of 40 %
Individual methods			
Tunable Resistive Pulse Sensing (TRPS) [30,44]	Resistive pulse sensing produced by passage of nanomaterials through a size tunable pore	<ul style="list-style-type: none"> - Simultaneous information about nanomaterial size and zeta potential on a nanomaterial-by-nanomaterial basis - Low sample consumption 	<ul style="list-style-type: none"> - Calibration needed to provide accurate evaluation of zeta potential
Nanoparticle Analysis [45]	Tracking of scattered light of individual nanomaterials in movement with a microscope onto which is mounted a camera	<ul style="list-style-type: none"> - Accuracy evaluation of zeta potential of dilute dispersions of nanomaterials 	<ul style="list-style-type: none"> - Needed to optimize parameters of the instrument to provide accurate evaluation of zeta potential
Separative methods			
Capillary electrophoresis [46,47]	Separation based on diameter and charge density of nanomaterials	<ul style="list-style-type: none"> - Quick analysis - Low sample consumption 	<ul style="list-style-type: none"> - Needed to optimize conditions of separation to avoid adsorption of nanomaterials on the internal surface of capillary

²Malvern, Technical Note, Zeta potential quality report for the Zetasizer Nano (2014).

<https://www.malvernpanalytical.com/en/learn/knowledge-center/technical-notes/TN101104ZetaPotentialQualityReportZetasizerNano> (consulted on March 2019).

2. Materials and methods

2.1. Materials

Zeta potential evaluation was performed in single-use folded capillary cells commercialized by Malvern Instruments (model DTS 1070). The RM (zeta potential: -42 ± 4.2 mV, other properties given in Appendix A) was purchased from Malvern and used as provided. Polymer-based nanomedicine were prepared with isobutylcyanoacrylate (IBCA) from Orapi, dextran sulfate (MW: 36 - 50 kD) from ICN Biomedicals, cerium (IV) ammonium nitrate from Fluka and nitric acid (purity: 61.5 - 65.5%) from Prolabo. For the metallic nanomedicines, titanium dioxide (TiO₂) nanopowder (rods, 10 x 40 nm, purity: 99.5%) and sodium hydroxide (purity $\geq 98\%$) were both obtained from Sigma-Aldrich. The solution of NaCl used to prepare dilute samples of polymer-based and TiO₂ particles was prepared with NaCl chloride provided by Sigma (purity $\geq 99.5\%$) and filtered with 0.22 μm filter (Roth). Liposomes were prepared with 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) and sodium salt of 1,2-dimyristoyl-*sn*-glycero-3-phospho-(1'-*rac*-glycerol) (DMPG) powders used without further purification (Avanti Polar Lipids), methanol and chloroform (HPLC grade from Carlo Erba Reagents) and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, highest purity from Merck). Aqueous buffer based on NaCl 1 mM and HEPES 10 mM used to prepare dilute samples of liposomes was prepared with NaCl chloride (highest purity from Carl Roth) and HEPES. The pH of this buffer was adjusted to pH 7.4 with concentrated NaOH (Carl Roth) and filtered with 0.1 μm filter (Millipore). Chemicals were used as purchased. Deionized and ultrapure water were obtained from Millipore apparatus.

2.2. Preparation of poly(isobutylcyanoacrylate) based nanomedicines coated with dextran sulfate

Poly(isobutylcyanoacrylate) (PIBCA) based nanomedicines stabilized with dextran sulfate at their surface were prepared as previously described [48]. Dextran sulfate (0.135 g) was dissolved in 8 mL of 0.2 N nitric acid at 40°C and degassed by bubbling argon. To initiate polymerization on the dextran sulfate chain, IBCA (0.5 mL) was added together with 2 mL of cerium (IV) ammonium nitrate ($8 \cdot 10^{-2}$ M in 0.2 N nitric acid). After 1 hour at 40°C, the dispersion was cooled in an ice bath and purified by dialysis against deionized water using a 100-kDa-cutoff membrane (Spectrum Laboratories). The dispersion of polymer nanomedicines dispersed in water was stored at 4°C until characterization.

2.3. Preparation of liposomes

Liposomes were prepared from DMPC or DMPC/DMPG (75:25 mol %) as described previously [49]. Lipids were dissolved in chloroform/methanol (2:1) after which the organic solvent was removed by evaporation in a stream of nitrogen followed by a vacuum overnight to form a lipid film. The films were hydrated with HEPES buffer to yield a final lipid concentration of 25 mg.mL⁻¹. After heating at 40°C, suspensions were vortexed to form multilamellar vesicles. Vesicle size was reduced by extrusion through polycarbonate membranes at 35°C under nitrogen pressure using a Lipex device. The suspension was passed once through a double 0.2- μm membrane followed by 5 passes through with 0.1 μm double membranes. Any aggregates were removed by centrifugation (10000 g, 30°C, 10 min with MF-20R centrifuge).

2.4. Preparation of rutile titanium dioxide based nanomedecines

The dispersion of TiO₂ nanomedicine was prepared according to the procedure described in [12,13] TiO₂ nanopowder was dispersed at a concentration of 10 mg.mL⁻¹ in ultrapure water and mixed by vortexing. After adjusting the pH to 11 with 1 M NaOH solution the nanoparticles were treated with ultrasound for 90 min (60 s pulses separated by 30-s gaps). They were then stirred in an Ultra-Turrax[®] mixer with a polycarbonate/PEEK paddle (S25D-14G-KS, IKA)) for 5 minutes at 24000 rpm, on ice. The method of dispersion of the nanoparticles aimed to minimize the size of aggregates in aqueous suspension, by the combined pH adjustment and the use of high energy dispersion techniques (ultrasonic rod and ultra-Turrax[®] mixer) [50,51]. Details about this method are published in [12] to compare toxicity of TiO₂ and PLGA on cell models or in [13] to investigate inflammatory response after intra-tracheal administration in mice. The obtained TiO₂ nanoparticles were stored at 4°C and vortexed just before characterization.

2.5. Measurement protocol

Zeta potential was evaluated with a Zetasizer Nano ZS (Malvern Instruments) working with a 633-nm laser and at a scattering angle of 13°. Settings of the instrument are given in Table 2. Samples were allowed to equilibrate at 25°C for 300 s before measurement to avoid artifacts from convection. The protocol validated in the present work was based on the application of the FFR mode. This corresponded to the monomodal model of the measurement instrument. Results were given as a mean value of zeta potential that corresponded to an apparent value of zeta potential of the system including the particles dispersed in the dispersing media including the nature and salt concentration of the dispersant used for the measurements as the surface conductivity was not taken into account in the calculation of the zeta potential. The validation of the protocol and in turn measurements on unknown particles was performed in the framework of the Smoluchowski's approximation consistently with measurement conditions requested with the RM. For comparisons, few measurements were performed applying both FFR and SFR modes called General purpose analysis model in the instrument used in the present work. This experimental measurement modality provided with a mean value of zeta potential and an evaluation of the distribution of zeta potential. The table 3 summarizes quality criteria and acceptance criteria used to ensure reliable results [38,52]. Measurement cells were washed with filtered ultrapure water through a 0.22 µm filter (Roth), followed by filtered ethanol (VWR) through a 0.2 µm filter (Roth) and finally filtered ultrapure water again and dried prior used and being filled with the sample following the instructions of the supplier [38,52].

^aThe Smoluchowski approximation was applied as it was the appropriate model for the evaluation of zeta potential of the RM. This model applies for the evaluation of zeta potential of nanomaterials with size larger than about 100 nm dispersed in polar electrolytes containing at least 10⁻⁵ molar salts [53].

^bThe attenuator modulates the intensity of the laser source and thereby the intensity of the scattered light. It allows much light to penetrate across samples of small nanomaterials or in samples with a low concentration of nanomaterials that do not scatter much light. In contrast, in samples containing large materials or at high concentration, the attenuator reduces the amount of light penetrating in the samples hence the intensity of the scattered light received by the detector.

Table 2. Overviewed of settings of the instrument.

Setting	Value
T_m (°C)	Fixed at 25 (Measured: 25.0 ± 0.1)
Laser wavelength (nm)	633
Number of samples	3
Number of measurements	3
Number of runs	Automatic
Delay between measurements (s)	0
Equilibration time (s)	300
Model for $f(\kappa a)$ selection	Smoluchowski ^a
Automatic voltage selection	Automatic
Automatic attenuation ^b selection	Automatic
Analysis model	FFR mode (Monomodal)

Table 3. Quality criteria and acceptance criteria of measurements performed with both analysis models.

Quality criteria	Acceptance criteria
<i>During measurements</i>	
Phase plot	Phase difference between measured and reference frequencies with time with well-defined and smooth alternative slopes <i>Phase difference proportional to electrophoretic mobility of nanomaterial hence zeta potential for a given medium.</i>
Count rate curve	Number of photons detected by the photomultiplier per second that should be stable for each run.
<i>After measurement</i>	
Final phase plot	Phase plot with well-defined and smooth alternative slopes for each measurement.
Frequency curve*	Frequency spectrum with smooth and unnoisy baseline for each measurement and obtained by Fourier transform analysis of SFR measurement providing zeta potential distribution.
Mean count rate	Average number of photons detected by the photomultiplier per second that should be within the range of intensity of signal indicated by the supplier of the instrument (20 - 500 kcps).
Result quality report	Tests carried out on raw data as quality of phase plot, conductivity of sample, control of the limits of zeta potential distribution, flare originating from cell wall and position of the attenuator that should meet specifications defined by the supplier of the instrument.

*Not applicable with the analysis model working with the FFR mode only.

2.6. Validation of the protocol for the evaluation of zeta potential using Fast Field Reversal mode

The validation of the protocol for the evaluation of zeta potential using FFR mode (monomodal analysis model in the measurement instrument) was carried out by investigating the zeta potential of the RM to study the influence of various factors that could potentially produce a bias in results. The investigated factors and the conditions used to study these are summarized in Table 4. The validation included robustness (evaluation of the influence temperature of the sample in the measurement cell 20.0 as, 22.5°C, of the batch of measurement cells and of the analyst), precision (evaluation of influence of time factor: repeatability, successive measurements; intermediate precision, measurements carried out over three days) and trueness studies (comparison between mean value obtained from evaluation of zeta potential under intermediate precision conditions and reference value [54]) and expanded uncertainty (interval for which the value can be found with confidence). The statistical methods developed in our previous work [38] were applied to the results obtained from zeta potential evaluation of the RM in the present study. These included experimental design, analysis of variance (ANOVA) tables and calculation of relative standard uncertainties of the studied factors, which were analyzed with the help of the statistical software package Minitab 16 [55]. The results of validation were compared to thresholds mentioned in the ISO standard [34]. The relative standard uncertainties of repeatability, intermediate precision and trueness should be less than 10, 15 and 10 % respectively.

Table 4. Investigated factors to validate a measurement protocol based on the use of the FFR mode.

Characteristics of the new protocol	Factor	Level	Number of samples per level	Number of measurements per sample
Robustness	Ts (°C)	20.0	3	3
		22.5		
	Batch of cells	A B	3	3
Precision (repeatability and intermediate precision)	Analyst	1	3	3
		2		
Precision (repeatability and intermediate precision)	Day	Day 1	3	3
		Day 2		
		Day 3		

2.7. Qualified zeta potential evaluation

Details of the procedure used to achieve qualified evaluation of zeta potential of unknown dispersions of particles were reported elsewhere [38]. Briefly, in the first step, absorption spectrum in the visible range of investigated nanomaterials, including RM, liposomes and TiO₂ nanoparticles, was recorded at 25°C (Perkin Elmer, Lambda 35 UV-Vis Spectrometer, wavelength range from 400 to 800 nm, no smooth). The absence of absorption band in the region of spectra corresponding to the wavelength of the laser source of the instrument used was controlled. Then, the optimal concentration of the dispersion of nanomaterials to be used to evaluate zeta potential with ELS and PALS was determined. As explained in our previous work [38], optimal concentrations of dispersions were chosen from curves giving the attenuation taken from the instrument and zeta potential values as a function of the nanomaterial concentrations in measured dispersions. Thus, dispersions were diluted to achieve the determination of the optimal concentration for zeta potential evaluation. Dilutions were performed following the equilibrium dilution procedure mentioned in the ISO standard [33]. This procedure consists in maintaining identical the composition of the dispersing

medium in all diluted samples. So, the dispersing media of the original dispersion is used to make the dilutions so that only the concentration in nanomaterials changed between diluted samples. The range of concentrations and composition of the medium of dilution used to prepare the dilutions are indicated in Table 5. Zeta potential was recorded with the validated protocol for each dilution of unknown particles analyzed in the present work. The value of the zeta potential and of the attenuation were then plotted against the concentration in nanomaterials of the measured dilution. Optimal concentrations of the different dispersions of nanomaterials were selected at the centre of plateaus of the curves showing the attenuation attenuator and zeta potential as a function of the nanomaterial concentration of the dilutions. They corresponded to a compromise between measurements performed at a low level of scattered light and in multiple scattering regimen. The optimal concentration depends on differences in refractive index between nanomaterials and the dispersing medium and of the size of the nanomaterials to be measured. If the concentration of nanomaterials is too high, the laser beam that penetrates across samples is attenuated hence the intensity of signal is reduced. So, attenuation is adjusted to allow the detector to receive more scattered light³. Having determined the optimal concentration to performed zeta potential measurements, zeta potential of the dispersions was evaluated under operational qualification of the instruments consistently with quality insurance procedures. The zeta potential of RM was investigated before and after evaluation of zeta potential of unknown nanomaterials to carry out operational qualification of the instrument used in this work. Zeta potential of tested nanomaterials were expressed as the mean value of zeta potential plus or minus the expended uncertainty determined during the validation.

Table 5. Dilution medium and range of concentrations tested to evaluate optimal concentration.

Tested nanomaterials	Medium of dilution	Range of concentrations (mg.mL ⁻¹)
PIBCA nanoparticles coated with dextran sulfate	NaCl 1mM	0.001 - 15
Rutile TiO ₂ nanoparticles	NaCl 1mM	0.0001 - 7.5
DMPC and DMPC/DMPG liposomes	Mixture composed of NaCl 1 mM and HEPES 10 mM, pH 7.4 ^a	0.3 - 26

^aThe two dispersions of liposomes were diluted in a mixture composed of NaCl 1 mM and HEPES 10 mM, pH 7.4. The HEPES buffer was used to control and maintain constant the pH of the dispersions that is important to insure to preserve the stability of liposomes. At a pH of 7.4, ester bonds of lipids composing liposomes are assumed to be stable hence stability of liposomes is preserved. This medium was also chosen as it is the preferred dispersion medium when liposomes are tested in biological studies. The concentration of the HEPES buffer was chosen consistently with the fact that we could not use large amounts of salt in FFR and SFR modes while the conductivity of the dispersion must be bellow than 5 $\mu\text{S.cm}^{-1}$.

2.8. Tunable resistive Pulse Sensing

³Malvern, Technical Note, Concentration Limits for Zeta Potential Measurements in the Zetasizer Nano (2017).

<https://www.malvernpanalytical.com/en/learn/knowledge-center/application-notes/AN160707-Concentration-Limits-Zeta-Potential-Zetasizer-Nano.html> (consulted on March 2019).

The evaluation of zeta potential of dispersions of nanomaterials was performed with an Izon qNano (Izon). The instrument was calibrated with polystyrene calibration nanoparticles with mode size at 115 nm and concentration set at $1.2 \cdot 10^{13}$ nanoparticles.mL⁻¹ obtained from Thermo Fisher Laboratories. All samples were dispersed in filtered aqueous solution of sodium chloride 150 mM with 0.22 µm filter (Roth). This solution was prepared with NaCl chloride provided by Sigma (purity ≥ 99.5 %) and 0.03 % of Tween 20 (Izon solution Q) were added. DMPC and DMPC/DMPG liposomes and TiO₂ nanoparticles were diluted with a factor of 1000. The PIBCA nanoparticles coated with dextran sulfate were prepared by diluting original dispersion with a dilution factor of 10000. Size and zeta potential were evaluated using Izon Control Suite Software v 3.2 with a minimum of 500 nanoparticle events.

3. Results and discussion

3.1. Validation

As shown previously, RM did not absorb light at the wavelength of laser source of the instrument, indicating that zeta potential of RM can be evaluated correctly with this instrument (see Appendix B). The zeta evaluation procedure was found to be robust because the results obtained with different batches of measuring cells, different analysts and different temperatures of sample preparation (20°C or 22.5°C) were not significantly different (p-values associated to each factor > 0.05 provided by ANOVA analysis of raw data). Performances of the new protocol performing measurements on the FFR mode are summarized in Fig. 1.

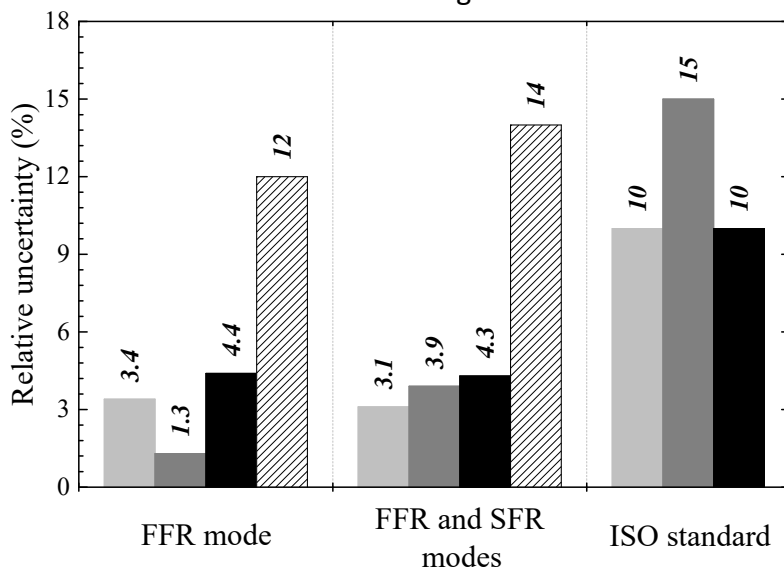


Figure 1. Relative standard and expanded uncertainties (%). Data obtained previously from the validation of the protocol based on the use of the FFR and SFR modes [38] and limits mentioned in the ISO standard [34] are given for comparison (light gray: repeatability; dark gray: intermediate precision, black: trueness and hashed: expanded uncertainty). The contribution of the factors replicates, cells and days to the total variance was not be estimated using the estimated mean square values and the equations for expected mean square described by Varenne et al. [49]. The method of estimation and the variances, expected to be positive, can come out with a negative value, which was not coherent. An alternative method based on pooled variances and described by Vander Heyden et al. can be used [56]. The variances were obtained by dividing the mean square of

a factor by the numbers of replicates within this factor according to equations described by Varenne et al. [49]. The contribution of factor at lower rank factor is eliminated from the calculation of variance of the factor above rank.

The expanded uncertainty was less than 15 %. The values obtained with this new protocol for determining zeta potential were within the limits set by the ISO standard [34] for the relative standard uncertainties of repeatability, intermediate precision and trueness. The performances of the protocol validated in this work were very similar to those obtained previously with a protocol using FFR and SFR modes [38]. These two protocols with similar performances can be used to evaluate zeta potential of dispersions of particles which characteristics fulfil the application of the Smoluchowski approximation (size higher than 100 nm, salt concentration of at least 1 mM) provided that criteria for evaluation quality are fulfilled.

3.2. Evaluation of zeta potential of various nanomaterials by ELS using the FFR mode

Prior zeta potential evaluation, it was checked that dispersions did not show an absorption band at the wavelength of the Laser source of the measurement instrument used (see [38] for PIBCA nanoparticles coated with dextran sulfate and appendix for liposomes and rutile TiO₂ nanoparticles). Then, the optimal concentration to evaluate zeta potential by ELS coupled with PALS using FFR mode with the protocol was determined for each type of nanomaterials. Zeta potential of each dispersion was evaluated under quality control analysis conditions, including a control of the instrument performance with RM. For comparison, the zeta potential of the same nanomaterials was also performed applying the protocol using the FFR and SFR modes. Consistently, optimal concentrations for the evaluation of the zeta potential were also determined using this protocol. Size properties were evaluated before and after zeta potential evaluation by DLS with validated protocol [39,49,57] to prove that no aggregation occurred during application of electric field showing that the nanomaterials were not modified by the electric field.

The scope of the application of the newly validated protocol was investigated by evaluating apparent zeta potential of series of potential nanomedicines including dispersions of nanomaterials of different nature (see Table 5). For comparisons, evaluation of zeta potential was also achieved with the already validated protocol suitable to apply the ELS coupled with PALS using FFR and SFR modes when applicable. Results were also compared with measurements made by TRPS. It is noteworthy that size properties were evaluated before and after zeta potential evaluation for TiO₂ nanoparticles.

3.2.1. Polymer-based nanoparticles

As shown in our previous work, the polymer particle dispersion to be analyzed did not show any absorption band at the wavelength of the laser source used in the measurement instrument [38]. The dispersed particles having a size above 100 nm (see Table 6) and the particles being dispersed in a solution of NaCl 1mM, the validated protocol can be applied to evaluate the zeta potential using the Smoluchowski approximation. An optimal concentration in particles of 1.5 mg.mL⁻¹ was determined to apply the protocol. It was chosen at the center of the plateau of the curves choosing the variation of attenuation and zeta potential for dispersions of different concentrations in

particles (Fig. 2). As it was expected, at the lowest concentrations of the dispersions, the attenuation was reduced and the intensity of signal was outside the acceptable range defined by the supplier of the instrument (20 to 500 kcps), so zeta potential values were not reliable. While the concentration of nanoparticles increased, the applied attenuation increased until it reached a plateau value at which the zeta potential values were stable. While particle concentrations in dispersions reached the highest values, the attenuation decreased because the intensity of signal was decreased due to multiple scattering phenomena. It is noteworthy that quality criteria defined to ensure reliable evaluation of zeta potential were not satisfied for the lowest and highest concentrations but they were fulfilled at the centre of plateau where the optimal concentration to perform zeta potential measurement was determined. Conductivity was systematically measured on each diluted sample. It is noteworthy that the values of conductivity were stable for plateaus of zeta potential and attenuation observed on data used to determine optimal concentration to perform evaluation of zeta potential. Evaluation of zeta potential at optimal concentration of PIBCA nanoparticles coated with dextran sulfate was performed and results are summarized in Table 6. The two protocols yielded similar values of zeta potential of the dispersion of PIBCA nanoparticles coated with dextran sulfate.

These results were compared to those obtained by TRPS. Results are presented in Table 7. The mean value of zeta potential obtained by TRPS was different from this obtained by ELS with PALS as the concentration of NaCl of the medium used to performed measurement were not the same (1 versus 150 mM for ELS with PALS and TRPS respectively). Reliable measurements with TRPS require higher concentrations of salt in dispersant medium in comparison to ELS with PALS. Nevertheless, the same trend was observed by comparing the series of nanomaterials.

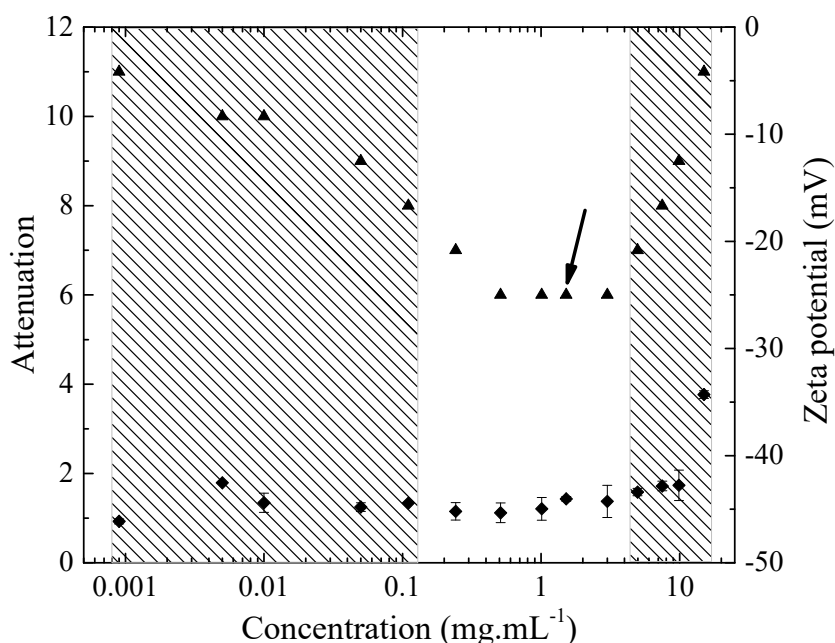


Figure 2. Evaluation of optimal concentration of PIBCA nanoparticles coated with dextran sulfate for carrying out zeta potential evaluation with ELS coupled to PALS using the FFR mode. Attenuation and zeta potential are indicated by up triangles and diamonds respectively. The selected concentration is indicated by an arrow. Quality criteria were not satisfied for the range of concentrations with a squared background.

Table 6. Zeta potential of tested nanoparticles provided with ELS with PALS. Operational qualification of the instrument was performed using the RM. Zeta potential values of the latter were within the range provided by the validation. Quality criteria of the measurements were fulfilled for both RM and the tested nanomaterials. Dispersant medium: aqueous solution of sodium chloride 1 mM.

Tested nanomaterials	Mode to evaluate zeta potential	Optimal concentration of nanomaterials to evaluate zeta potential (mg.mL ⁻¹)	Zeta potential (mV)	Optimal concentration of nanomaterials to evaluate size properties (mg.mL ⁻¹)	Size properties ^a			
					Before zeta potential evaluation		After zeta potential evaluation	
					Z-average diameter (nm)	Polydispersity index	Z-average diameter (nm)	Polydispersity index
PIBCA nanoparticles coated with dextran sulfate	FFR	1.5	-46 ± 12 % ^b	0.2	110 ± 6.8 %	0.128 ± 0.021	110 ± 6.8 %	0.132 ± 0.011
	FFR and SFR	1.0 [38]	-45 ± 14 % ^c [38]				110 ± 6.8 %	0.127 ± 0.012
DMPC liposome	FFR	2.0	-1.7 ± 12 % ^b	0.25	128 ± 6.8 %	0.050 ± 0.012	128 ± 6.8 %	0.047 ± 0.016
	FFR and SFR	Na ^d	Na ^d				128 ± 6.8 %	0.054 ± 0.015
DMPC/DMPG liposome	FFR	2.0	-53 ± 12 % ^b	0.25	115 ± 6.8 %	0.065 ± 0.018	117 ± 6.8 %	0.087 ± 0.034
	FFR and SFR	Na ^d	Na ^d				115 ± 6.8 %	0.065 ± 0.016
Rutile TiO ₂ nanoparticles	FFR			0.01	Nd ^e	Nd ^e	Nd ^e	Nd ^e
	FFR and SFR	Na ^d	Na ^d					

^aDetermined with DLS using validated protocol of size measurement (Cumulants method) [49]; ^bThe expanded uncertainty determined from evaluation of zeta potential of RM using FFR mode was applied to zeta potential results obtained for tested nanomaterials; ^cThe expanded uncertainty determined from evaluation of zeta potential of RM using FFR and SFR modes was applied to zeta potential results obtained for tested nanomaterials [38]; ^dNot applicable; ^eNot determined as the DLS method used was not suitable for measuring the size of non-spherical particles [58].

Table 7. Zeta potential of tested nanoparticles provided with TRPS. Calibration of the instrument was performed with polystyrene calibration nanoparticles with mode size at 115 nm and concentration set at $1.2 \cdot 10^{13}$ nanoparticles.mL⁻¹. Dispersant medium: aqueous solution of sodium chloride 150 mM.

Tested nanomaterials	Zeta potential (mV)
PIBCA nanoparticles coated with dextran sulfate	-24.3
DMPC liposome	12.1
DMPC/DMPG liposome	-18.5
Rutile TiO ₂ nanoparticles	13.3

3.2.2. Liposomes

Liposomes dispersions did not show absorption bands at the wavelength of the Laser found in the zeta potential measurement instrument. The size of the liposomes and the composition of the dispersing media were included in the domain of application of the Smoluchowski approximation. Pre-requirements for the application of the validated protocols included both the FFR and the SFR and SFR modes were fulfilled. The optimal concentrations were determined for the two dispersions of liposomes and the two validated protocols for the evaluation of zeta potential (Fig. 3). None of the quality criteria used to evaluate the reliability of zeta potential investigation was satisfied using the FFR and SFR modes. Indeed, phase plot curves obtained for DMPC liposomes did not show smooth alternative slopes. The associated frequency curves did not show a smooth baseline and were noisy. The intensity of signal was outside the intensity range mentioned by the instrument supplier. For DMPC/DMPG liposomes, the baseline of the frequency curves was noisy and not smooth and the result quality report for each measurement was not satisfied, whatever the concentration. Therefore, the protocol using this analysis model was not suitable to evaluate zeta potential of the liposomes considered in the present work. Performing measurements with the protocol working with the FFR model, an optimal concentration for performing zeta potential measurements was determined for each type of liposome (Fig. 3 and Table 6).

The newly validated protocol based on the application of the FFR mode only during measurements provided with a mean value of zeta potential of the two dispersions of liposomes (Table 6). Conductivity was systematically measured on each diluted sample. It is noteworthy that the values of conductivity were stable for plateaus of zeta potential and attenuation observed on data used to determine optimal concentration to perform evaluation of zeta potential.

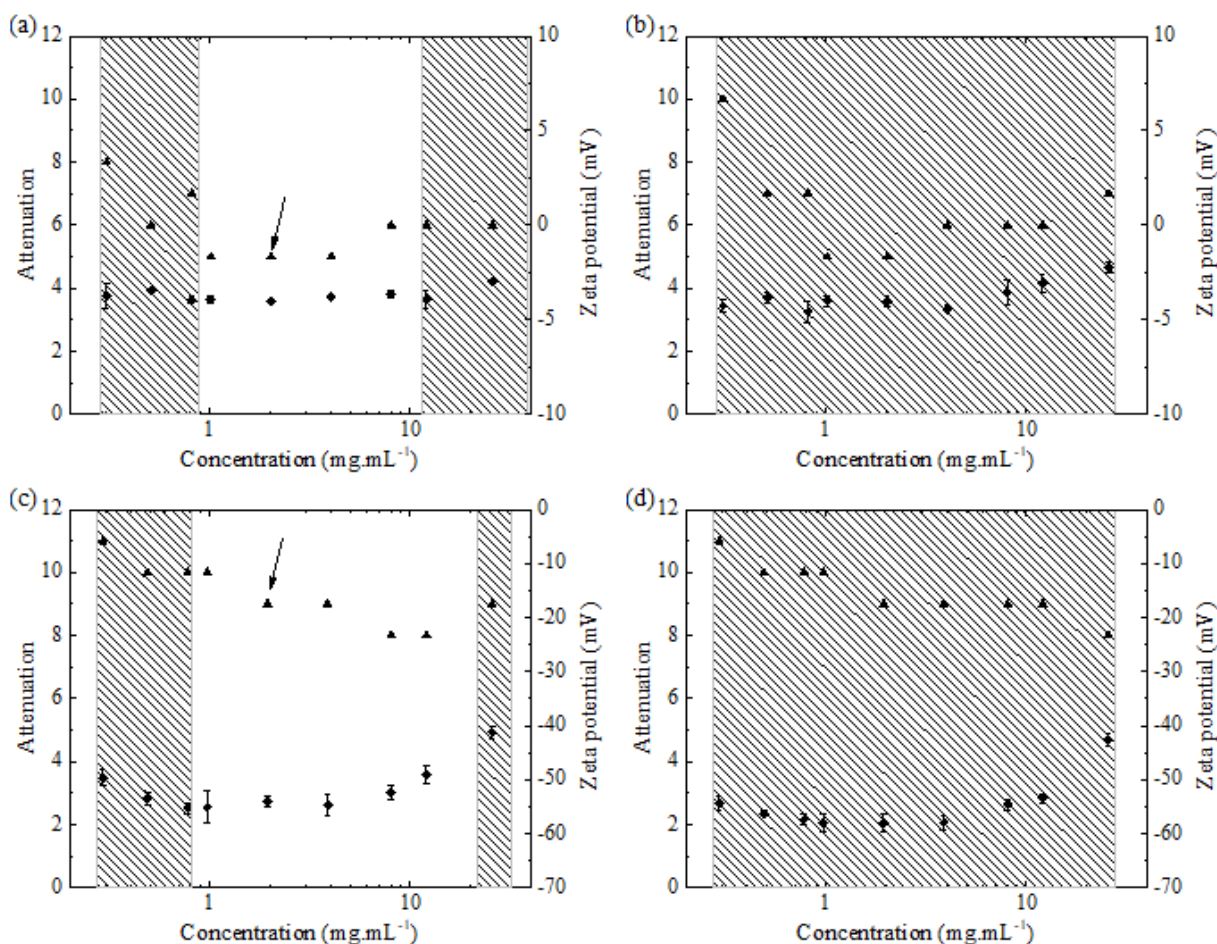


Figure 3. Evaluation of optimal concentration of liposomes for carrying out zeta potential evaluation with ELS coupled to PALS. DMPC liposome: (a) FFR and FFR and SFR (b) modes. DMPC/DMPG liposome: FFR (c) and FFR and SFR (d) modes. Attenuation and zeta potential are indicated by up triangles and diamonds respectively. The selected concentration is indicated by arrow. Quality criteria were not satisfied for the range of concentrations with a squared background.

The lack of RM or CRM in the case of soft neutral materials points out the difficulty to evaluate trueness of protocols to evaluate zeta potential of nanomaterials by ELS with PALS. The method was validated for a negatively charged RM which is easily measured with FFR or FFR and SFR modes. It is possible to support the results obtained by the FFR mode by comparing the mean zeta potential with an orthogonal measurement of the same sample with TRPS. Results are summarized in Table 7. It is noteworthy that the values of zeta potential obtained by TRPS were different from those obtained by ELS with PALS as the concentration of NaCl of the medium used to performed measurement were not the same (1 versus 150 mM for ELS with PALS and TRPS respectively). Reliable measurements with TRPS require higher concentrations of salt in dispersant medium in comparison to ELS with PALS. Nevertheless, the same trend was observed by comparing the series of nanomaterials.

3.2.3. Rutile titanium dioxide nanoparticles

Attempt to evaluate the zeta potential of rutile TiO₂ nanoparticles by ELS with PALS was performed using the protocol validated in the present work. As shown in the Fig. 4, quality criteria obtained

from these measurements were unmet. Several hypotheses may explain the noisy frequency plot shown during measurements which hampered reliable determination of zeta potential of this dispersion. It was checked that the nanoparticles were not modified during measurement which requires the application of an electric field. As the size of these nanoparticles cannot be easily determined by DLS, TRPS was performed to highlight any change of the size of the nanoparticles. Observations confirmed that particles were unchanged after they have been submitted to the electric field applied during zeta potential evaluation (100 nm before and after applying electric field). TiO₂ nanoparticles are dense hence they may sediment during analysis. A sedimentation of the nanoparticles was macroscopically observed in the sample at the end of the analysis. This hypothesis was privileged to explain the failure of the application of the protocol to the evaluation of zeta potential for the dispersion of TiO₂ nanoparticles. The previous validated protocol working in the FFR and SFR modes also did not applied to the evaluation of zeta potential of aqueous dispersions of these dense nanoparticles. The determination of the zeta potential of such dispersions by ELS with PALS appeared difficult because the density of the dispersing medium compared to that of the nanoparticles is too low to prevent sedimentation of the particles in the course of the measurement. Since zeta potential evaluation is very sensitive to the composition of the dispersing media in which the nanoparticles are dispersed, if a mean value and a distribution of zeta potential are needed for nanoparticles dispersed in an aqueous medium at a low salt concentration, results from our work suggest that another method of evaluation of zeta potential should be used. Results from assays performed using tunable resistive pulse sensing have demonstrated the feasibility of the evaluation of the zeta dispersion of the TiO₂ tested in the present work.

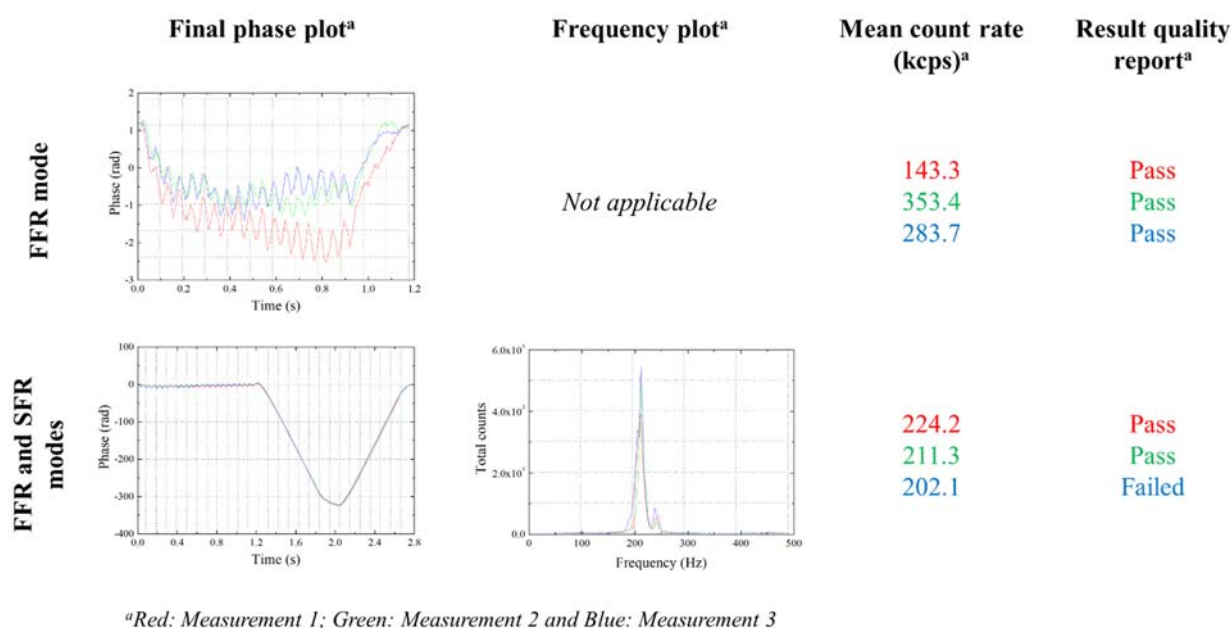


Figure 4. Quality criteria recorded for raw data obtained for zeta potential evaluation of rutile TiO₂ nanoparticles with ELS coupled to PALS using FFR and FFR and SFR modes ($C = 0.05 \text{ mg.mL}^{-1}$ for both analysis models). Conductivity was systematically measured on each diluted sample. It is noteworthy that the values of conductivity were stable for concentrations of TiO₂ nanoparticles within the range from 0.001 to 1 mg.mL⁻¹.

The lack of RM or CRM in the case of non-spherical nanoparticles points out the difficulty to evaluate trueness of protocols to evaluate zeta potential of nanomaterials by ELS with PALS. The mean zeta potential with an orthogonal measurement of the same sample with TRPS. Results are summarized in Table 7. The zeta potential of TiO₂ particles was positive in considered dispersant medium e.g. aqueous solution of NaCl 150 mM.

4. Conclusion

A new protocol for zeta potential evaluation of nanomaterials by ELS with PALS has been validated. This protocol is based on the use of the FFR mode corresponding to monomodal model on the instrument used. The protocol was found to be robust since there was no significantly difference between results obtained with different batch of cells, at sample temperatures of 20.0 and 22.5 °C and performed by various analysts. The performance of this protocol in terms of repeatability, intermediate precision and trueness was within the limits of the ISO standard and consistent with that of a previously validated protocol performing measurements using both SFR and FFR modes corresponding to General purpose on the instrument used. This protocol was used to evaluate the zeta potential of nanoparticles, using Smoluchowski's approximation to calculate zeta potential from the electrophoretic mobility. From the study of the scope of application of the present protocol, it could not be applied to evaluate the zeta potential of aqueous dispersions of dense nanoparticles such as titanium dioxide nanoparticles. Quality criteria of measurements were not met due to bias created by the sedimentation of the nanoparticles. Another method such as TRPS can be used to evaluate zeta potential of such dispersions. In contrast, the protocol can be applied to determine an average value of zeta potential of dispersions containing soft nanomaterials including polymer-based particles and liposomes dispersed in aqueous media. With this new validated protocol, the range of nanomedicines for which zeta potential can be evaluated by ELS with PALS under conditions compatible with quality assurance procedures was extended and the applicability of the TRPS method was demonstrated for an application to the evaluation of the zeta potential of dispersions of nanoparticles that are outside the scope of the ELS with PALS method. .

Acknowledgement

This work was supported by BPI France (Project NICE). The authors thank Larissa Antoniacomi from Universidade Federal do Paraná (Brazil) and Sofiane Mokrani from University Paris Descartes (France) for their participation in this work. The authors acknowledge Dr Camille Roesch (Izon Science Europe Ltd, Lyon, France) for zeta potential evaluation of dispersions of nanomaterials with tunable resistive pulse sensing.

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