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A systematic comparison of different techniques to determine the zeta potential of silica nanoparticles in biological medium†

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The surface charge density of nanoparticles plays an important role in the way they interact with biological systems. The ability to measure the surface charge density of nanoparticles in biological media is therefore of importance in understanding the magnitude of such interactions. There are a number of methods which may be used to assess surface charge density through the measurement of electrophoretic mobility. In order to better understand the comparability of these methods, the ζ-potential of silica nanoparticles in water, buffer and serum-based biological medium was measured by one ensemble and two particle-by-particle techniques: electrophoretic light scattering (ELS), tunable resistive pulse sensing (TRPS) and zeta particle tracking analysis (z-PTA). To allow the comparability of results from different techniques, test samples were prepared according to an established protocol, although some variations were necessary to meet specific instrument requirements. Here we compare, for the first time, measurement results from the different techniques and discuss how modifications related to parameters such as environmental pH, dilution factor and presence of biomolecules influence the charge measurements.

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1. Introduction

The ability to reliably measure the surface charge density of nanoparticles (NPs) and determine how this may change upon exposure to complex biological media is of importance in many areas of applications, such as drug delivery[1–3] and bio-sensing.[4] For example, since most cellular membranes are negatively charged, the surface charge density of NPs will influence their ability to permeate membranes. It has been shown that cationic NPs cause more toxic effects associated with cell wall disruption.[5,6] The surface charge density of NPs dispersed in a liquid is influenced by the solution conditions (*i.e.* pH, temperature and ionic strength) and adsorption of charged moieties from the surrounding medium onto the particles' surfaces. It is believed that the surface of NPs is immediately decorated with proteins when brought into physiological environments, and this adsorbed layer plays a fundamental role in the way NPs interact with each other and with cells, ultimately determining their fate.[7,8]

The surface charge density of particles dispersed in a liquid medium cannot be directly measured. However, the ζ-potential is often used interchangeably with the term surface charge and is a useful experimental parameter that can be theoretically related to the surface charge density by solving the Poisson–Boltzmann equation.[9] The ζ-potential is defined as the electric potential at the shear or slipping plane, which is a notional boundary that separates the (bulk) liquid showing normal viscous behavior from the diffuse double layer which is predominantly composed of counter ions and is considered to move with the particle.[9,10] The ζ-potential is directly related to the electrophoretic mobility through the Henry equation and the Smoluchowski or Hückel models,[9] and provides an indication of the stability of the colloidal system. NPs with a ζ-potential between −30 mV and +30 mV are considered electrostatically unstable and prone to agglomeration/aggregation and flocculation[5] due to the dominant attractive van der Waals interactions. Agglomeration may be inhibited by modifying the environmental conditions of the liquid medium or by adding non-ionic moieties for steric stabilisation.[11]

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† Electronic supplementary information (ESI) available: Particle size measurements at different pH values (Fig. S1), DLS normalised scattered light intensity-weighted particle size distributions (Fig. S2), technical requirements for ζ-potential measurements (Table S1), and ζ-potential measured in 150 mM Tris–HCl by z-PTA (Table S2). See DOI: 10.1039/c5ay02014j

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The method that is most commonly used to determine ζ-potential makes use of micro-electrophoresis in combination with the detection of scattered light.[12] This technique, which is known as electrophoretic light scattering (ELS), has the great advantages of requiring minimal sample preparation, analysing large ensembles of particles thus providing results with good statistics, and preventing cross contamination among samples through the usage of disposable capillary cells. However, it is not suitable for heterogeneous samples. For example, in a sample of NPs which are heterogeneous in size, the light scattered by larger NPs may mask that of smaller NPs, resulting in the measured ζ-potential being affected by a sub-population of the NPs. Similar bias will occur in an ensemble of NPs made of a range of materials with different optical properties which cause them to scatter light more or less effectively. In this respect, techniques measuring ζ-potential on a particle-by-particle basis provide a more detailed understanding of sample behaviour. In this work we have performed, for the first time, a systematic comparison of measurement techniques, to reliably determine the ζ-potential of plain and aminated silica NPs dispersed in water, buffer and biological media. Measurements were conducted on a particle-by-particle basis using TRPS and z-PTA, and the results obtained with these techniques are compared with the results from ELS.

2. Experimental

2.1 Development of test materials

Representative test materials (RTMs) of plain and aminated silica NPs dispersed in an aqueous solution were prepared as previously described.[13,14] The RTMs were produced in the context of the NEW03 NanoChOp project[15] of the European Metrology Research Programme aiming at the development of analytical methods for the assessment[13] of NPs in biological media. Both materials (NanoChOp-05, plain silica and NanoChOp-06, aminated silica) with a nominal sphere-equivalent hydrodynamic particle diameter of 82 nm, as measured by small-angle X-ray scattering (SAXS), were supplied in Ar-flushed and flame-sealed amber ampoules filled with 9 mL (NanoChOp-05) or with 2.5 mL (NanoChOp-06) of the aqueous suspensions of nominally 2.5 g kg^{-1} particle mass fraction. The silica NPs used for the development of both RTMs originate from the same starting material (Klebosol 30R50, AZ Electronic Materials France SAS, Trosly Breuil, FR).[16] The homogeneity of the RTMs was quantified in terms of particle size and ζ-potential (ELS), indicating a between-unit variation of less than 2 mV and 1 mV for the plain and aminated silica materials, respectively. A small, but significant trend was observed for the ζ-potential of the aminated silica (−5 mV over a period of 18 months when stored at 4 °C). These variations were taken into consideration when analysing the outcome of the experiments reported in this paper.

2.2 Sample preparation

All solutions were prepared using ultrapure water (Milli-Q water, Merck Millipore Corporation, Darmstadt, Germany and ELGA LabWater, PURELABS flex, High Wycombe, UK). The solution pH was measured in each institute using portable pH meters (IoLine IL-Micro-pH-A, SI Analytics, Mainz, Germany; 744 pH Meter, Metrohm, Herisau, Switzerland; HI-1083B electrode, Hanna Instruments, Rhode Island, US; B-712 pH meter, Horriba, Kyoto-Shi, Japan). Before opening, all ampoules were gently inverted several times to ensure the homogeneity of the suspension. Samples for ELS were prepared by diluting both as-received materials to 1 g L^{-1} in purified water (resistivity 18.2 MΩ cm at 25 °C), 50 mM Tris–HCl at pH 7.4 (Sigma-Aldrich, Dorset, UK) and in 10% foetal bovine serum (FBS, PAA Laboratories GmbH, Colbe, Germany) in Eagle's minimum essential medium (EMEM, ATCC® 30-2003™, Manassas, US). Measurements were performed immediately after NP dilution and after 24 h incubation.

For TRPS, purified water and 50 mM Tris–HCl buffer did not have sufficient electrical conductivity to establish a stable signal and baseline during the measurements. The base material was therefore diluted in 150 mM Tris–HCl to 38 mg L^{-1} in order to achieve a concentration of $\sim 6.8 \times 10^{10}$ particles per mL recommended by the instrument manufacturer. For measurements in biological media, both NP samples were diluted to 1 g L^{-1} in 10% FBS in EMEM. 24 h incubation was also performed under these conditions. Further dilution in EMEM was performed immediately before the measurements to reach the recommended NP concentration, where the final concentration of serum proteins and NPs was 0.38% and 38 mg L^{-1}, respectively. Control experiments were performed using FBS diluted in EMEM at the same concentration and no significant signal was measured. All calibration samples were dispersed in 150 mM Tris–HCl and EMEM and subjected to 5 min bath sonication prior to measurements.

For measurements with z-PTA, dilutions in purified water and 50 mM Tris–HCl at pH 7.4 were performed gravimetrically with a dilution factor around 1500×. For measurements in biological media, both NP samples were diluted in 10% FBS. Further dilution in EMEM was performed to reach the final concentration of serum and NPs 0.02% and 1.66 mg L^{-1}, respectively, immediately before measurement. A portion of the sample of at least 0.25 mL was loaded into the sample chamber using built-in pumps controlled by the instrument's software, for each individual measurement.

2.3 Characterisation techniques and measurement procedure

ELS and DLS. ELS is the most common technique used to measure the electrophoretic mobility of dispersed NPs. The electrophoretic mobility is converted to an average ζ-potential value measured within a sample. The principle of this technique is based on laser Doppler electrophoresis, where the charged NPs, in the presence of an electric field, migrate towards the oppositely charged electrode and are illuminated with a laser light beam. The particle velocity is determined from the frequency shift in the scattered light and the mobility is obtained from the ratio of velocity to electric field strength. ELS and dynamic light scattering (DLS) measurements were performed using Malvern Zetasizer Nano ZS instruments (Malvern

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Instruments Ltd., Worcestershire, UK), equipped with a titration device MPT-2 (Malvern Instruments Ltd., Worcestershire, UK) and a 4 mV He–Ne laser emitting at 633 nm. To ensure laser stability, the instruments were turned on at least 30 min before measurements. Disposable polycarbonate folded capillary cells with gold plated beryllium-copper electrodes (Malvern DTS1061 or DTS1070) were used to perform the measurements. The capillary cells were pre-rinsed with analytical grade ethanol and then excessively rinsed with purified water prior to sample loading. Additionally, the cells were preliminary rinsed with the sample before filling the cell to avoid dilution effects from remaining water. When analysing protein-based media, proteins can denature due to resistive heating of the electrodes. The resulting degradation products can deposit on the gold plated electrodes, with the risk of contamination of the subsequent samples. To minimise this risk, new capillary cells were used for each single measurement.

The use of the titration device allows ζ-potential and particle size measurements to be performed at each titration point in an automated routine which titrates the solution between two pH values set by the operator. The titration results were used to study the stability of both materials in water at different pH values, by performing acidic and basic titration. The pH meter probe of the titrator was calibrated at the beginning of each day using buffer solutions (EDT direct-ion, Dover, UK) with certified pH values of 4, 7 and 10. The system was cleaned with purified water (3 cycles) and primed before each titration. The pH meter probe was immersed in 10 mL of the NP sample which was kept under continuous stirring. The measurement cell was automatically filled with the sample prior to the measurement. The pH of the suspension was increased stepwise to pH 10 using 0.1 M and/or 0.01 M NaOH, and subsequently decreased again in integer pH steps to pH 3–4 using 0.1 M and/or 0.01 M HCl. All the DLS and ELS measurements were performed at 25 °C with an equilibration time of 120 s (ref. 17) and forward scattering angle of 13°. The refractive index (n) and extinction coefficient (k) of the silica NPs were assumed to be 1.46 and 0, respectively.[13] A dynamic viscosity (η) of 0.8872 mPa s and n of 1.330 (measured in house) were used for measurements in water and Tris–HCl buffer (dielectric constant $\varepsilon = 79$) while η of 1.09 mPa s and n of 1.335 (measured in house) were used for measurements in serum. A ζ-potential standard (polystyrene latex particles in aqueous buffer pH 9 with an assigned ζ-potential value of (-42 ± 4) mV, Malvern Instruments DTS1235) was used to verify the performance of the instrument. DLS size measurements are expressed as the scattered light intensity-weighted harmonic mean diameter, as calculated by the instrument software according to the cumulant method.[18] For ELS, the ζ-potential values reported were calculated using Smoluchowski approximation and are the averages of the values obtained from 2 (plain silica) or 3 (aminated silica) different laboratories. The precision is expressed as a standard deviation from the results.

TRPS. TRPS is based on the Coulter principle[19,20] for measuring size, concentration and ζ-potential of NPs larger than ~50 nm in diameter. It measures the reduction in ionic current across a pore on a membrane due to the temporary occlusion as a particle traverses it. A detailed analysis of the magnitude and duration of the blockade event, combined with a calibration routine enables the estimation of the particle volume and ζ-potential. The frequency of events can be related to the concentration of particles in suspension. The pulse duration, measured as the full width at half-maximum of the pulse signal is a function of particle velocity and length. The velocity of the particle is a combination of three effects: fluid velocity (convection), electroosmotic flow and electrophoretic mobility. The first two can be predicted by using calibration particles of known size and ζ-potential and by measuring the pore ζ-potential respectively. The electrophoretic mobility is estimated using the Smoluchowski approximation.[21]

TRPS measurements were performed with a qNano instrument (Izon Science Ltd, Christchurch, NZ). The TRPS system requires calibration by using particles of known size and ζ-potential. The relationship between the TRPS pulse signal and particle size was established by calibrating the system with monodisperse carboxylated polystyrene NPs (CPC100, diameter mode nominally 115 nm, supplied by Izon) certified for the size and concentration against other standards that have NIST-certified property values. The instrument was calibrated to ELS results on CPC100 calibration particles in 150 mM Tris and EMEM buffer and the surface potential of the nanopore membrane was set to −10 mV according to the manufacturer's advice.

The pore membrane (NP100, supplied by Izon) used for these experiments is recommended for particles with equivalent spherical diameters between 70 nm and 200 nm to pass through. All measurements were performed at room temperature (21 ± 1) °C. 40 μL of the NP suspension was loaded into the top fluid cell above the pore membrane. An electric field was applied across the pore and a pressure of 0.1 kPa to 0.2 kPa was applied to the top fluid cell using the Izon variable pressure module. The blockade signals were collected and exported for analysis using Izon Control Suite software V2.2. A minimum of 500 events were recorded for each measurement condition. The TRPS results are expressed as an arithmetic mean and precisions reported here are the standard deviations calculated from three repeats.

z-PTA. PTA operates by imaging particle movements in solution using a video camera. To enable this technique to also work for NPs, the suspension in the measurement cell is illuminated with a laser light beam, perpendicular to the camera axis. The camera records the bright spots of light scattered by the illuminated particles. The recorded movies are then analysed to locate and track individual NPs on a frame-by-frame basis. The ζ-potential for each particle is measured from the mean velocity in the direction of an applied electric field. The total velocity of each tracked particle is actually a sum of electrophoretic mobility and Brownian motion. These two components can, however, be separated by determining the total velocity at different depths within the closed sample chamber, and assuming a zero net flow over the entire chamber depth.

Measurements and analysis of the recorded movies were performed with an NS500 particle tracking analyser (NanoSight, Malvern Instruments Ltd, Worcestershire, UK) and equipped with a light source (violet diode laser, 405 nm, CW,

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power < 60 mW), Electron Multiplying Charge Coupled Device (EMCCD) camera and video analysis software version NTA2.2. The instrument was switched on at least 30 min before the measurements in order to ensure the stability of the optical system. The temperature was set and maintained at 25 °C (±1 °C) throughout the measurements. The applied voltage was 24 V for water and Tris–HCl and 12 V for biological medium. Movies were recorded over 30 s to 60 s depending on the depth of view, with 30 s equilibration time prior to each measurement. Camera levels were set to a value of 9 for water and Tris–HCl and a value of 16 for biological media. No long- or short-pass cut-off fluorescent filters were used. The performance of the instrument was checked daily with NIST RM 8013 (Au NPs, nominal average diameter 60 nm) diluted ~50 times with purified water. The following parameters were fixed: viscosity was set to 0.8905 mPa s, detection threshold was set to 25 and minimum particle size was set to 30 nm, blur and minimum track length was set to automatic. A minimum of 700 completed tracks were recorded per measurement. The ζ-potential for each particle was calculated using the Smoluchowski approximation. The values reported are the arithmetic mean of three measurements; the precisions reported here are the standard deviations of those measurements. The instrument was calibrated for the size and ζ-potential by the instrument manufacturer during yearly services against the manufacturer's own standard material (NanoSight, Malvern Instruments Ltd., Worcestershire, UK).

3. Results

The possible impact of the dispersion media water, Tris–HCl and FBS/EMEM on the colloidal stability of the plain (NanoChOp-05) and aminated (NanoChOp-06) silica NPs was investigated. Measurements were conducted by ensemble (ELS), and particle-by-particle based techniques (TRPS and z-PTA). The influence of environmental pH, dilution factor and presence of biomolecules on the ζ-potential measurements is discussed in the following sections.

3.1 pH of NP dispersions

The pH of the media in which the NPs were dispersed is one of the most important parameters that affect the ζ-potential of particles. A summary of the pH of the different suspensions is shown in Table 1. For TRPS and z-PTA the mean values are reported with the corresponding standard uncertainty, for ELS the values reported are the mean of the values obtained from 2 or 3 different laboratories. The pH of dispersions was measured at the NP concentration that was considered to be optimal for each technique. For each medium, no significant variations in pH for samples prepared for the different techniques were observed, except for aminated silica NP dispersions that were intended for ELS experiments. The pH for this material in purified water and Tris–HCl buffer was below 4 and about 5, respectively. This is due to the traces of acetic acid which was used to stabilise the aminated NPs.[13] For TRPS and z-PTA, the pH values of the more diluted dispersions were found to be closer to that of the dispersing media: about 5 for purified water (z-PTA) and about 7 for Tris–HCl buffer.

3.2 ELS and DLS

Table 2 shows the summary of the ζ-potential results that were obtained with the three techniques.

According to ELS measurements in purified water, a clear difference between silica NPs with and without (plain) surface amination was observed. Negative ζ-potentials were obtained for the plain silica NPs, whereas the aminated particles had slightly positive ζ-potentials. Measurements performed in Tris–HCl showed that both materials had a negative ζ-potential, with plain silica NPs again exhibiting the most negative value. The difference in ζ-potential between the two media is due to the increased number of counter-ions in the buffer and the difference in pH. In 10% FBS-EMEM, despite the slightly lower pH of NanoChOp-06, all samples had negative ζ-potential values which agreed within their stated measurement uncertainties.

It is well-known that changes in pH can significantly alter the degree of agglomerates/aggregates, ultimately compromising

Table 1 Measured pH of dilutions used for the different techniques

Technique (NP dilution)			ELS (1 : 2.5)	TRPS (1 : 65)	z-PTA (1 : 1500)
Material	Medium		pH	pH	pH
NanoChOp-05 (plain silica)	Purified water	0	7.3 ± 0.2	—	5.6 ± 0.1
		24 h	7.4 ± 0.2	—	—
	Tris–HCl	0	7.4 ± 0.1	7.4[a] ± 0.1	7.5 ± 0.1
		24 h	7.4 ± 0.1	7.4[a] ± 0.1	—
	FBS-EMEM	0	7.6 ± 0.1	7.6 ± 0.1	7.7 ± 0.2
		24 h	7.8 ± 0.1	7.6 ± 0.2	—
NanoChOp-06 (aminated silica)	Purified water	0	3.6 ± 0.1	—	5.2 ± 0.1
		24 h	3.6 ± 0.1	—	—
	Tris–HCl	0	5.0 ± 0.2	7.3[a] ± 0.1	7.5 ± 0.1
		24 h	5.2 ± 0.2	7.3[a] ± 0.2	—
	FBS-EMEM	0	6.3 ± 0.1	7.5 ± 0.1	7.6 ± 0.1
		24 h	6.3 ± 0.2	7.6 ± 0.1	—

[a] Measured in 150 mM Tris–HCl.

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Table 2 ζ-potential values of plain and aminated silica NPs. For measurements in FBS EMEM, the content of serum was 10% (ELS), 0.38% (TRPS) and 0.02% (z-PTA), respectively[b]

Material	Medium	Time	ELS (mV)	TRPS (mV)	z-PTA (mV)
NanoChOp-05 (plain silica)	Purified water	0 h	−48 ± 9	N/A	−39 ± 4
		24 h	−45 ± 10		−24 ± 4
	Tris–HCl	0 h	−30 ± 5	−24[a] ± 6	−21 ± 3
		24 h	−31 ± 5	−22[a] ± 2	−21 ± 2
	FBS EMEM	0 h	−15 ± 3	−15 ± 2	−7 ± 1
		24 h	−14 ± 3	−15 ± 1	—
NanoChOp-06 (aminated silica)	Purified water	0 h	+8 ± 2	N/A	−4 ± 1
		24 h	+7 ± 2		−13 ± 2
	Tris–HCl	0 h	−8 ± 4	−14[a] ± 1	−19 ± 2
		24 h	−9 ± 4	−17[a] ± 4	−20 ± 2
	FBS EMEM	0 h	−10 ± 3	−12 ± 1	−15 ± 3
		24 h	−11 ± 3	−12 ± 5	—

[a] Measured in 150 mM Tris–HCl. [b] N/A = not applicable for measurements in purified water due to the low ionic conductivity.

the colloidal stability of suspensions. The possible impact on ζ-potential and particle size was examined by performing ELS and DLS measurements upon sequentially adjusting the pH of the NP samples. The pH of the dispersions was modified in a controlled and automated manner by adding specific volumes of acid or base. The plain silica NPs were shown to be stable at all pH values (Fig. 1A) during titration to pH 10 (black circles) and back to pH 4 (white circles). No significant change in particle size was observed at any pH (Fig. S1A†), with an average NP diameter of (91 ± 2) nm. These measurements indicate that the plain silica NPs are expected to be stable and monodisperse over a wide pH range.

Titration of the aminated silica material (Fig. 1B) from pH 3 to pH 10 (black circles) shows that the NPs had slightly positive ζ-potential values at pH ∼ 3 and that the curve had passed the iso-electric point (zero net surface charge) near a pH of 3.5. At pH > 4, the ζ-potential was slightly negative and at pH ≥ 5 the ζ-potential was ≤−25 mV. A similar trend (white circles) was observed when the pH was reduced from 10 to 3. During the titration from the initial pH 3 to pH 10 some agglomeration/aggregation of the NPs occurred around pH 4–5 (Fig. S1B†), where the average NP diameter by DLS was measured to be (98 ± 9) nm. A similar agglomeration/aggregation at around pH ≤ 5 was observed when the pH was changed from 10 to 3.

DLS size measurements (see Fig. S2†) showed that both materials exhibit a monomodal size distribution in all media. In water and Tris–HCl plain silica was stable for 24 h, where a slight increase in size was observed for aminated silica NPs. The presence of serum significantly altered the width of the particle size distributions; both materials exhibited some level of agglomeration/aggregation.

3.3 TRPS

TRPS measurements were performed in 150 mM Tris–HCl and in 0.38% FBS-EMEM medium. As shown in Table 2, where the average ζ-potential values measured across the samples are reported, plain and aminated silica NP materials had negative ζ-potential values when dispersed in both media. The ζ-potential of NPs dispersed in purified water could not be determined due to the low ionic conductivity of the dispersing medium.

Fig. 2 shows representative TRPS results for aminated silica NPs in 0.38% FBS-EMEM measured immediately after dispersion. Each point in the graph relates to a single NP of which both the size and the ζ-potential are simultaneously measured.

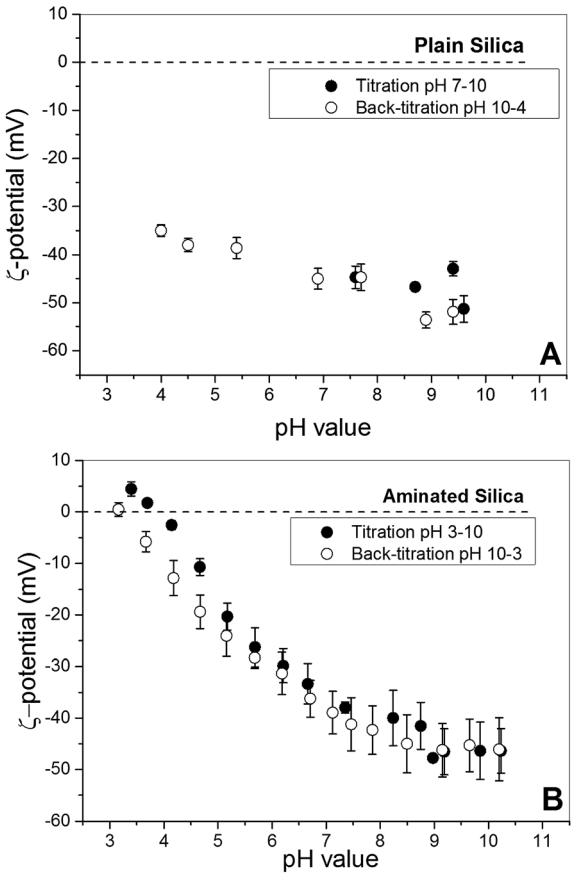

Fig. 1 Particle ζ-potential of plain (A) and aminated (B) silica at different pH values.

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Compared to DLS (Fig. 2S†), TRPS provides more detailed size distribution of NPs that can be related to their ζ-potential and different sub-populations (*i.e.* dimers, agglomerates) may be distinguished. The size histogram shows a major population peak at ~95 nm which represents the monomers or non-clustered particles, whereas a second population likely indicates the presence of clustered primary particles at ~115 nm. The ability of TRPS to measure the size and ζ-potential at the same time allows the observation of a trend indicating that NPs with more negative ζ-potentials are generally monodisperse, while NPs with high level of agglomeration/aggregation exhibit less negative ζ-potentials. The homogeneity of the sample can be related back to the width of the size and ζ-potential distribution.

3.4 z-PTA

The results of z-PTA measurements are shown in Table 2.

In purified water at about pH 5, both materials exhibited negative ζ-potentials, with plain silica NPs exhibiting more negative values than aminated NPs. After an incubation period of 24 h, the ζ-potential of the plain and aminated NPs conversely changes; *i.e.* it is less negative for plain silica and more negative for aminated silica. In Tris–HCl, both materials had similar negative ζ-potential values which did not change over time. These results indicate that the materials were more stable in this environment. Consistently, for both materials suspended in water and in Tris–HCl no change in diameter ~80 nm was observed. In biological media, with the serum content of ~0.02% both silica NP materials exhibited negative ζ-potentials with an increase in diameter to ~105 nm.

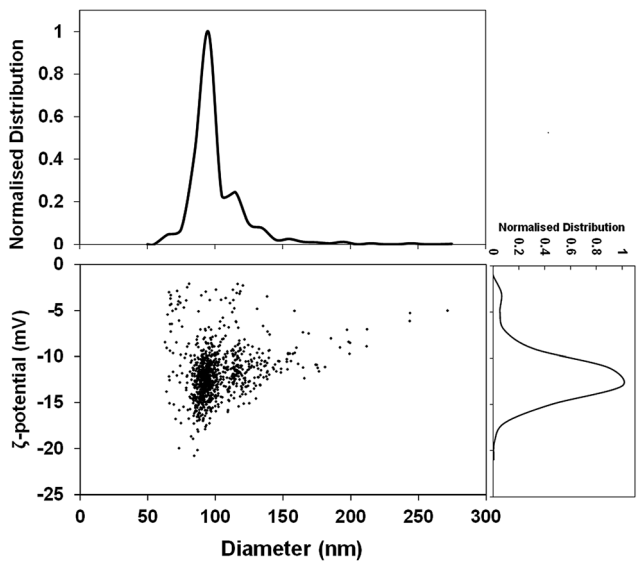

Fig. 2 Representative particle-by-particle ζ-potential and size distributions of aminated silica NPs in 0.38% FBS-EMEM measured by TRPS. Each measurement point represents a ζ-potential and size of an individual particle. The associated size (top) and zeta-potential (right) histograms show the distribution of these properties over the whole suspension.

3.5 Comparison of the results of the three ζ-potential measurement methods

Fig. 3 shows representative ζ-potential distributions obtained for TRPS and z-PTA and average mean values measured by ELS. The ζ-potential distributions for both materials and media obtained by z-PTA are broader than TRPS. In Tris–HCl, the mean ζ-potential value of plain silica measured by ELS was more negative than the values obtained by TRPS or z-PTA (Fig. 3A), while less negative for aminated silica (Fig. 3B). The latter is due to the moderate dilution factor used for ELS measurements and hence a lower pH value. For measurements in serum all techniques measured consistently negative ζ-potential values for both the plain (Fig. 3C) and aminated silica NPs (Fig. 3D). The ELS mean ζ-potential shows less negative values for aminated silica, which may be due to lower pH or the presence of a small population of larger NPs with a less negative ζ-potential as shown in Fig. 2. Such particles will scatter light more intensely and will contribute disproportionately to the ELS measurement, resulting in the measured ζ-potential being skewed by a sub-population of the NPs.

4. Discussion

All techniques used in this work have been assessed for their ability to measure ζ-potential in a range of media, using near-spherical silica NPs as model systems. The analysis of dispersion properties by simultaneous size and ζ-potential measurements was performed using particle-by-particle based techniques, which permit the study of variations in particle surface properties within homogeneous and heterogeneous samples. Ensemble measurements, which provide average values across a sample, lack resolution and selectivity within heterogeneous samples and as a result the shape and position of the signal peaks of the distributions can be significantly affected by a small sub-population of particles (*e.g.* of larger size), making it difficult to gain a detailed understanding of the sample. Furthermore, we investigated the challenges in characterising such materials in complex media such as serum. This is of particular interest for many biological applications of NPs, where in the presence of free proteins, the NP's surface is often spontaneously coated with adsorbed proteins. Even more than some other properties of NPs, ζ-potential intrinsically relates to the dispersion conditions, not only in terms of the type of medium, but also the pH and NP concentration[22] (Table S1†). Consequently, details of the NP environment need to be defined when measuring their ζ-potential. More importantly, these details need to be reported and documented when such measurements are part of a larger study and compared to other measurements performed in different media, with different instruments or different NP samples.

The comparison of results among different techniques is not straightforward for several reasons. One reason might be the differences in samples used in the different laboratories and at different points of time. However, the homogeneity and stability data provided with the test materials[13] are much smaller than the experimentally observed differences. For the purposes of the

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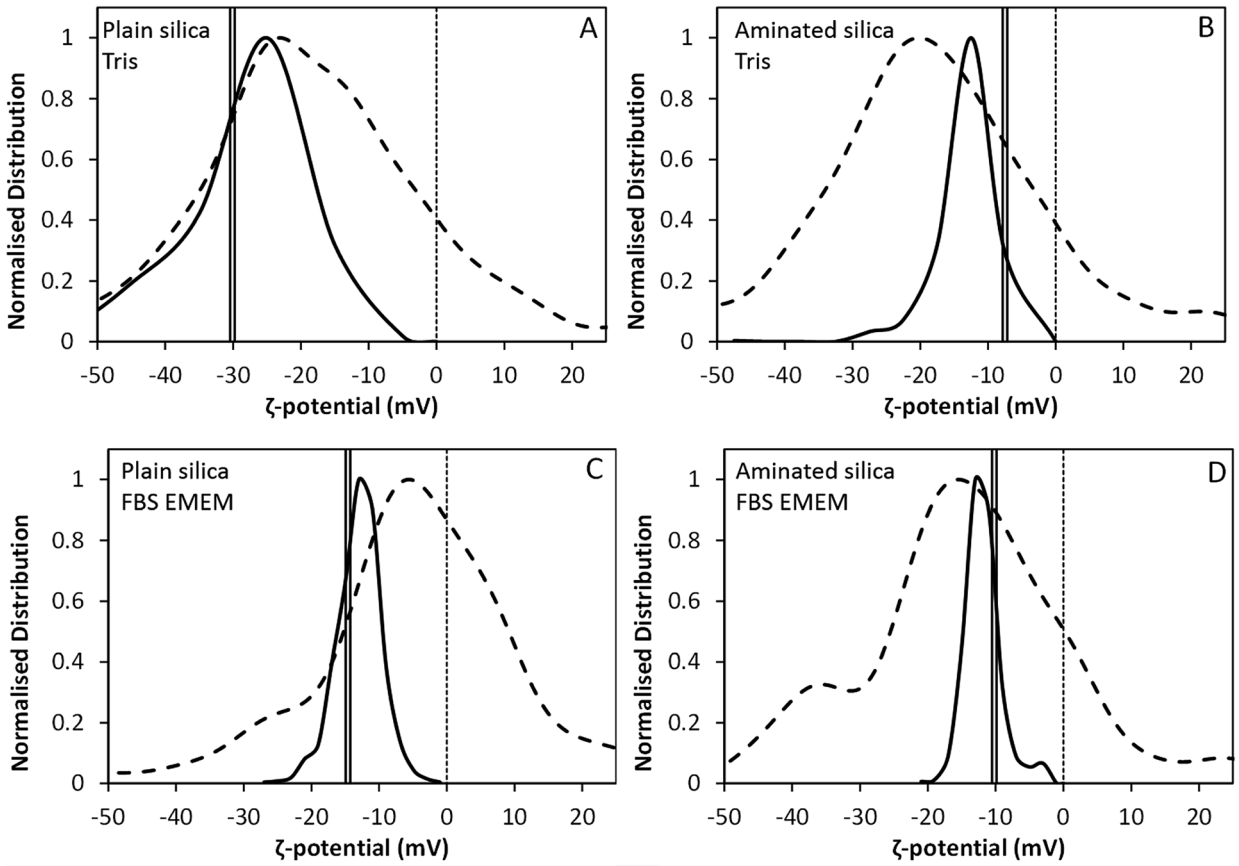

Fig. 3 The normalised ζ-potential distributions of plain (A and C) and aminated (B and D) silica NPs determined by TRPS (continuous line) and z-PTA (dashed line). The mean value measured by ELS is shown as a vertical bold line. Representative graphs were obtained at $t = 0$ after dispersion in Tris–HCl (A and B) and biological medium (C and D), respectively.

performed study, the test materials can be considered as being reliable reference materials.

A second reason for variation between the measured values can be due to the intrinsic differences between the measurement principles of the applied methods. Both z-PTA and ELS are light scattering-based techniques. For the latter, the particle velocity is determined from the frequency shift utilizing the Doppler effect, whereas for z-PTA both electrophoretic and Brownian motions of each particle is extracted by analysing the track of a particle in time. Drift velocities are calculated on a particle-by-particle basis and is not intensity-weighted towards larger NPs. TRPS provides measurements which are independent of the light scattering properties of the particles and the velocity of each NP can be calculated by analysis of their transit time through an aperture. The results obtained by TRPS indicate that particle size and ζ-potential properties can be observed and correlated.

Thirdly, experimental scatter can be due to variations in sample preparation protocols, which need to be optimised for each instrument according to their technical requirements. This can be seen in the results of the measurements performed by ELS and z-PTA in water in the case of aminated silica NPs. These results seem to disagree, ELS measuring slightly positive ζ-potential values and z-PTA providing negative ζ-potentials. However, samples for z-PTA measurements were considerably more dilute in purified water than those prepared for ELS measurements. A direct consequence of such dilution was the change in pH of the solution: where ELS measurements were performed at pH 3.6, z-PTA measurements were performed at pH 5.2. Titration studies (Fig. 1B) show that, when taking the environmental pH under consideration, the ELS ζ-potential results (in water at pH 5) are comparable to the z-PTA results.

The type of buffer and its molarity is an additional factor to be considered during ζ-potential measurements. For example, TRPS measurements need to be performed in an electrolyte solution of a certain ionic strength and therefore ζ-potential measurements in purified water or 50 mM Tris–HCl could not be performed, unlike ELS and z-PTA. When z-PTA measurements were repeated in 150 mM Tris–HCl buffer, no significant difference in ζ-potential has been observed (Table S2†) and therefore, a comparison among the techniques of the measurements performed in Tris–HCl can still be attempted. Both NP materials in Tris–HCl buffer exhibited a negative ζ-potential as measured by all techniques, also in agreement with previously published studies.[7,23,24] While the three techniques were in broad agreement for the ζ-potential of plain silica NPs, that of aminated silica measured by ELS was significantly less negative than that measured by both TRPS and z-PTA. It is

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possible that ELS ζ-potential measurements are skewed by the presence of agglomerates or agglomerates/aggregates.

When NPs are introduced in serum, some of the proteins may adsorb at the surface of NPs. According to Tenzer and co-workers[25] at physiological pH protein-coated silica NPs display a negative surface charge density, irrespective of the original NPs' surface charge density or duration of their exposure to human plasma proteins. All techniques consistently measured negative ζ-potential values for both the plain and aminated silica NPs in FBS EMEM regardless of the difference in the concentration of NPs and serum content used due to the instrumental requirements. ζ-potential values were similar for the two NPs, although not identical, and did not significantly change with time.

5. Conclusions

In summary, in this work we have systematically compared measurement strategies based on the use of ensemble (ELS) and particle-by-particle techniques (TRPS and z-PTA) for the measurement of ζ-potential of plain and aminated silica NPs dispersed in different media, including a complex serum matrix. Despite differences between the basic measurement principles of the three methods, the results are overall in good agreement. It is clear that particle-by-particle techniques provided additional information in terms of distribution of ζ-potential over the whole sample and hence allow us to better understand the fundamental behaviour of silica NPs in biological media. This work also highlighted some of the challenges related to the measurement of ζ-potential of NPs with focus on the influence of biological media on this parameter. To allow comparison of the results among the different techniques, efforts were made to prepare the samples according to similar protocols. However, some variations had to be introduced in order to meet specific instrument requirements. When diluting the sample, factors that need to be considered are (i) the incompatibility of some media with a measuring technique; (ii) the possible change in pH of the suspension due to different dilution factors; (iii) the change in the relative excess of proteins to NPs. We therefore recommend that when ζ-potential values of NPs are presented contextual details are also reported, such as type, ionic strength, viscosity and pH of the dispersant, along with the NP concentration and dilution factor in media.

Author contributions

AS acquired and evaluated the ELS and TRPS measurements. DB acquired and evaluated the z-PTA measurements. DG, VK, YR acquired and evaluated the ELS data. GR organised the production of the test materials. ZV and MP performed the amination of the plain silica NPs. HGI conceived the study of the framework of the NanoChoP project and research design. AGS and CM contributed to the concept and research design. All authors reviewed and contributed to the manuscript.

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