This protocol assumes an intermediate level of scientific competency with regard to techniques, instrumentation, and safety procedures. Rudimentary assay details have been omitted for the sake of brevity.
Method written by:
Jeffrey D. Clogston, Ph.D.

Reviewed by:
Anil K. Patri, Ph.D.
1. Introduction

This protocol describes the measurement of zeta potential for nanomaterial samples in aqueous solutions. Particle surface characteristics and charge play an important role in the particle’s physical state, stability in different media, agglomeration tendencies, and interaction with biological systems. Zeta potential measurement provides an indirect measure of the net charge and as a tool to test batch-to-batch consistency.

A particle in solution with a net charge has a layer of ions (of opposite charge) strongly bound to its surface; this is referred to as the Stern layer. A second diffuse outer layer is comprised of loosely associated ions. These two layers are collectively called the electrical double layer. As the particle moves (due to gravity, kinetic energy, and/or under an applied electrical field), there exists a boundary between the ions in the diffuse layer that move with the particle and ions that remain with the bulk dispersant. The electrostatic potential at this “slipping plane” boundary is the zeta potential.

In zeta potential measurements, an electrical field is applied across the sample and the movement of the particles (electrophoretic mobility) is measured by the light scattering of the particles. The Henry equation is then used to calculate the zeta potential, \( z \):

\[
U_e = \frac{2 \varepsilon \ z \ f(\kappa a)}{3 \eta}
\]

where \( U_e \) is the electrophoretic mobility, \( \varepsilon \) is the dielectric constant, \( \eta \) is the absolute zero-shear viscosity of the medium, \( f(\kappa a) \) is the Henry function, and \( \kappa a \) is a measure of the ratio of the particle radius to the Debye length.

This assay protocol outlines the procedure for sample preparation and determination of zeta potential. Guidelines for making successful zeta potential measurements are provided, as well as a discussion of relevant standards and data analysis. Examples of zeta potential results are illustrated for 30 nm nominal size colloidal gold and a G5 PAMAM amine-terminated dendrimer.
2. Reagents, Materials, and Equipment

Note: The NCL does not endorse any of the suppliers listed below; their inclusion is for informational purposes only. Equivalent supplies from alternate vendors can be substituted.

2.1 ZetaSizer Nano ZS (Malvern Instruments)
2.2 Folded capillary cell (referred to as zeta cells, polycarbonate cell with gold-plated electrodes; Malvern Instruments, DTS1060C)
2.3 Caps for zeta cells (2 per cell)
2.4 Zeta Potential Transfer Standard (Carboxylated polystyrene latex dispersed in pH 9.22 buffer; Malvern Instruments, DTS0050)
2.5 10 mM NaCl

3. Methods

3.1 Cleaning and Handling of Zeta Cells

3.1.1 Zeta cells should be rinsed thoroughly before use. This involves rinsing the zeta cells with water, followed by ethanol, and finally water again. As the zeta cells have two ports, it is recommended to flush a minimum of 1.0 mL of each rinsing solvent through each port to thoroughly rinse each electrode. This can be achieved by either using a 1 mL disposable syringe or a wash bottle filled with the corresponding rinsing solvent.

3.1.2 After the zeta cells are rinsed, visually check the electrodes both inside and outside of the cell and the measuring window for any manufacturing defects such as scratches on or non-transparent measuring windows, dirty or non-homogenous surface coating of electrodes (electrodes are gold-plated), or any residual polycarbonate (from the manufacturing process) in the cell or on the electrodes. New zeta cells should not be used if any of these defects are found.

3.1.3 The zeta cells, after rinsing, should be dried using a gentle stream of nitrogen attached to a filter to remove any remaining solvent and physically re-examined as above.
3.1.4 The zeta cells should be capped until use to prevent any dust contamination.

3.1.5 Take care not to touch the measuring window of the zeta cells as well as the electrodes. Use of gloves is highly recommended to avoid any residual oil deposits.

3.1.6 Zeta cells are to be used once per sample and then disposed of. It is highly advised to not re-use or re-clean the zeta cells for future measurements.

3.2 Sample Preparation

3.2.1 Samples should be prepared in a biological safety hood to protect the sample from particulate and to minimize exposure; appropriate safety precautions and protective gear such as gloves, lab coat and goggles must be worn.

3.2.2 Samples should be prepared in a low ionic strength medium; 10 mM NaCl is highly recommended. Suspending medium should be filtered prior to sample preparation using a 0.1 μm or smaller pore size membrane.

3.2.3 Sample concentration is particle-dependent. Ideal sample concentration is determined by the nature (light scattering properties) of the analyte nanoparticle. Metallic nanoparticles scatter light more strongly than hydrocarbon-based ‘soft’ nanoparticles such as dendrimers, proteins and polymers. As a result, the concentration for metallic nanoparticles will be lower. A representative measurement on two classes of nanoparticles (colloidal gold and dendrimers) is provided in this protocol.

3.2.4 Zeta potential is dependent on pH and therefore the sample pH should be measured before and after the zeta potential readings. Care and precautions should be taken, such as rinsing the electrode with filtered buffers and working on a clean bench/biological safety cabinet, to minimize introduction of any new particulate into the sample during the pH measurement as this will effect the zeta potential measurement.

3.3 Loading Sample into Zeta Cells

3.3.1 The zeta cell can accommodate a minimum of 750 μL of sample. Transfer the sample to a 1 mL disposable syringe, dislodge any air bubbles, and
insert the syringe to one of the ports on the zeta cell. Fill the zeta cell with sample by gently depressing the syringe plunger. Remove the syringe from the zeta cell after the entire sample has been transferred.

3.3.2 Check for any air bubbles in the zeta cell or on the electrodes; gently tap the zeta cell to dislodge them. Visually check that both electrodes are submerged. Add more sample to the zeta cell if necessary by using a 1 mL disposable syringe; add sample in a drop-wise manner to one port and gently tap the zeta cell until the sample drop clears the port.

3.3.3 Cap both ports simultaneously to ensure even sample levels on both sides of the zeta cell.

3.4 Measurement Procedure

3.4.1 The zeta cell contains a weld line (a small notch along the top of the cell) on one side; this is the front of the zeta cell and should be facing the front of the instrument. Before loading the zeta cell into the instrument, carefully wipe the measuring windows with lens paper. Once the zeta cell is placed in the instrument, check that the electrodes on the zeta cell are in contact with the leads in the instrument.

3.4.2 The temperature should be controlled and measured with an accuracy and precision of 0.3°C or better. For a maximum sample volume of 1.0 mL and operation close to room temperature (ΔT ± 1°C), an equilibration time of 2 minutes is recommended prior to starting measurements.

3.4.3 Perform a minimum of three runs per sample to establish measurement repeatability. Measurement duration (i.e. # of sub-runs per run) should be set according to the instrument manufacturer's recommendations, and will differ depending on particle size and scattering characteristics.

3.4.4 The applied voltage can be set to automatic or manual mode. In automatic mode, the best voltage is determined by the software. However it is recommended to run in manual mode and start at a low voltage, typically 80 V, and increase the voltage gradually for optimal results (maximum voltage is 150 V).
3.5 Data Analysis

3.5.1 A detailed description of the data analysis will not be given here. It is left for the reader to check the instrument’s user manual to identify the data analysis algorithm. However, regardless of the instrument used, the Henry equation will be ultimately employed to calculate the zeta potential, $z$: 

$$U_e = \frac{2 \varepsilon z f(\kappa a)}{3 \eta}$$

where $U_e$ is the electrophoretic mobility, $\varepsilon$ is the dielectric constant, $\eta$ is the absolute zero-shear viscosity of the medium, $f(\kappa a)$ is the Henry function, and $\kappa a$ is a measure of the ratio of the particle radius to the Debye length. The electrophoretic mobility is determined experimentally and used to calculate the zeta potential. The remaining terms, dielectric constant, viscosity, and Henry function are user input values. Table 1 lists the viscosities at three different temperatures for 10 mM NaCl and the dielectric constant at $25^\circ$C (1). The Henry function can be approximated as 1.5 (Smoluchowski model) for measurements made in aqueous medium with salt concentrations $\geq$ 10 mM.

<table>
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<th>Aqueous Medium</th>
<th>Absolute Viscosityy (mPa ∙ s)</th>
<th>Dielectric Constant</th>
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<td>20$^\circ$ C</td>
<td>25$^\circ$ C</td>
<td>37$^\circ$ C</td>
</tr>
<tr>
<td>10 mM NaCl</td>
<td>1.003</td>
<td>0.891</td>
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</table>

3.5.2 The zeta potential, at a minimum, is reported with its standard deviation (report number of runs) along with the measurement temperature, the pH and concentration of the sample, and the dispersion medium composition. Additional information should be reported and include: Henry function approximation employed, viscosity of dispersing medium, dielectric constant, applied voltage, instrument make and model, and type of zeta
cell used. Examples of zeta potential results are shown for commercial 30 nm nominal size colloidal gold and commercially available G5 amine-terminated dendrimer in Figures 1 and 2, respectively.

3.5.3 Zeta potential values in the range between -10 to +10 mV are considered neutral.

4. Precautions and Guidelines

4.1 All safety precautions per you lab SOPs should be followed for the nanomaterial that is being tested. At a minimum, one should wear a lab coat, gloves and protective goggles and work in a biological safety cabinet or a chemical fume hood to minimize exposure to the worker.

4.2 Zeta potential measurements are based on first principles and hence no calibration is required. However, the instrument can be validated by running an appropriate standard. It is recommended to run such a commercial standard along with the samples. One such a standard is available from Malvern Instruments (Zeta Potential Transfer Standard, DTS0050) and has a zeta potential value of -50 ± 5 mV at 25°C.

4.3 Zeta potential is dependent on sample concentration and pH, and the conductivity of the dispersing medium. Different values for zeta potentials are obtained depending on these factors and thus it is important to accurately measure and report them.

4.4 The pH of the sample is reported along with the zeta potential. Sometimes it is useful to adjust the native sample pH or change ionic strength. Care should be taken in this process. Addition of strong acid or base too rapidly may compromise the sample’s integrity and result in a polydispersed sample. The zeta potential distribution may contain multiple peaks arising from the presence of differently-charged species. It is recommended to titrate with 0.1 M HCl or 0.1 M NaOH in a drop-wise (~1-3 μL) manner while stirring and monitor the pH throughout. Alternatively, an autotitrator can be used to adjust the sample pH. The sample should be physically examined to see if it is unstable under different pH or buffer conditions. Particle instabilities may lead to phase separation,
precipitation, agglomeration or aggregation. If polydisperse, part of the sample may precipitate under certain pH conditions and the data reported may not be accurate. Precautions should be taken to understand these phenomenon for the test material.

4.5 The quality of data can be assessed by examining several parameters. Check that the scattering intensity count rate is acceptable based on the instrument manufacturer’s specifications. The phase plots should have alternating slopes with time followed by either a smooth positive or negative peak (see Figures 1A, 2A). Review the frequency and phase plots; the baseline in the frequency plots should not be noisy but rather smooth (see Figures 1B, 2B). The zeta potential should not change with measurement duration (i.e. number of runs) or with different applied voltages.

4.6 Unreliable results as defined by the criteria above can be due to several reasons. Periodically check that air bubbles do not form on the electrode surface as a result of the applied voltage. Gently tap the zeta cell to dislodge them before proceeding with the measurement. Check that the zeta cell is placed in the correct orientation and that the measuring window is clean. Samples should be optically clear and not turbid. Inconsistent zeta potential values with time can be a result of sample or electrode degradation. Blackening of the electrode surface or visual evidence of precipitate formation or change of sample color verifies such degradation. Lower applied voltages are recommended in these cases. Blackening of the electrodes also occurs if the dispersing medium has a high salt concentration (high conductivity). The authors highly recommend using 10 mM NaCl as the dispersing medium. Poor phase plots can be often remedied by increasing the number of sub-runs. Sample concentration is particle-specific and depends on the particle’s scattering properties. Low concentrations may seriously degrade the signal-to-noise ratio and will result in noisy and inconsistent results. High concentrations can lead to multiple scattering effects and particle interactions, both of which can produce changes in the measured zeta potential. Typically, zeta potential measurements are made after DLS has been performed.
If this is the case, normally the optimal concentration for DLS works well for zeta potential measurements.

4.7 A final note on zeta potential values is worth mentioning. The zeta potential value obtained from the measurement is an average value. Thus if the sample is monodispersed, this value corresponds to the zeta potential of the single component in the sample. However, if the sample is polydispersed (i.e. contains several different charged species), the zeta potential distribution plot will contain multiple peaks corresponding to the multiple components. The zeta potential in this case can be reported as either an average across all charged species or (if the instrument software allows) the zeta potential for each peak. In the former case, it should be noted that the sample is polydispersed.
Figure 1. The averaged (n = 5) phase (A), frequency (B), and zeta potential distribution (C) for 30 nm colloidal gold. Sample concentration was 1.3 μg/mL in 10 mM NaCl with a pH value of 6.5. The sample was transferred to a zeta cell (Malvern Instruments, DTS1060C) and measured at 25°C using a Malvern ZetaSizer Nano ZS and an applied voltage of 150 V. The zeta potential was -33.0 ± 0.5 mV (n = 5). A viscosity of 0.891 cP, a dielectric constant of 78.6, and Henry function of 1.5 were used for the calculations.
Figure 2. The averaged $(n = 4)$ phase (A), frequency (B), and zeta potential distribution (C) for a G5 PAMAM amine-terminated dendrimer. Sample concentration was 2 mg/mL in 10 mM NaCl with a pH value of 7.4. The sample was transferred to a zeta cell (Malvern Instruments, DTS1060C) and measured at 25°C using a Malvern ZetaSizer Nano ZS and an applied voltage of 120 V. The zeta potential was $41.9 \pm 5.5$ mV $(n = 4)$. A viscosity of 0.891 cP, a dielectric constant of 78.6, and Henry function of 1.5 were used for the calculations.
5. Waste Disposal Information

Follow your facility's recommended disposal procedure for your specific nanomaterial and solvents.

6. References


7. Abbreviations

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<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>C</td>
<td>Celsius</td>
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<td>cP</td>
<td>centipoise</td>
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<td>DLS</td>
<td>dynamic light scattering</td>
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<td>ε</td>
<td>dielectric constant</td>
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<tr>
<td>f(kα)</td>
<td>Henry function</td>
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<td>G5 PAMAM</td>
<td>fifth-generation polyamidoamine</td>
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<tr>
<td>HCl</td>
<td>hydrochloric acid</td>
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<td>kα</td>
<td>ratio of the particle radius to the Debye length</td>
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<td>M</td>
<td>molar</td>
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<td>mL</td>
<td>milliliter</td>
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\( \eta \)  
absolute zero-shear viscosity

NaCl  
sodium chloride

NaOH  
sodium hydroxide

nm  
nanometer

s  
seconds

T  
temperature

\( U_e \)  
electrophoretic mobility

V  
volts

z  
zeta potential