Determination of zeta potential via nanoparticle translocation velocities through a tunable nanopore: using DNA-modified particles as an example

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Metadata Record: https://dspace.lboro.ac.uk/2134/23101

Version: Accepted for publication

Publisher: Journal of Visualized Experiments (JoVE)

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Title: Determination of zeta potential via nanoparticle translocation velocities through a tunable nanopore: Using DNA-modified particles as an example.

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Key words: Biosensor; TRPS; Nanopore; Zeta potential; DNA; Superparamagnetic particles.

Short abstract:
Here we use a polyurethane tunable nanopore integrated into a resistive pulse sensing technique to characterise nanoparticles surface chemistries via the measurement of particle translocation velocities, which can be used to determine the zeta potential of individual nanoparticles.

Long abstract:
Nanopore technologies, known collectively as Resistive Pulse Sensors, RPS, are being used to detect, quantify and characterize proteins, molecules and nanoparticles. Tunable resistive pulse sensing (TRPS) is a relatively recent adaptation to RPS that incorporates a tunable pore that can be altered in real time. Here, we use TRPS to monitor the translocation times of DNA-modified nanoparticles as they traverse the tunable pore membrane as a function of DNA concentration and structure (i.e. single-stranded to double-stranded DNA).

TRPS is based on two Ag/AgCl electrodes, separated by an elastomeric pore membrane that establishes a stable ionic current upon an applied electric field. Unlike various optical-based particle characterization technologies, TRPS can characterize individual particles amongst a sample population, allowing for multimodal samples to be analyzed with ease. Here, we demonstrate zeta potential measurements via particle translocation velocities of known standards and apply these to sample analyte translocation times, thus resulting in measuring the zeta potential of those analytes.

As well as acquiring mean zeta potential values, the samples are all measured using a particle-by-particle perspective exhibiting more information on a given sample through sample population distributions, for example. Of such, this method
demonstrates potential within sensing applications for both medical and environmental fields.

Introduction:

Functionalized nanoparticles are becoming increasingly popular as biosensors in both medical and environmental fields. The ability to alter a nanoparticle’s surface chemistry, with DNA for example, is proving useful for targeted drug delivery systems\(^1\) and monitoring DNA-protein interactions\(^2-4\). An increasingly common nanoparticle property being utilized in bioassays and in the delivery of therapeutics is superparamagnetism\(^5\). Superparamagnetic particles (SPPs) are extremely useful in identifying and removing specific analytes from complex mixtures and can do so with the simple use of a single magnet. Once removed, the analyte-bound particles can be characterized and analyzed fit for purpose.

Previous methods used for the detection and characterization of nanoparticles include optical techniques such as dynamic light scattering (DLS), otherwise known as photon correlation spectroscopy. Although a high throughput technique, DLS is limited to being an averaging based technique and when analyzing multimodal samples without the addition of specialist software, the larger particles will produce a much more dominant signal, leaving some of the smaller particles completely unnoticed\(^6,7\). Particle-by-particle characterization techniques are therefore much more favourable to analyze nanoparticle and functionalized nanoparticle systems.

RPS based technologies are based around applying an electric field to a sample and monitoring the transportation mechanism of the particles through a synthetic or biological nanopore. A relatively recent nanoparticle detection and characterization technique based on RPS is tunable resistive pulse sensing (TRPS)\(^8-16\). TRPS is a two electrode system separated by an elastomeric, tunable pore membrane. A tunable pore method allows for analytes of a range of shape\(^17\) and size to be measured via their transport mechanisms through the pore. Tunable pores have previously been used for the detection of small particles (70-95 nm diameter) producing comparable results to other techniques such as transmission electron spectroscopy (TEM)\(^10\). When an electric field is applied, an ionic current is observed and as particles/molecules pass through the pore, they temporarily block the pore, causing a reduction in the current that can be defined as a ‘blockade event’. Each blockade event is representative of a single particle so that each particle within a sample can be characterised individually based on the blockade magnitude, \(\Delta i_p\), and full width half-maximum, \(FWHM\), as well as other blockade properties. Analyzing individual particles as they pass through a nanopore is advantageous for multimodal samples as TRPS can successfully and effectively distinguish a range of particle sizes amongst a single sample. Tunable resistive pulse sensing completes size\(^10\), zeta potential\(^12,18\) and concentration\(^15\) measurements simultaneously in a single run and can therefore still differentiate samples of similar, if not the same, size by their surface charge\(^19\); an advantage over alternative sizing techniques.

Zeta potential is defined as the electrostatic potential at the plane of shear\(^20\), and is calculated from particle velocities as they traverse a pore\(^19\). Zeta potential measurements of individual particles thus gives insight into the translocation mechanisms and behaviour of nanoparticle systems in solution, valuable information
for the future of nanoparticle assay designs for a range of applications. Particle-by-particle analysis of such nature also allows for the spread and distribution of zeta potential values amongst a sample population to be explored, allowing for more information on reaction kinetics (single-stranded to double-stranded DNA, for example) and particle stabilities in solution to be attained.

Here, we describe a technique that detects and characterizes both unmodified and DNA-modified SPP surfaces. The protocol described herein is applicable to a range of inorganic and biological nanoparticles, but we demonstrate the procedure using DNA-modified surfaces due to their wide range of applications. The technique allows the user to distinguish between single-stranded and double-stranded DNA targets on a nanoparticle surface, based on particle translocation velocities through a pore system and thus their zeta potentials.

**Protocol:**

For all materials and equipment used within this protocol, please see the table of materials and equipment published alongside this article.

1. Making the Phosphate buffered saline with tween-20 (PBST) buffer.

   1.1. Dissolve one PBS tablet (0.01 M phosphate buffer, 0.0027 M Potassium Chloride, 0.137 M Sodium Chloride, pH 7.4) in 200 mL deionised water (18.2 MΩ cm).

   1.2. Add 100 µL (0.05 (v/v)%) Tween-20 to the 200 mL buffer solution as a surfactant.

2. Preparing the carboxyl polystyrene particle standards.

   2.1. Vortex the calibration particles for 30 seconds before sonication for 2 minutes at 80 watts to create monodispersity of the particles.

   2.2. Dilute the calibration particles 1 in 100 to a concentration of 1x10¹⁰ particles/mL in PBST buffer and vortex for 30 seconds.

3. Preparing streptavidin coated particles.

   3.1. Vortex the particles for 30 seconds before sonication for 2 minutes at 80 watts to ensure monodispersity.

   3.2. Dilute the streptavidin coated particles 1 in 100 in PBST buffer to achieve a resulting concentration of 1x10⁹ particles/mL and vortex for 30 seconds. A typical sample volume is 200 µL, if you are investigating five DNA concentrations, for example, 1 mL of diluted streptavidin coated particles should be prepared.

4. Preparation of oligonucleotides:

   4.1. Reconstitute oligonucleotides with deionised water to a resulting concentration of 100 µM.
5. **Addition of capture probe (CP) DNA to the streptavidin coated particles.**

5.1. Prior to DNA binding, vortex the streptavidin coated particles (200 µL sample volume) for 30 seconds followed by a 2 minute sonication at 80 watts.

5.2. Based on the binding capacity provided by the supplier (4352 pmol/mg), add the appropriate concentration of DNA to the particles for resulting concentrations of 10, 20, 30, 40, 47, 95, 140, and 210 nM DNA.

5.3. Vortex the samples for 10 seconds and place on a rotary wheel at room temperature for 30 minutes to allow for the DNA to bind to the particle surfaces via a streptavidin-biotin interaction.

5.4. Once the capture DNA has been added and incubated with the streptavidin coated particles, remove the excess DNA in solution via magnetic separation by placing the samples onto a magrack for 30 minutes.

5.5. Remove the supernatant, taking care not to disturb the newly formed cluster of particles closest to the magnet, and replace with the same volume of new PBST buffer.

6. **Hybridizing complementary DNA to the CP-particles.**

6.1. Add the required amount of target DNA (in excess at 500 nM) to ensure the maximum possible target binding was reached.

6.2. Vortex the samples for 10 seconds and place on a rotary wheel at room temperature for 30 minutes.

6.3. Once the hybridization is complete, remove the excess target DNA via magnetic separation by placing the samples onto a magrack for 30 minutes.

6.4. Remove the supernatant, taking care not to disturb the newly formed cluster of particles closest to the magnet, and replace with the same volume of new PBST buffer.

6.5 Repeat steps 6.1 to 6.4 for duplicate samples and place these samples on a rotary wheel at room temperature for 16 hours to investigate DNA hybridization times.

7. **TRPS setup.**

7.1. Plug in the instrument into a computer system with Izon Control Suite software in place.

7.2. Calibrate the initial stretch using callipers by measuring the distance between the outside of two parallel jaws and input into the software by typing the stretch in the ‘stretch’ in the ‘Instrument Settings’ tab and clicking ‘Calibrate stretch’ underneath the tab.
7.3. Laterally fit a polyurethane nanopore membrane of appropriate sizing for analysis onto the jaws with the nanopore ID number facing upward. Then, stretch the jaws to the stretch required for analysis using the stretch adjustment handle on the side of the instrument. The stretch should be between 43 and 48 mm and the exact value is determined alongside applied voltage so that calibration particle blockades are at least 0.3 nA in size. The stretch is already inputted into the software in step 7.2 and will automatically adjust as you stretch the jaws.

7.4. Place 80 µL of PBST buffer in the lower fluid cell, beneath the nanopore, ensuring there are no bubbles present that may affect the measurement. If there are bubbles seen, remove and replace the buffer.

7.5. Click the upper fluid cell into place and place 40 µL of buffer into it, again ensuring there are no bubbles present. If bubbles are present in the upper fluid cell, remove them by replacing the liquid.

7.6. When a reproducible baseline current has been reached from replacing the upper fluid cell with buffer, add 40 µL of the sample to the upper fluid cell and measure by clicking ‘start’ in the ‘Data Acquisition’ tab on the software screen. The data acquisition is completed at a frequency of 50 MHz with a blockade magnitude lower limit of 0.05 nA, although this can be altered using the Izon Control Suite software on the ‘Analyse Data’ tab (under ‘Analysis Settings’ and ‘Resistive Blockades’).

7.7. Place a Faraday cage over the top of the fluid cell system to reduce electrical background noise on the measurements.

7.8. The variable pressure module (VPM) is used to apply a pressure or vacuum to the samples. To apply an external pressure the nozzle should be connected to the upper fluid cell, the pressure arm is then rotated and clicked into place depending on whether you want to apply a positive pressure (PRE) or a vacuum (VAC). Pressure can be applied in a ‘cm’ or ‘mm’ scale that is determined by the pressure stage knob situated on the top of the VPM. If the knob is pressed down the pressure applied is on the ‘cm’ scale and if it is pulled upward it is on the ‘mm’ scale.

8. Preparing samples for TRPS analysis.

8.1. Vortex samples for 30 seconds and sonicate for 2 minutes at 80 watts prior to TRPS analysis.

9. Calibrating the nanopore for zeta analysis.

9.1. After placing 40 µL calibration particles (1x10¹⁰ particles/mL) into the upper fluid cell, complete a TRPS measurement (setup as in section 7) at 3 applied voltages, the voltage is altered by clicking on the ‘+’ and ‘-’ buttons on the voltage scale in the ‘Instrument Settings’ tab on the software. The 3 voltages should return background currents of approximately 140, 110, and 80 nA. At the medium voltage the calibration particles, should produce an average blockade magnitude of at least 0.3 nA. The voltages chosen should have runs completed using an applied pressure and the highest voltage should be run at two pressures. Apply a pressure so the
average full width half maximum (FWHM) durations of the calibration particles are at least 0.15 ms, manually using the pressure arm attached to the variable pressure module. Select pressure (PRE) or vacuum (VAC) by rotating the arm until it clicks in the desired position and apply accordingly following set up instructions in step 7.8.

9.2. Once these conditions have been achieved, each run is started by clicking ‘start’ on the software in the ‘Data Acquisition’ tab. The run is completed by pressing the ‘stop’ in the ‘Data Acquisition’ tab when at least 500 particles have been measured (see ‘Particle Count’ at the bottom of the software screen during the measurement) and the run has exceeded 30 seconds (see ‘Run Time’ also toward the bottom of the screen).

9.3. Calibrate the system by completing a calibration run every time a new nanopore is introduced or for each new day of analysis by completing step 9.1.

10. Running a sample.

10.1. Run the samples at the highest or second highest voltage the calibration samples were run at ensuring a similar (±10 nA), if not the same, baseline current. Once the appropriate baseline current is achieved, the electrolyte in the upper fluid cell is replaced with 40 µL of sample. When a sample is introduced, you will see blockades on the signal trace. Start the sample run by clicking ‘start’ in the ‘Data Acquisition’ tab and record a minimum of 500 particles (check ‘Particle Count’ situated under the signal trace) and ensure the run time is a minimum of 30 seconds (see ‘Run Time’ also situated below the signal trace). To complete the measurement, click ‘stop’ in the ‘Data Acquisition tab’ and save the data file. The file is saved after the file information has been inputted in the following format; ‘Investigation’ is the folder the file will be saved in, ‘Nanopore ID’ is the serial number of the pore being used, ‘Part #’ is the type of pore (i.e. NP150/NP200), ‘Sample ID’ is the name of the sample, ‘Calibration or sample’ details whether it is a calibration or sample measurement, ‘Dilution’ is used if the sample was diluted (type 100 if the sample was diluted 100-fold), ‘Pressure’ is the applied pressure to the sample (in cm – see section 7.8), ‘Electrolyte ID’ is the name of the buffer the sample is made up in, and ‘Notes’ are any personal notes you want to make about the sample or run.

10.2. Between each sample run, wash the system by placing 40 µL of PBST buffer into the upper fluid cell several times and applying various pressures (usually at -10, -5 cm (vacuum), and 5 and 10 cm (positive pressure)) until no more blockade events are present, ensuring there are no residual particles remaining in the system and therefore no cross contamination between samples. Samples are recommended to be run in triplicate with this wash step completed between each repeat sample run as well as between different samples.

Results:

[Place Figure 1 here]

The removal of any excess DNA that has not bound to the particles surface from the samples, as to not report any ‘false-positive’ results, is important prior to TRPS analysis. The ability to use a magnet to extract and wash the SPP’s is a huge benefit for TRPS. Figure 1B describes a basic example of a TRPS measurement and an
example ‘blockade event’ achieved as a particle traverses the pore. Firstly, we have demonstrated that TRPS is a high throughput technique that can distinguish between samples of a similar size but of a considerably different charge with its ability to complete both size and charge analysis simultaneously in a single measurement, data of which can be seen in Figure 2. Figure 2 is an example of a) size and b) zeta potential analysis of streptavidin coated particles with no modifications (light pink data set) and streptavidin coated particles saturated with single-stranded DNA on the surface (blue data set). Although both samples were of a similar size, the zeta potential was significantly different and much larger when DNA was functionalised onto the particle’s surface.

[Place Figure 2 here]

Not only can the technique differentiate between particles unmodified and modified with DNA, TRPS can also differentiate between samples with different concentrations of DNA hybridised to the particle surface. Figure 3 shows the size and zeta potential data exhibited for samples with the lowest (10 nM, light green data set) and highest (210 nM, blue data set) concentrations of DNA hybridised to the streptavidin coated particles. A larger zeta potential value is recorded for particles hybridised with a higher concentration of DNA.

[Place Figure 3 here]

It is useful to note that each data point in the scatter plot represents a single particle amongst a sample population, allowing for in depth particle-by-particle analysis with every sample. Figure 4 supports the technique’s effectiveness in determining minor changes in DNA structure (single-stranded and double-stranded DNA), as well as identifying differences in samples with target DNA of the same size, but bound to a different area of the capture probe demonstrating high levels of sensitivity (i.e. Middle binding and End binding targets shown in Figure 4). The particles shown in Figure 4 are those with the following surface modifications, from left to right; capture probe (CP) DNA only, CP and a fully complementary DNA target, CP and a middle binding DNA target, CP and an end binding DNA target, CP and an overhanging DNA target.

[Place Figure 4 here]

**Figure Legends:**

**Figure 1 – Schematic representation of the processes of magnetic purification and a TRPS measurement.** A) Example of magnetic purification of sample starting with a sample containing excess, unbound capture probe DNA. B) TRPS measurement example i) Particle passing through the nanopore and ii) Blockade event produced from particle temporarily occluding ions in the pore causing a temporary decrease in current; Information from which is used to calculate particle translocation velocities.

**Figure 2 – Size and Zeta potential analysis of DNA-modified and unmodified streptavidin coated nanoparticles.** The light pink bars represent unmodified streptavidin coated particles and the blue bars represent DNA-modified particles. A)
Frequency, %, vs particle diameter (nm) B) Frequency, %, vs zeta potential (mV). Figure adapted from supplementary data in Blundell et al.¹⁹.

**Figure 3 – Simultaneous size and zeta potential data captured from a single TRPS measurement.** The blue bars/data points are representative of streptavidin coated particles hybridised with 210 nM CP DNA and the light green bars/data points represent streptavidin coated particles hybridised with 10 nM CP DNA. Figure adapted from Blundell et al.¹⁹.

**Figure 4 – Relative change in zeta potential measured for DNA-modified particles with a range of DNA targets.** Change in zeta potential, mV, from i) CP functionalised particle to a range of DNA targets; ii) Fully complementary, iii) Middle binding, iv) End binding, v) Overhanging target. The error bars represent standard deviation where n=3. Figure adapted from Blundell et al.¹⁹.

**Discussion:**
The calculation for the zeta potential used a calibration based method related to work by Arjmandi et al.²¹. The duration of the translocation of particles as they traverse a nanopore is measured as a function of applied voltage, using an average electric field and particle velocities over the entirety of a regular conical pore. The electrophoretic mobility of a particle is derived from 1/T, where T is the blockade duration and voltage multiplied by the square of the sensing zone length, l, included in a calibration constant. Average velocities at multiple reference points through the sensing zone are measured to allow for minimal errors in calculating zeta potential using this method.

The calibration of the pore is based on the linearity of 1/T vs voltage, V, at each reference point in the sensing zone. The particle velocities of calibration and sample particles, (vₓ)el Cal and (vₓ)i Sample respectively, are related to their zeta potentials, ξ net Cal and ξ net Sample, as shown in equation 1, assuming a linear relationship between the two as given in the Smoluchowski approximation¹²,²⁰. The net zeta potential values for both calibration and sample are the differences in particle zeta potential and the membrane zeta potential, ξ m. The zeta potential of the polyurethane pore was measured using streaming potential techniques¹²,¹⁸ as -11 mV in PBS for this study.

\[
\frac{(v_x)_{el Sample}}{(v_x)_{el Cal}} = \frac{\xi^i_{net Sample}}{\xi^i_{net Cal}}
\]

(1)

The zeta potential of each individual particle, i, ξ i Sample, is measured from the respective zeta potentials calculated at various reference points within the pore (equation 2), where lₓ is the position of the particle within the pore after time, t=Tₓ, and v x Sample is the sum of the particle velocity components at relative positions, l x, within the pore; v x Cal V, v x Cal P, P, and V are electrokinetic velocity per unit voltage, convective velocity per unit pressure, applied pressure and voltage for the sample runs respectively, a full derivation of this equation can be found in work by Blundell et al.,¹⁹.
\[ s_{\text{Sample}}^i = \frac{\sum x s_{\text{Sample}}}{\sum x} = \frac{\sum x (v_{\text{Sample}}^x - v_{\text{Cal}}^x \times P)}{\sum x (v_{\text{Cal}}^x \times V)} \times \xi_{\text{net Cal}} + \xi_m \]  

(2)

When binding the capture probe DNA to the streptavidin coated nanoparticles, it is vital that the researcher removes excess, unbound capture probe DNA left in solution. This is done easily using the SPPs and a simple magnet allowing the rapid and easy replacement of the supernatant with new PBST buffer. If excess capture DNA is left in solution and target DNA added, the target DNA may bind to the free capture DNA in solution, rather than that on the SPP surface. A change in particle velocity and zeta potential will only be observed if the target DNA binds to the capture probe present on the particle’s surface.

Analysis and comparison of a large number of samples across many days using TRPS may require the use of more than one pore membrane. Some pores can have some minor differences in their size due to the manufacturing process and in these cases, the user must ensure the baseline current remains identical across all runs. If the same baseline current is observed, the results obtained are comparable between pores. Once the baseline is the same as previous runs, it is imperative that the user keeps the stretch unchanged between calibration and sample runs to allow for accurate determination of particle translocation velocities as they traverse the pore.

The TRPS technology has a relatively simple set up, which can be disassembled easily and quickly during an experiment. If troubleshooting problems, this can make the process a lot easier. For example, it is important not to allow any bubbles in the lower fluid cell or upper fluid cell when undertaking analysis, this will lead to an unstable baseline current. If bubbles are present in the upper fluid cell, the sample may be removed and replaced. If bubbles appear in the lower fluid cell, the buffer should be removed and replaced with fresh buffer. If the bubbles are a persistent problem, then there may be too much surfactant in the solution so this may have to be reduced (we only use 0.05% Tween-20). Some samples may block the pore if their size exceeds the pore size or if the concentration of the sample is too high. To rectify this, the pore size can be increased by increasing the stretch or the sample can be diluted to a lower particle concentration. For single particle analysis the sample may also block the pore if there are a lot of large aggregates present, it is important to vortex and sonicate the sample before running it through TRPS.

Amongst other methods, TRPS has various advantages including the ability to complete size and charge measurements of individual particles simultaneously; allowing for multimodal samples to be analyzed effectively using this method. One advantage is the signal/blockades produced can be optimized in minutes for a particular sample by simply changing the stretch and voltage to obtain a blockade magnitude, \( \Delta i_p \), significantly larger than the background noise (blockades are of nA scale in comparison to the background noise <10 pA). Being able to alter the stretch of the pore makes the method more versatile over solid-state pore techniques as the pore size can be adjusted with respect to the size of the analyte in question; particularly useful when investigating effects such as aggregation and DNA-protein binding that may result in analyte sizes exceeding the original solid-state pore size range. Another advantageous aspect of TRPS is the level of sensitivity from the technique. The ability to detect subtle differences in DNA binding (where the same amount of DNA has been added (same amount of added charge) and the samples are of the same size) based on the position of target DNA binding is quite profound.
in this area of analysis and will be of great use for future nanoparticle-assay design platforms. Each subtle difference can be detected and isolated using a particle-by-particle nature of TRPS technology. This analysis exceeds that of ensemble techniques such as dynamic light scattering or photon correlation spectroscopy that will merely gage an average of the sample population analyzed and can't differentiate in the cases of multimodal samples6, 7.

Small solid-state nanopores (100-200 nm) have also been used to monitor particle dynamics and have found that particle mobility can be affected as the diameter of the particle begins to approach that of the nanopore22, 23. Nanopores much larger than the particles being analysed (as used in this study) have less of an effect on the particle mobility and thus the translocation dynamics within the pore. The pores used in this study are however limited to their analyte size ranges, an NP150 for example has a size range of 60-480 nm so if a multimodal sample consisted of particles within and exceeding this limit, they cannot be analyzed on the same pore as the pore may then become blocked. It is also important to note that measuring a bimodal sample containing 60 and 480 nm particles (those at the absolute lower and higher limits of the pore), for example, will require different stretch and voltage conditions, although both are within the size analysis range of the pore. This is because the stretch required for the larger particles will result in the smaller particles having a particularly small blockade magnitude (based on the reduced resistance) that could be regarded as background noise and thus not necessarily measured during a sample run.

Bubbles can be a problem with the sample measurements as bubbles in the lower or upper fluid cell will create an unstable baseline current, to which sample runs cannot be completed. Electrolytes of an effervescent nature (some highly concentrated biological media, for example) may be difficult to run and thus samples requiring suspension in these specific mediums may prove problematic. Most samples however, can be vastly diluted or suspended into alternative buffers prior to TRPS analysis.

The method is adaptable and can be used to analyse a range of nanoparticle-based analytes, including the analysis of proteins, DNA, small molecules, aggregation assays17, 24 and biologically relevant particles. The versatility of TRPS in characterizing a vast range of analytes shows the techniques potential in a range areas such as drug delivery1, 25, biosensing26-28, and environmental testing.

Acknowledgments:
The authors thank Izon Science Ltd for their support. The work was supported by the European Commission for Research (PCIG11-GA-2012-321836 Nano4Bio). E.L.C.J.B. is supported by Izon Science Ltd.

Disclosures:
The authors declare that they have no competing financial interests.

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