Measuring zeta potential using tunable resistive pulse sensing: applications in biosensing

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Measuring Zeta Potential using Tunable Resistive Pulse Sensing: Applications in Biosensing

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Sponsored by Izon Science Ltd and Loughborough University
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List of Abbreviations

TRPS – Tunable Resistive Pulse Sensing
DNA – Deoxyribonucleic Acid
JoVE – Journal of Visualised Experiments
RPS – Resistive Pulse Sensing
SPP – Superparamagnetic Particles
RNA – Ribonucleic Acid
EDC - N-(3-Dimethylaminopropyl)-N’-ethylcarbodiimide hydrochloride
ELISA – Enzyme-Linked Immunosorbent Assay
IgY/G – Immunoglobulin Y/G
DLS – Dynamic Light Scattering
PALS – Phase Analysis Light Scattering
NTA – Nanoparticle Tracking Analysis
SIOS – Scanning Ion Occlusion Spectroscopy
TEM – Transmission Electron Microscopy
PBS(T) – Phosphate buffered Saline (Tween-20)
ELS – Electrophoretic Light Scattering
VP – Virus Particles
PCR – Polymerase Chain Reaction
FWHM – Full Width Half Maximum
VPM – Variable pressure Module
SEM – Scanning Electron Microscopy
NP - Nanopore
ss/ds – single-stranded/double-stranded
CPC – Carboxylated Polystyrene Calibration (particles)
Btn – Biotin
VL – Varied Length
CP – Capture Probe
cDNA – Complementary DNA
MidT – Mid-binding Target
EndT – End-binding Target
OverT – Overhanging Target
MES - 2-(N-Morpholino)ethanesulfonic acid
VEGF – Vascular Endothelial Growth Factor
BioCPC – Biotinylated (Carboxyl polystyrene calibration (particles))
Strept – Streptavidin
E.coli - Escherichia Coli
C.Difficile – Clostridium Difficile
LbL – Layer-by-Layer
PEI – Poly(ethyleneimine)
L/HMW – Low/High Molecular Weight
PAAMA – Poly(acrylic acid co-maleic-acid)
BSA – Bovine Serum Albumin
HC/FC – Half Coverage/Full Coverage
CD – Circular Dichroism
TPU – Thermoplastic Polyurethane
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Abstract

The aim of this PhD was to develop and optimise an analytical method that incorporated zeta potential measurements within tunable resistive pulse sensing (TRPS) for biosensing. Modern society is dependent upon the accurate and rapid quantification of biological analytes within solution (biological or environmental) and on materials (clothing, skin, food). If the characterisation of particles within biological samples such as blood, plasma and serum is done simply by optical methods such as light scattering or microscopy, the various particulates and molecules, many of which are similar in size may not be able to be identified. TRPS is a label-free, non-optical based technique that can complete size, concentration, and more recently aided by the work in this thesis, zeta potential measurements in real time. Zeta potential could be a powerful analytical tool, as it is relative to the charge on an analyte and can be measured by monitoring the velocities of analytes as they traverse a nanopore in an electric field. Monitoring translocation velocities through the pore and thus zeta potentials could allow for an extra signal to help characterise analytes.

Following a literature review in chapter 1 which focuses on the use of nanoparticles and their characterisation within bioassays, a general theory chapter (chapter 2) covers common theory and experimental setup used throughout the research. Chapter 3 contains theory specific to zeta potential measurements using TRPS developed with an industrial sponsor to which chapter 4 is the application of this theory. It contains details on applying the method of inferring zeta potential from particle velocities to measure the change in zeta potential of nanoparticles as their surfaces are functionalised with DNA of varying packing density, length, structure, and hybridisation times to also determine the sensitivity of the method. As described the zeta potential is determined via the particle velocities as they traverse a pore that are determined from the signal produced using a TRPS measurement, a blockade. The blockade gives information on the particle velocities at relative positions within the pore as well as information on the size and charge of the particle.

TRPS is an evolving analytical platform that can differentiate samples of similar and the same size by their charge in a range of electrolyte solutions. This is important for whole blood and biological samples, for example, as there will always be other biomolecules or contaminants present, of similar size that may not be the target of
interest. A large part of this PhD was the incorporation of DNA aptamers onto nanoparticles as recognition elements to a specific target. They were of particular interest as aptamers are ssDNA (single-stranded DNA) strands of high affinity and specificity to a target analyte. Nanoparticles can be functionalised with DNA aptamers or proteins as a means to capture a target analyte. TRPS was used to monitor the binding of DNA aptamers to their target proteins, aided by zeta potential measurements. The results showed that a smaller zeta potential value was observed when a target protein was bound to the aptamer-modified particles.

As well as protein detection and quantification, a new assay using nanoparticles as ‘tags’ was investigated, chapter 5. TRPS was used to monitor controlled particle aggregation in the presence of target bioparticles mimicking a streptavidin-biotin assay at first. It was found that when two differently sized particles, one functionalised with biotin and the other streptavidin (70 nm and 115 nm at a 10:1 ratio), the particles in excess saturated the larger particles resulting in a large change in size and zeta potential that could be monitored using the tunable pores.

This method was then applied to nanoparticles in complex biological media, including plasma, serum, and biological buffers used to suspend bacteriophage samples, two examples are given in the thesis; the first in chapter 5 and second in chapter 7. In chapter 5, as well as sub 150 nm particles, bacteriophages of similar sizes were investigated to test the technique to biologically relevant particles. State of the art methods of counting bacteriophage via optical techniques have proven difficult, or inconsistent. In preliminary work shown in chapter 6, the characterisation of phage samples in their respective media is demonstrated. TRPS has overcome some of these challenges and preliminary data has been obtained for the size and charge characteristics of different phage types including Salmonella phage and coliphage. The study has also progressed to the size and concentration analysis of Clostridium difficile phage that has gained interest in recent decades due to their uses in therapeutics.

As an alternative to nanoparticle based assays, the pores themselves were modified with DNA aptamers, see chapter 6, for direct detection of a target analyte without the need for a particle ‘label’. Pore surface modifications have been completed to enable pores to be easily functionalised with DNA and this work has enabled current
rectification properties of conically shaped pores to be explored. Limits of detection for DNA-modified pores were found to be similar to that of a particle-based assay (5 pM and 18 pM, respectively) but the particle assays are more versatile and may be used in future for multiplexing experiments.

Finally, in chapter 7, the technique and methodology were able to monitor changes in the behaviour of nanoparticles as they were immersed in protein rich solutions, to mimic an *in vivo* environment. Here the protein corona around the nanoparticles was investigated as a function of temperature (25°C and 37°C). The kinetics and binding mechanism of high and low affinity proteins forming a protein corona could be monitored in real time as well as displacement reactions between various proteins, showing the advantages of TRPS technology.

In summary, from working with a commercial partner and collaborating with other institutions, we have delivered 4 papers (plus one JoVE paper) including a review of applications of TRPS technology and work detailed in this thesis, presented at 14 conferences and user meetings, and facilitated the development and implementation of zeta potential into bioassays.
1 Literature Review

1.1 Introduction

Nano and micron-sized particles in bioassays have seen an increase in use within both research and diagnostic laboratories especially in recent years, see figure 1.1.

![Figure 1.1 - The frequency of papers relating to detection and quantification of proteins, DNA and nanoparticles in the last 6+ years.](image)

Nanotechnology in biological and medical applications has allowed for rapid advancements in real-time particle analysis\textsuperscript{1–5}. Rapid and effective nanoparticle characterisation relative to size, shape, and surface charge, for example, would help gain valuable knowledge on a variety of biological processes and biologically relevant particles.

Particle detection and characterisation is important in many applications of science and technology and earlier literature describes the use of flow cytometry\textsuperscript{6–8} and light scattering techniques\textsuperscript{9–11}. For example, dynamic light scattering (otherwise known as photon correlation spectroscopy) measures the diffusivity of small particles undergoing Brownian motion\textsuperscript{9}. More novel approaches to particle detection, quantification, and characterisation incorporate nanotechnology using nanopore
sensors and resistive pulse sensing (RPS)\textsuperscript{12–16} that is based on a Coulter counter technology, which allows for colloidal characterisation of nanoparticle-based systems.

The ideal particle/molecule detection and characterisation method should enable direct analysis of entities in their natural environment without the need for radio or fluorescent labels at high resolution and sensitivity. This can be a challenge in bioscience as biological media, blood, and/or protein rich solutions are complex matrices for particle analysis and cause hindrances to the majority of techniques readily available. Whilst many sensor platforms, and nanoparticles that are used for bioassay we will focus on magnetic particles due to the advantages described below.

### 1.2 Magnetic Nanoparticles in Bioassays

A common particle chosen to facilitate sample handling either via purification or preconcentration are superparamagnetic particles (SPPs). These have attracted a lot of interest in advancing particle-based assays for drug delivery\textsuperscript{17–20} and biosensing applications\textsuperscript{21–24}. SPPs are commonly used as nanomaterials that can be surface-modified and are particularly favoured due to the ease of purification for the resulting samples. The main advantage of SPPs is the simple and rapid removal of specific analytes from complex sample matrices using nothing more than an external magnetic field or a hand held magnet\textsuperscript{22,25–28}. This is particularly useful in biological samples such as blood, plasma, urine and biological media as these are the more likely of samples to contain several particles and molecules amongst a given sample population as well as the particles of interest. Modification of nanoparticles with biological molecules and organic ligands is becoming more popular using the nanoparticles themselves as a ‘label’ in particle-based assay systems\textsuperscript{24,29–33}.

Analysing samples without the need for purification or isolation is preferred when studying chemically rich environments (enriched waste plants) or protein rich environments (the human body) to get an idea of sample behaviour in natural complex environments. To account for this, various work has been carried out to analyse protein ‘coronas’ that are formed as a result of contact with nanoparticles\textsuperscript{34–39}. The particle surface does impact these effects and previous work has been carried out using silica and carboxylated nanoparticles\textsuperscript{34–37,39–43}. Understanding the behaviour and characteristics of the nanoparticles with and without surface modification is
important for particle analysis in monitoring the effects of the target analytes themselves as a result.

One set of powerful techniques for the characterisation of SPP-target interaction are optomagnetic methods. These are extremely sensitive and quick in characterising and detecting biological molecules using a small sample volume incorporating magnetic particles. An example of an optomagnetic set up is displayed in figure 1.244.

![Optomagnetic set up for a nanoparticle-based assay, figure reproduced from Ranzoni et al](image)

SPPs emit different light scattering effects based on different surface properties and size. Park et al. introduced a rotating external field ($H_{ex}$) to superparamagnetic beads and established that at a constant $H_{ex}$, the amplitude of light transmittance increased when longer superparamagnetic bead chains were present. The recorded amplitude was correlated with analyte concentration, displaying that an increased concentration resulted in bead aggregation. Light scattering techniques can characterise particle colloids and aggregates. The light scattering intensity of particles can be reduced because of intra-aggregate interference and frictional resistance causing aggregates to appear a smaller size than they actually are.

### 1.3 Particle Surface Modifications

Modifying a nanoparticle’s surface can alter its interactions with analytes in solution and therefore have inherent effects on particle velocity in an electric field, zeta potential and surface charge. DNA, RNA, proteins, antigens, and cells are all
examples of entities that can be bound onto a particle's surface through a variety of methods including streptavidin-biotin interactions and EDC (N-(3-Dimethylaminopropyl)-N'-ethylecarbodiimide hydrochloride) crosslinking chemistry, for example.

Figure 1.3 – Example surface modifications and optional functionalities that can be adhered to a nanoparticle surface for analyte detection and characterisation.

Aggregation assays, measured via light scattering have been detected and thus binding capabilities of select targets monitored using magnetic separation techniques. This was shown in a one hour DNA affinity magnetic separation producing a product of higher purity than from simply using traditional chromatographic procedures. Functionalising SPP surfaces has also led to advancements in DNA sequencing, proteins such as streptavidin has allowed for solid phase sequencing of DNA, for example.

Medical research is currently being carried out with the use of gold nanoparticles and quantum dots to specifically detect and target cancer tumours, and silica nanoparticles to allow for drug delivery to cancer cells only. Nanoparticles are not only extremely useful in diagnostics, they are also used in electronic mechanisms, packaging and wastewater treatment, and biosensors. Biosensor technologies have advanced in recent decades with the use of nanomaterials to reduce the time and resources required between sampling and obtaining a result. Nanoparticle synthesis
has more recently developed so that intentional adaptations can not only be made to their size and shape, but also their compositions.

The immobilisation of nucleic acids (RNA/DNA) onto particle surfaces has an impact on the particle’s behaviour in an electrolyte solution, taking into account factors such as the electrical double layer surrounding the particle and subsequently these ion-based interactions. Functionalising nucleic acids onto a surface has been a widely used technique leading to interesting findings in genomics and biosensing. They are becoming extremely useful for investigating fundamental properties of charged polymer chains and modelling their behaviour at solid-liquid interfaces. Relative to this, DNA aptamers are becoming particularly useful in monitoring DNA-protein and DNA-DNA interactions. Aptamers are single-stranded DNA or RNA molecules that bind to a target analyte with high affinity and specificity. They are robust structures that allow for a specific target (even at low concentration in a complex mixture) to be detected, isolated and thus characterised.

Although a range of polymer coatings can be assembled onto colloidal particles, the polymers that form a spherical effect on the particle are of particular interest. Spherical brush polyelectrolytes are bound to the particle by one end and the other left free standing, like with conjugated DNA strands. Immobilising oligonucleotides onto surfaces is a key design to many technologies within DNA sequencing, DNA-protein interactions, biosensing and targeted drug delivery. The functionalisation of DNA onto nanoparticle surfaces is now a common practice, and within the field of biosensors alone the number of strategies for immobilisation, type of nanomaterial, and detection platform are varied enough to fill several reviews.

As well as DNA, nanoparticles and various other nanomaterials can be modified with other biological molecules such as proteins and bacteria, and organic molecules for biomedical and environmental applications.

### 1.4 Particle Detection and Characterisation Techniques

#### 1.4.1 Optical Techniques

##### 1.4.1.1 Dynamic Light Scattering (DLS) and PALS

Optical particle analysis can be carried out using the high throughput technique of dynamic light scattering, otherwise known as photon correlation spectroscopy.
Although this technique is high throughput, temperature control is required to maintain a consistent particle diffusion rate relative to their size\(^9,82\). DLS is limited in comparison to some of the other modern analysis tools as multimodal solutions become a challenge as larger particles will dominate the signal produced\(^84\), leaving some of smaller particles completely unnoticed. DLS is an averaging based technique\(^85\) and is therefore not as efficient, but is capable of measuring a number of particles of any shape as long as an appropriate capture probe (antibody or aptamer) is present for the particular analyte.

Dynamic light scattering has been used with gold nanoparticles to detect the influenza A virus. The virus was detected via a specific influenza A antibody as a capture probe conjugated to gold nanoparticles, followed by DLS\(^85\). The DLS monitored and measured the aggregation effects of the labelled nanoparticles. The mean (as DLS is an averaging based technique) hydrodynamic diameter was relative to the concentration of the target virus\(^85\) and hence the analyte was quantified successfully within the gold nanoparticle assay.

Phase analysis light scattering (PALS)\(^86\) is similar to DLS in respect to them both being averaging techniques and will only report values based on the mean of a whole sample population. This is of course not as useful in studying complex mixtures with a range of particulates that could be present in a single sample.

### 1.4.1.2 Flow Cytometry

Flow cytometry is a laser based technique used in diagnostics and has evolved in recent years to feature magnetic immunoassays\(^8,87\). In the last decade, micro flow cytometry has been employed to analyse fluorescent-labelled cells and investigate protein binding properties. Figure 1.4 displays the various steps carried out for the detection of virus-bound magnetic particles. (a) shows mixing the biological samples with the magnetic particles, (b) illustrates purification of the target viruses through washing, (c) is the capture of antibodies onto the magnetic particles conjugated with specifically developed antibodies, (d) shows the purification of the developed antibodies, (e) is the fluorescent detection of the analyte as the magnetic particles pass through the optical detection region (photo multiplier tube), and (f) displays magnetic particle sorting with the use of microvalves\(^87\).
Figure 1.4 - Schematic representation of integrated microfluidic chips and the use of virus-bound magnetic particles. (a) biological sample and magnetic beads mixing, (b) purification of virus bound magnetic particles via washing steps, (c) capture of custom developed antibodies onto the virus bound magnetic particles, (d) purification of magnetic particles bound to custom developed antibodies and excess custom developed antibodies, (e) fluorescent detection of the virus analyte as the particles pass through a photo multiplier tube, and (f) magnetic particle sorting using microvalves. Figure reproduced from Yang et al. \(^87\).

1.4.1.3 Nanoparticle Tracking Analysis (NTA)

An emerging optical technique for particle detection is nanoparticle tracking analysis (NTA). This technique gathers data from real-time particle-by-particle analysis by measuring the particle diffusion coefficients using a finely focused laser beam through a prism\(^2\). Nanoparticle tracking has been found to be useful for applications such as drug delivery and protein aggregate analysis. Fluorescent labelling can be incorporated into NTA to allow for complex, impure samples, such as micro and nanovesicles to be analysed\(^2\). NTA could be used to identify smaller biomolecules as it has been found to visualise and analyse particles ranging from 30 nm to 1000 nm\(^88\). Although small particle analysis can be achieved, the particle concentration that can be analysed is limited to 10\(^7\)-10\(^9\) particles/mL\(^88\).
1.4.2 Pore-based Technologies

In nanopore systems, two types of pores can be investigated, synthetic and biological. Biological pores are made up of proteins and peptides and have been utilised in studying different properties of water\cite{89, 90} as well as nanoelectronics, medicine and sensing. In biotechnology, the characterisation of DNA and RNA can be accomplished via natural pores as well as cell tumour targets activating multimeric pores to kill the appropriate cells\cite{91}. The delivery of therapeutic molecules via a plasma membrane is a challenging aspect of biological advancement but can be overcome by biological pores both \textit{in vivo} and \textit{in vitro}\cite{92, 93}. These investigations have also been analysed in cancer therapies where nanoparticles have been used as vectors. Synthetic pores, with a polyurethane membrane are more established in these fields as they are more durable and can have higher resolutions to individual proteins and bases\cite{94}. Biological pores however, have been known to have a better controlled geometry and chemical structure that can be identified at atomic precision\cite{95}.

Tunable pores have recently been incorporated into RPS technologies (TRPS)\cite{15} and are more widely used than fixed sized pores because having a fixed sized pore allows less versatility as the particles being analysed are restricted to being a certain size. Tunable pores of varying sizes have been incorporated in scanning ion occlusion spectroscopy (SIOS)\cite{96} in order to detect and differentiate particles of different sizes. Individual particles can be distinguished from a sample containing a mixture of nanoparticles using real-time scanning of the pore conductivity. Demonstrations of distinguishing individual particles through SIOS was observed through DNA-modified particles displaying a longer blockade duration\cite{97}. A variety of other surface modified particles have also been analysed using tunable pores\cite{98}. The main advantage of using a tunable pore is the ability to explore multimodal particle size systems\cite{97, 99} over light scattering techniques that focus more on colloidal particles.

1.4.2.1 Resistive Pulse Sensing (RPS)

Small particle analysis has been recently executed through resistive pulse sensing using a pore sensor. Synthetic pores are effective tools in small particle analysis and have been incorporated into emerging technologies, such as resistive pulse sensing. This is a key area of nanotechnology to be explored that is extremely beneficial for diagnostic and biomedical fields. Vogel \textit{et al.} successfully detected and sized a virion 70-95 nm in diameter, producing highly reproducible data in agreement with both
optical methods and TEM\textsuperscript{12}. Resistive pulse sensing is an effective particle detection and characterisation technique that consists of an electric field being applied to a sample, and monitoring the transportation of particles through a synthetic or biological nanopore. Polyurethane membrane based pores are the more readily established as they have increased durability and have a higher resolution to individual proteins and bases\textsuperscript{94}.

When an electrical field is applied, the buffer solution (i.e. phosphate buffered saline, PBS) is always conductive and a baseline current is observed. A blockade (pulse) in the baseline is observed as a particle passes through the nanopore and therefore displaces the buffer solution. The blockade magnitude is relative to particle size and can be optimised for size and charge analysis through a series of parameters. These include the pore stretch, applied voltage, and external pressures/vacuums that may be introduced. The parameters can be altered to aid in monitoring the electrical and convective movements of particles through the pore, refining the analytical result obtained. The pore stretch can be altered due to the elastomeric membranes within the pore and can be used many times and reversibly modified to a range of micro and nano-sized geometries.

Aggregation effects can be monitored using resistive pulse sensing methods by monitoring the blockade magnitude. For example, if an aggregate of two particles is observed, the blockade magnitude will be double that of an individual particle passing through. This observation is accountable for dimers, trimers and other sized particle aggregates. The aggregates that form are dependent on the binding sites available on the analyte.

Pressure can also be introduced to the pore and increases the particle rate through the pore and is therefore relative to the blockade rate. The background current is also dependent on the pore size, the larger the pore size, the higher the background current\textsuperscript{97}. The particle size is determined from a current vs time relationship. The pulse frequency is equivalent to the bead concentration.

\textbf{1.4.2.2 Tunable Resistive Pulse Sensing (TRPS)}

A more recent adaptation of resistive pulse sensing is the incorporation of an elastomeric tunable pores to create the renowned technology of tunable resistive pulse sensing (TRPS)\textsuperscript{15,100,101}. The elastomeric membranes used to create tunable
nanopores can be used many times and can be reversibly altered to a range of micro and nano-sized geometries. Blockages can occur in the pore and are most commonly due to impurities, aggregation, or surface adhesion. These blockages are removed by increasing the membrane stretch to allow possible aggregates or larger particles to pass through, or by applying pressure to force the oversized particles through\textsuperscript{15}. Although there are a few minor difficulties with blockages in the pore at times, resistive pulse sensing is a technique with some of the highest resolution recorded in the detection of multiple particle sizes in a mixture. This was discussed by Anderson \textit{et al.} comparing the technique to dynamic light scattering and other light scattering based techniques\textsuperscript{84}. There is negligible bias towards any sized particles using tunable resistive pulse sensing (TRPS), unlike in dynamic light scattering techniques.

TRPS technology has been utilised to analyse size and charge characteristics of micron and nano-sized particles. TRPS is currently the only technique that can simultaneously measure size and charge in a single measurement\textsuperscript{33,102}. The Izon qNano (Izon Science Ltd) is a portable instrument used to detect, quantify, and characterise individual particles via TRPS. The device can efficiently determine particle size and concentration from a small sample volume (40 μl) when calibrated with a known standard. The technology is label-free with respect to fluorophores gaining advantages over immunodiffusion techniques and ELISA particle analysis. The qNano completes real-time particle-by-particle size and charge analysis rapidly. Another advantage of TRPS via the qNano is background noise is reduced by placing a Faraday cage over the upper fluid cell.
Size, concentration, velocity, and zeta potential measurements can be completed simultaneously using TRPS technology. Charge measurements using zeta potential are a vital addition to nanoparticle analysis when investigating analytes of similar, if not the same, sizes. These measurements can also be used to monitor DNA interactions to a target analyte effectively\textsuperscript{12,32,103}, as DNA binding to an analyte can result in conformational or physical property changes of the DNA itself or the target analyte.

### 1.5 Charge Analysis and Zeta Potential Measurements

In depth charge analysis of nano and micron-sized particles has become increasingly popular over the last decade providing data for a wide variety of applications, including nanomedicine and diagnostics. Charged particles have been found to be of great use to medical sciences, particularly towards cancer therapeutics\textsuperscript{104,105}. 

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**Figure 1.5** – a) Izon qNano instrument, b) a schematic of sample particles translocating a pore membrane as a result of an applied electric field, and c) an example of the blockades produced as TRPS outputs that can be used to determine the size, concentration, velocity and charge of a sample.
The charge characteristics of a particle set are studied through the use of a measured zeta potential and can be obtained from the Izon qNano\textsuperscript{13,33,102,106}. Zeta potential, $\zeta$, is defined as the electrostatic potential at the plane of shear between the particle-associated and the stationary part of the ionic double layer\textsuperscript{107}.

The compact layer is also known as the Stern layer, which contains strongly bound charged particles to the surface, see figure 1.6. Zeta potential is mathematically defined through the Smoluchowski approach\textsuperscript{107,108}, $\zeta = \frac{\eta \mu}{\varepsilon}$, where $\eta$ is dynamic viscosity of the fluid, $\mu$ is the electrophoretic mobility and $\varepsilon$ is the dielectric constant of the electrolyte solution. The zeta potential is relative to surface charge of particles in suspension. A larger zeta potential value is exhibited from particles with excellent electrical stability.

![Figure 1.6 - Schematic illustrating the electrical double layer surrounding a nanoparticle suspended in an electrolyte solution. The zeta potential can be defined as the electrical potential at the slip/shear plane.](image-url)
The first calculations of zeta potential were completed by manually extrapolating plots using Ferguson analysis\textsuperscript{109,110} and applying the data to Henry’s equation\textsuperscript{111}. The vast majority of zeta potentials have been calculated using light scattering techniques such as dynamic light scattering and electrophoretic light scattering (DLS and ELS, respectively)\textsuperscript{112–116}. Pore-based methods, whether it be pyramidal pores\textsuperscript{117} or conical pores\textsuperscript{13,33,102,118}. Calculating the zeta potential from tunable resistive pulse sensing allows for the combination of small particle analysis with charge analysis.

1.6 Current Rectification using Nanopores

Current rectification properties have been investigated for a variety of pore shapes, sizes, and materials. A range of materials have been used to create synthetic including graphene\textsuperscript{119–122}, polymers\textsuperscript{97}, and silicon nitride\textsuperscript{123}, but it is glass\textsuperscript{124–127} and polymer-based\textsuperscript{128–130} pores where more prominent studies have been carried out. The vast majority of the studies have been completed using conically shaped pores\textsuperscript{128–131}. Current rectification effects are largely influenced by the charges of pore walls and as such modifying pore surfaces will have inherent effects on the current rectification effects produced. It is important to understand these changes to increase the level of certainty that it is the detection of a target analyte being monitored and not that of a change to the pore surface by a ligand or DNA capture probe, for example. Polymeric conical nanopores are particularly useful for understanding these effects as they have been reported as successful ion-current rectifiers\textsuperscript{132–134} with mechanisms reported for quartz materials\textsuperscript{135} that have been applied to polymeric nanopores at this stage. It has previously been difficult to process a mechanism for polymeric conical pores based on the level of uncertainty present for the vague data available for the chemistry and charge of polymer-based membranes.

Current rectification studies are being investigated to monitor the effects of ions surrounding the pore surface and the effects of particle flux through charged pore systems. The molarity of electrolyte being used in each study is an important consideration in this research as this will inevitably change the ion concentration and thus current rectification effects observed. The effects are pH dependent and electrolyte concentration dependent\textsuperscript{136,137}. The current effects are stronger at low electrolyte molarities.
1.7 Challenges: Viruses and Bacteriophage

Particles of sizes sub 150/100 nm can be a challenge for many nanoparticle characterisation techniques, hence why emerging technologies such as pore-based technologies are a great benefit to biomedicine and nanotechnology. These analytical technologies are allowing for further research to be carried out, specifically investigating bacteria, viruses, and bacteriophages. A wider variation of pathogenic targets is being established forcing the need for additional technologies to be created\textsuperscript{138–140}.

Viruses are small infectious particles that are considered to be detrimental to living organisms through their abilities to cause disease. Viruses and virions (virus particles, VPs) have robust structures as the viral nucleic acids are encapsulated in a protein shell, known as a capsid. VPs are self-assembled protein structures with a relatively identical, if not completely identical, structure to their resident virus\textsuperscript{141}. Bacteriophages are made up of proteins encapsulating a genome and are considered viruses that infect and thus replicate within a bacterium. There is much interest in analysing and characterising viruses and phage samples, but they can be significantly smaller than whole bacteria/cells so optical and microscopic analysis has proven difficult. The ability to effectively analyse small biologically relevant molecules will allow for valuable knowledge that would contribute greatly to vaccine and drug delivery system developments. Bacteriophage are of particular interest as they are gaining interest as possible alternatives and improvements for antimicrobial treatments\textsuperscript{142,143}.

Traditional detection methods include transmission electron microscopy (TEM)\textsuperscript{144,145}, as well as fluorescent and non-fluorescent protein assays\textsuperscript{146–148}. Analytical tools for the detection and characterisation of biomolecules such as viruses are constantly being developed and improved. Popular virus detection methods for single viruses are electrical measurement methods that use nanowire field transistors\textsuperscript{149}. Microfluidics has been used to deliver the virus sample to the nanowire array allowing for selective virus sensing, and the electrical measurements giving results relating to the surface charge of the analyte and conductance changes\textsuperscript{149}. The more modern approaches to virus detection and quantification include flow cytometry, enzyme-linked immunosorbent assay (ELISA)\textsuperscript{150}, dynamic light scattering (DLS)\textsuperscript{9}, electrochemical methods (electrophoretic titration curves\textsuperscript{151}), resistive pulse sensing (RPS)\textsuperscript{152}.
including tunable resistive pulse sensing (TRPS)\textsuperscript{12}, and the most commonly carried out technique being variants of the polymerase chain reaction (PCR)\textsuperscript{153,154}. Other modern nanoparticle detection approaches and characterisation methods include flow cytometry\textsuperscript{155}, nanoparticle tracking analysis (NTA)\textsuperscript{88} and TRPS, however, the selectivity and sensitivity levels do vary. Dynamic light scattering, for example, is an averaging based technique and isn't as effective as particle-by-particle TRPS analysis. A great advantage of TRPS technology is that it is a non-colour based technique that doesn't require a fluorophore or enzyme detection molecule. Tunable resistive pulse sensing displays the most versatility and can determine charge characteristics of a small particle through zeta potential measurements, as well as particle size and concentration determination. Although nanoparticle tracking analysis is also showing some versatility in its measurements, some parameters (such as particle concentration) are limited and some NTA measurements could take up to an hour. This is significantly longer than a TRPS measurement on the Izon qNano that can be completed in minutes.

Previously, phage concentrations have been determined using plaque assays, a technique widely used to isolate phages\textsuperscript{156}, and reverse transcription PCR\textsuperscript{157}. As per section 1.2.2, magnetic particle-based assays functionalised with specific capture probes have also been used to detect phage contamination\textsuperscript{158} through an electrochemical detection method using electric chips\textsuperscript{159}. In this example, the capture probe bound to the particle is selective of specific phage genes/DNA and an assay time as low as 25 minutes could detect DNA-phage binding that was a result of phage infection and this study was expanded to multiplex detection\textsuperscript{158}. There are currently no techniques that can complete concentration measurements alongside activity analysis of phage. Currently, techniques such as flow cytometry, epi-fluorescence, electron microscopy, and plaque assays are combined to relay information on a given sample\textsuperscript{116}.

1.8 Conclusions

Of the particle detection technologies discussed, tunable resistive pulse sensing is the most versatile of all techniques as it can distinguish between multimodal particles in a complex mixture\textsuperscript{97,99} and does not incur any bias towards different sized particles. Other techniques such as dynamic light scattering and various immunoassay
techniques are sufficient for particle characterisation but not always as selective or sensitive. Synthetic tunable pores allow for complete control over which particle size and shape can pass through as well as producing accurate and reliable data quickly. The pores are very stable and cost-effective to analyse a number of particle mixtures and samples repeatedly. The pores can be modified using simple cross linking chemistries and current rectification properties can thus be exploited. TRPS also discards the need for a colorimetric analysis as it does not require fluorophores, enzymes, or radioactive labels, lowering the toxicity risk when carrying out the analysis. The radio-labelled immunoassays have a particular disadvantage as there is the problem with radioactive waste being produced. Resistive pulse sensing is a simple and cost effective method to quantify proteins and DNA if the appropriate particle surface chemistry is established.

The main advancements in recent decades have evolved from the extended use of magnetic beads and surface modification of nanoparticles. Manipulation of their sizes and shape is very practical and has allowed for greater sensitivities and selectivity, in comparison to more conventional analytical methods. The advancements that have been made over recent years are definitely encouraging and have already had an impact on biomedical analysis and nanoparticle bioassays. Development in refining particle detection and characterisation methods is however, still needed. Tunable resistive pulse sensing can simultaneously complete zeta potential, concentration and size measurements of a multimodal sample containing dispersed particles. In the last few years, combining magnetic beads with TRPS has become an impressive emerging field\textsuperscript{15,100}. This combination allows for effective and more accurate particle characterisation in relation to particle size, surface charge, and aggregation properties. Modifying a particle’s surface chemistry and functionality in relation to biomolecules enables characterisation of both the surface functional groups and charge. Further work in this field will broaden the applications for current technologies, such as tunable resistive pulse sensing.

Challenges continue to lie with particles and molecules on the smaller end of the nanoscale (sub 100 nm) including viruses, cells, and bacteriophages. There are no current technologies that can quantify and monitor the activity of small pathogenic targets. TRPS is an advancing biosensing method with the potential to help resolve some aspects of these current challenges for biomedical analysis. There is still the
need for further progress and optimisation of current particle detection and characterisation techniques to develop an elevated analytical platform for the detection of the more challenging target analytes.

1.9 References


2 Theory

2.1 Introduction

This section aims to give an insight into the common theory used throughout the thesis. It will define the theory of the technique used within all of the subsequent studies described (tunable resistive pulse sensing) including a background to conically shaped pores, theory of behaviours and transport of particles, biomolecules, and other examples, as well as a brief introduction of zeta potential relative to particle surface charge.

2.2 Tunable Resistive Pulse Sensing

Resistive pulse sensing, RPS, can be used to determine size, concentration and charge characteristics of particles in an electrolyte solution\(^1\)-\(^8\). Tunable resistive pulse sensing (TRPS) incorporates a tunable, synthetic pore membrane\(^1\) allowing for versatility in the particle size ranges that can be analysed and is therefore more favourable for determining the sizes of an unknown particle set. These elastomeric membranes can be used many times and can be reversibly altered to fit a range of nano and micron-sized geometries. TRPS utilises the tunable pore as a biosensor and uses the portable Izon qNano instrument to complete rapid, real-time measurements and particle-by-particle analysis. The qNano instrument setup is shown in figure 2.1.
Figure 2.1 - Image of the TRPS electrochemical cell. a) Lower fluid cell that holds 80 µL electrolyte solution, b) Polyurethane nanopore membrane placed on jaws and stretched appropriately for analysis, c) Upper fluid cell that holds 40 µL solution, d) Faraday cage placed over the fluid cell to reduce background electrical noise.

The instrumentation consists of two electrodes and although the qNano is susceptible to background electrical noise, this is reduced by a Faraday cage that is placed over the upper fluid cell (figure 2.1d). TRPS can complete particle-by-particle analysis of complex mixtures and solutions. The technology enables accurate monitoring of a particle’s electrophoretic mobility, as well as particle characteristics such as particle size and surface charge\textsuperscript{2,3,5,9–11}.

The electrolyte buffer solution is placed in an upper and lower fluid cells (40 µl and 80 µL, respectively, were used for all studies described in this thesis) and when an external electric field is applied through the two Ag/AgCl electrodes, a baseline current is observed. This current is dependent on the pore size, applied potential and ionic strength of the electrolyte\textsuperscript{12} and is shown through equation 2.1 describing Ohm’s law and equation 2.1a\textsuperscript{12}.

\[
I = \frac{V}{R} \quad (2.1)
\]

Where \( R = \frac{4L \rho}{\pi D_f D_s} \) \quad (2.1a)
When a particle approaches the pore, there is a significant increase in resistance observed and as a particle translocates the pore membrane, it displaces the electrolyte solution and creates a blockade (pulse) in the baseline current as shown in figure 2.2. The properties of the blockade itself can be used to determine the characteristics of the passing particle and are defined in figure 2.2.

The magnitude of the blockade, $\Delta i_p$, is the size (volume) of the analyte, FWHM (full width half maximum) is the particle translocation time through the nanopore, and the blockade frequency is relative to the particle concentration (particles/mL). The blockade magnitude is relative to the electrolyte resistivity, $\rho$, particle diameter, $d$, and pore diameter, $D$, as shown in equation 2.2:\(^8,13\).

$$\Delta i_p = \frac{(4\rho d^3)}{\pi D^4}$$  \hspace{1cm} (2.2)

The TRPS system has an inherent pressure head of approximately 50 Pa due to the 40 $\mu$L of liquid in the upper fluid cell\(^3\). An external pressure difference can also be introduced across the pore; applying a positive pressure increases the fluid flow from the upper fluid cell to the lower fluid cell and therefore increases the particle rate\(^3\). An external pressure is applied using the variable pressure module (VPM) as an accessory to the qNano, see figure 2.3.
The VPM uses a pressure scale of mm and cm where applying 1 cm of positive pressure is equivalent to 100 Pa. Introducing a vacuum, or negative pressure, slows the flow of liquid through the pore, therefore slowing the particle speeds and can even induce a reversed translocation effect back up through the pore in the opposite direction and can also be applied externally using the VPM. If a particle passes through the pore in the opposite direction, a reversed blockade would be observed. An example of a reversed blockade is shown in figure 2.4.

Blockages can occur in the pore and are most commonly due to impurities, aggregation, or surface adhesion. These blockages are removed by increasing the membrane stretch to allow possible aggregates or larger particles to pass through, or
by applying pressure to force the oversized particles through\textsuperscript{3,14}. Although there are a few minor difficulties with blockages in the pore at times, resistive pulse sensing is a technique with some of the highest resolution recorded in the detection of multiple particle sizes in a mixture. This was discussed by Anderson \textit{et al.}, comparing the technique to dynamic light scattering and other light scattering based techniques\textsuperscript{15}. There is negligible bias towards any sized particles using tunable resistive pulse sensing (TRPS), unlike in dynamic light scattering techniques.

### 2.2.1 TRPS Setup

All measurements were conducted using the qNano (Izon Science Ltd, NZ) combining tunable nanopores with proprietary data capture and analysis software, Izon Control Suite. The lower fluid cell always contained the electrolyte buffer (80 µL). The upper fluid cell always contained 40 µL of sample (that was suspended in the electrolyte) when a measurement was being completed with an inherent pressure on the system (47 Pa). The nanopore is placed laterally onto 4 jaws with the pore ID number facing up (as described in chapter 2, section 1) and is situated between the upper and lower fluid cells. Prior to TRPS analysis, most samples were vortexed and sonicated. During each sample run, the system was washed by placing electrolyte buffer (40 µL) into the upper fluid cell several times with various pressures applied to ensure there were no residual particles remaining and therefore no cross contamination between samples. A detailed description of such a tunable resistive pulse sensing device can be found in Willmott \textit{et al.}\textsuperscript{16} and Vogel \textit{et al}.\textsuperscript{2}.

### 2.2.2 Calibration Method for Zeta Potential Measurements

The nanopore chosen for each study is dependent on the size of the sample particles being analysed as well as the size of the calibration particles; the most appropriate pore to use will be of a size range that can measure both the calibration and sample particles. The different size ranges for each nanopore size provided by Izon Science Ltd (NZ) are detailed in table 2.1.
<table>
<thead>
<tr>
<th>Nanopore</th>
<th>Analysis Size Range (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NP80</td>
<td>40-255</td>
</tr>
<tr>
<td>NP100</td>
<td>50-330</td>
</tr>
<tr>
<td>NP150</td>
<td>70-420</td>
</tr>
<tr>
<td>NP200</td>
<td>85-500</td>
</tr>
<tr>
<td>NP250</td>
<td>110-630</td>
</tr>
<tr>
<td>NP300</td>
<td>150-900</td>
</tr>
<tr>
<td>NP400</td>
<td>185-1100</td>
</tr>
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<td>NP600</td>
<td>270-1570</td>
</tr>
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<td>NP800</td>
<td>385-2050</td>
</tr>
<tr>
<td>NP1000</td>
<td>490-2900</td>
</tr>
<tr>
<td>NP2000</td>
<td>935-5700</td>
</tr>
<tr>
<td>NP4000</td>
<td>1990-11300</td>
</tr>
</tbody>
</table>

Table 2.1 – Description of analysis size ranges of each nanopore ID supplied by Izon Science Ltd.

The calibration particles are measured at 3 applied voltages that are dependent on the applied stretch and consequent baseline current observed. Each sample measurement should be performed using a baseline current of 100+ nA, to allow us to compare data sets across several runs and pores. To account for variation in the pore size from the manufacturing process, the stretch and voltage were adjusted to achieve a similar baseline current for each experiment. As well as matching the baseline current each sample blockade signal was greater than 0.05 nA, compared to a background noise of circa 10 pA. Finally, when performing an experiment a calibration was performed on particles of known size and zeta potential. For the purpose of measuring and comparing zeta potential, it was imperative that the stretch of the nanopore and the applied potential were not changed during a sample or calibration measurement of a particular dataset. The sample measurements were all completed at the highest or second highest voltage that the calibration measurements were
carried out at. Calibration measurements were completed on each new day analysis was completed and when a new nanopore was introduced. An external applied pressure can be used with the calibration and sample measurements to allow for more dilute/less concentrated samples or samples containing a complex mixture of particles (with a potentially wide range of zeta potential values, including negative, neutral, and positively charged particles). A minimum of two applied pressures are applied at the highest voltage at which the calibration particles were measured.

2.3 Theory of Transport

The theory of transport of particles through a nanopore depends on a number of factors, including external influences, such as an electric field and pressure, nanopore geometry, and particle behaviour in an electrolyte solution. Particle flux is determined from Nernst-Planck theory that defines electrophoretic forces, electroosmotic flow, and pressure driven flow as contributions towards particle flux, \( J \). \( J \) is also relative to the particle concentration, \( C_s \), as well as the velocity of the translocating particle, \( \nu_P \), resulting in equation 2.3.

\[
J = C_s \times \nu_P \quad (2.3)
\]

The magnitude of other forces present, such as electrophoresis and electroosmosis means any diffusion contributions can be ignored at this point. \( \nu_P \) is defined in equation 2.4.

\[
\nu_P = \frac{Q}{\frac{\pi}{2} D_S^2} + \frac{\varepsilon \zeta_{\text{particle}}}{\eta} E - \frac{\varepsilon \zeta_{\text{pore}}}{\eta} E \quad (2.4)
\]

Where:

\[
Q = \frac{3 \pi D_p^3 \Delta P}{2 \eta \left( \frac{L}{D_L-D_S} \right)} \quad (2.4a)
\]

\( \varepsilon \) is the medium permittivity and \( \eta \) kinematic viscosity, \( \Delta P \) is the pressure across the pore, \( \zeta_{\text{pore}} \) and \( \zeta_{\text{particle}} \) are the zeta potentials of the channel pore and particle respectively, \( E \) is the applied electric field, \( L \) is the pore length, and \( D_S \) and \( D_L \) are the diameters of the entry (small) and exit (large) openings of the pore respectively.

Combining equations 2.1 and 2.2, where \( O \) is the cross-sectional area of the pore, and \( Q_p \) is the pressure driven flow as defined in equation 2.4a, leads to equation 2.5.
$$\frac{J}{\zeta_s} = \varepsilon \frac{(\zeta_{\text{particle}}-\zeta_{\text{pore}})}{\eta} E + \frac{Q_p}{0} \tag{2.5}$$

When there is no pressure applied across the system, electrophoresis dominates over electroosmosis\textsuperscript{16}. Particle mobility in electrophoretic transport only, $\mu$, is defined by equation 2.6\textsuperscript{16}.

$$\mu = \frac{aD}{k_B T} \tag{2.6}$$

The particle velocity is a total of the following velocities; fluidic, $v_F$, electrophoretic, $v_{EP}$, and electroosmotic, $v_{EO}$, as well as diffusion, $D$, shown in equation 2.7\textsuperscript{13}. The effect of each force on a particle is shown in figure 2.5.

$$v_P = v_F + v_{EP} + v_{EO} + D \tag{2.7}$$

Figure 2.5 - Representation of the three forces acting on a particle as it translocates the nanopore that result in total particle velocity.

Figure 2.5 shows the effect each force has on a particle as it translocates the pore membrane. Fluidic and electrophoretic forces have a more dominant effect on the particle velocity than the electroosmotic forces in a conical pore system, with electrophoresis being the most dominant factor\textsuperscript{16}.

### 2.3.1 Electroosmosis

A counter-charge present in an electrolyte solution compensates a particle’s surface charge and an ion flow occurs as a result of an applied potential or electric field. The ion flow will also be influenced by the net charge of the pore membrane.
Electroosmotic velocity, $v_{EO}$, is defined in equation 2.8 and electroosmotic flux, $J_{EO}$, is defined in equation 2.9, where $\varepsilon\varepsilon_0$ is the molar permittivity of solution.

$$v_{EO} = \frac{\varepsilon\varepsilon_0 E\varepsilon_{\text{particle}}}{\eta} \quad (2.8)$$

$$J_{EO} = v_{EO} \eta \quad (2.9)$$

### 2.3.2 Electrophoretic Mobility

The electrophoretic mobility of a particle is the movement of a particle through solution with the influence of an applied electric field. The movement of a charged particle through solution can be hindered by a dragging effect caused by the interference of counterions in solution that are moving in the opposite direction to the charged particle itself causing a resistance to the particle’s movement (see figure 2.5 for an example of counterions in solution surrounding a particle). This reduces the speed of the particles in electrolyte solution and results in electrophoretic retardation. Electrophoretic retardation can be observed when a small particle incorporates a thick double layer that results in a large influence on the movement of the small particle. A thin double layer surrounding a large particle will have a lesser effect on the mobility of the particle itself.

The Helmholtz-Smoluchowski equation (equation 2.10) defines electrophoretic mobility, $U_{EP}$, when taking electrophoretic retardation into account.

$$U_{EP} = \frac{\varepsilon\varepsilon_0 E\varepsilon_{\text{particle}}}{\eta} \quad (2.10)$$

### 2.4 Particle Stability

A particle’s stability is a key factor to its mobility through a pore and is affected by particle size and surface properties. Derjaguin, Landau, Verwey and Overbeek introduced DLVO theory describing the interactions that cause coagulation in a colloid system. If there is a potential energy barrier larger than the average kinetic energy of the particles, the system is considered stable. When colliding particles have sufficient energy to overcome this barrier, the system becomes less stable and aggregation can occur. The rate of coagulation is slowed by a “stability ratio”, denoted $V_T$, the total energy of particles interacting and is the sum of $V_A$ and $V_R$. $V_A$ is the sum of the Van der Waals attractive forces, and $V_R$ is the sum of the electrostatic repulsive forces of
the double layer\textsuperscript{20}. From this information, it can be expected that smaller particles undergo flocculation more rapidly than larger particles and are therefore less stable in solution.

Both steric and electrostatic repulsion affect colloidal stability. The electrostatic forces acting on each particle are determined from the charge distribution of the particle. The more even the charge distribution in the electrical double layer, the stronger the electrostatic forces. Steric repulsive forces however, arise from any polymers that adsorb onto the particle surfaces. The larger the polymer, the more steric hindrance there is that prevent Van der Waals forces causing coagulation.

The zeta potential describes the stability of a colloidal system as it is related to those particular repulsive forces, $V_R$, depicted in equation 2.11\textsuperscript{20}, in the double layer. $\varepsilon$ is the medium permittivity, $a$ is the particle radius, $\zeta$ is zeta potential, $\kappa$ is the Debye length, and $h$ is the particle separation.

\[ V_R = 2\pi \varepsilon a \zeta^2 \ln(1 + e^{-\kappa h}) \]  

(2.11)

### 2.5 Zeta Potential

For charge stabilised particles, a typical measurement used to represent the surface charge, and infer stability, is zeta potential. The zeta potential represents the value of the electrostatic potential at the plane of shear of a colloidal system (figure 2.6) and typically for nanoparticle systems, zeta potential values of ±30 mV are representative of stabilised particles\textsuperscript{21,22}. A smaller zeta potential value is observed when the particles in suspension are less stable and flocculating more rapidly. Therefore, a larger zeta potential value indicates excellent electrical stability in an electrolyte solution.
Figure 2.6 - A schematic definition of zeta potential and the electrical double layer surrounding a particle in an electrolyte solution including the effect of high (red) and low (green) ionic strengths. The electrical potential at the slip/shear plane defines the zeta potential.

Figure 2.6 shows the effect of ionic strength on zeta potential, a high ionic strength results in an increased concentration of counterions that compresses the diffuse double layer thus increasing the electrokinetic/electrical potential gradient as a function of distance from the particle’s surface, reducing the zeta potential (red curve, figure 2.6). A low ionic strength however, will decrease the counterion concentration that allows for the diffuse double layer to become extended and thus decreasing the electrokinetic/electrical potential gradient as a function of distance from the particle’s surface, resulting in a larger zeta potential and is indicated by the green curve in figure 2.6.

The Smoluchowski approach defining zeta potential is displayed in equation 2.12:\(^\text{23}\):

$$\zeta_{\text{particle}} = \frac{4\mu}{e}$$  \hspace{1cm} (2.12)
The Smoluchowski approach supports that a particle’s zeta potential can be
determined from a particle’s velocity taking into account convective and electroosmotic
forces, as well as the electrophoretic mobility of the particle. In TRPS, the translocation
speeds of particles in solution can infer their electrophoretic mobilities through the pore
due to the attraction to the oppositely charged electrode in the TRPS system.

Zeta potential measurements can be extracted from the electrophoretic information in
a given sample. Translocation mobility, $\mu_{tr}$, is defined in equation 2.13:\n
$$\mu_{tr} = \frac{\delta \nu}{\delta E}$$  \hspace{1cm} (2.13)\n
Where $\bar{\nu} = \frac{l_s}{t_{tr}}$ and $E = \frac{V}{l_e}$ representing the average velocity and average electric field
applied. $l_s$ and $l_e$ are the length of the sensing zone and the length the applied voltage
drops, $t_{tr}$ is the translocation duration and $V$ is the applied voltage. $l_s = l_e$ in most cases
as the voltage drops over the sensing zone.

Following Smoluchowski’s approximation, translocation mobility can be defined as follows;

$$\mu_{tr} = \frac{\varepsilon \varepsilon_0 \zeta}{\eta}$$  \hspace{1cm} (2.14)\n
A combination of equation 2.14, the average translocation velocity, the applied
voltage, and zeta potential results in equation 2.15:\n
$$\bar{\nu} = l \left( \frac{1}{t_{tr}} \right), \quad \mu_{tr} = l^2 \frac{\delta (\zeta)}{\delta V}, \quad t_{tr} = \frac{\eta l^2}{(V+B) \varepsilon \varepsilon_0}$$  \hspace{1cm} (2.15)\n
And therefore zeta potential can be determined by combining equations 2.12-2.15;

$$\zeta = \frac{\eta l \delta (\frac{1}{t_{tr}})}{\varepsilon \varepsilon_0 \frac{\delta V}{\delta V}} = A \frac{\delta (\frac{1}{t_{tr}})}{\delta V}$$  \hspace{1cm} (2.16)\n
Where $B$ is an integration constant that is the voltage offset (although is generally an
unknown parameter that can vary between experiments, $\frac{\delta (\frac{1}{t_{tr}})}{\delta V}$ can be easily measured
as the applied voltage and translocation duration are known, $\frac{\eta l^2}{\varepsilon \varepsilon_0}$ however, includes
parameters that are more difficult to calculate exactly. From equation 2.16, the
following equation can be extracted:
\[ A = \frac{\eta \ell^2}{\varepsilon \varepsilon_0} \quad \text{(2.17)} \]

From equation 2.17, \( A \) can be calculated from the calibration experiments run with particles of a known zeta potential. The zeta potential of a sample set can then be calculated exactly using equation 2.16 as the applied voltage and translocation duration can be obtained from the TRPS output.

### 2.6 Effect of Surface Modifications on Surface Charge

Polyelectrolyte brushes, such as DNA have been previously studied as surface modifications and it has been found that if the outer region of the brushes is open to any extent, the electroosmotic flow may have an influence and change the measured charge of the brush (the zeta potential may appear lower)\(^{25}\). Spherical brush polyelectrolytes include DNA strands that are bound to the particle surface by one end, leaving the other free standing into solution\(^{26}\). It has been found that increasing the ionic strength causes the brushes to collapse due to electrostatic attractions\(^{26}\). If a thinner, denser layer is surrounding the particle, the hydrodynamic resistance of the brush coated particle is reduced\(^{26}\).

When using nanomaterials in bioassays, the material must remain suspended in the solution for it to capture the analyte. A particle’s surface chemistry design is important to avoid sedimentation of irreversible aggregation; there are two mechanisms available to prevent this. First is the use of steric stabilisation by placing a neutral polymer onto the particle surface, and the second depends upon charge stabilisation whereby the repulsive coulombic forces overcome the attractive Van der Waals forces, as described previously\(^{18,27}\).

When a polyelectrolyte, such as DNA, is immobilised onto the surface of the nanomaterials the DNA can take on two roles. The first is the more natural of the two as a capture probe, designed to hybridise to target DNA. The second is a passive role where the inherent charge on the phosphate back bone can act as a stabiliser by creating a high charge density on the particle surfaces, helping suspend them in solution\(^{28}\).
2.7 Ionic Effects of Conical Pores

The surface charge of the pore walls is important as it can affect the translocation events of nanomaterials as they traverse small pores and thus the pore material/pore coating material can be of interest. Such effects could manifest themselves as changes in ionic current, and become current rectifiers. Conical pores will typically exhibit this effect to a larger extent based on the pore opening diameter being equivalent to the electrical double layer thickness\textsuperscript{29,30} and when the pore walls are negative in charge.

Conventionally, the preferential direction of the flow of cations, for a negatively charged pore, is observed to be from the small pore opening to the larger pore opening in a conical pore setup\textsuperscript{30}. When the pore walls are negative in charge a smaller ionic current is observed at positive potentials and a larger current is observed at negative potentials and is due to mainly cations being able to enter a pore, whereas anions are often rejected\textsuperscript{31}. Opposite effects are seen when the pore walls are positively and indicated that current rectification effects originate from electrostatic interactions between ions traversing the pore and the pore walls\textsuperscript{30}.

The current flow through a conical pore has two contributing factors; the electroosmotic flow across the pore surface and migration of ions through the centre. For a negatively charged pore and a positive potential, the small pore opening will have the highest concentration of cations and thus anions are more likely to be rejected, the cations are thus transferring from a region of high cation concentration to a region of lower cation concentration and will give smaller current values. When a negative potential is applied to the pore, the cations will transfer from a low concentration region to a higher concentrated region resulting in a higher concentration of ions in the transition region causing a larger (more negative) ion current\textsuperscript{30,32}.

The effects of ionic strength and pH on current rectification are particularly important as these have direct implications on the cation/anion concentration throughout the pore and the pore wall surface charge, respectively. As you increase the ionic strength of the electrolyte, you increase the concentration of cations at the pore opening and throughout the pore system and thus a more prominent ion transfer process, albeit at positive or negative potentials\textsuperscript{30}. The lower the ion concentration amongst the pore,
the smaller the recorded ionic currents, an increased number of ions would result in a more ohmic current-voltage response.

2.8 Acknowledgements

I would like to thank Dr Robert Vogel for his input and help on the zeta potential derivation.

2.9 References


3 Zeta Potential Measurements via Tunable Resistive Pulse Sensing

3.1 Introduction

This chapter will provide the theory for the calculation of zeta potential using tunable resistive pulse sensing (TRPS). Zeta potential is a measure of surface charge and can give information on the charge effects of a sample in solution. This theory was developed in collaboration with Dr Robert Vogel (Izon Science Ltd) and was derived using a large series of example experiments and respective data analysis tools. The experiments were conducted by myself to help in the development of the method and applying it to real samples and assays.

3.2 Aims and Objectives

The aim of this chapter is to describe the current theory used to determine zeta potential that can be verified using tunable resistive pulse sensing technology. The electrokinetic and convective velocity effects are used to calculate zeta potential as a function of applied voltage and pressure.

3.3 Theory

Zeta potential measurements can be completed using tunable resistive pulse sensing technology and provides information on the charge of a sample. The zeta potential represents the value of the electrostatic potential as the plane of shear of a colloidal system (see chapter 2). The method for determining zeta potential is similar to that by Arjmandi et al.¹ who describes a calibration based method using resistive pulse sensing to measure particle zeta potentials. We adapt the method by considering the effect of pressure on the system as this is important in completing zeta potential measurements.

This method is based on measuring the duration of the translocation of particles through a nanopore as a function of applied voltage, $V$, with particle velocities and the electric field being averaged over the entire sensing zone of a regular conical pore, $l$. The electric field, $E$, can be determined using the calculation of pore resistance, so that the electric field is entirely parallel to the z-axis (see equation 3.1)
\[ E_z(z) = -I_0 \frac{dR}{dz} \] (3.1)

Where \( E_z, I_0, \) and \( R \) being the electric field component along the pore axis (z), electric current, and resistance, respectively. For a voltage, \( V_0 \), of 0.5 V, a small pore opening diameter of 0.8 µm, a large pore opening diameter of 40 µm, and a membrane thickness of 250 µm, the maximum electric field is approximately \( 10^5 \) V/m. The above pore dimensions are estimates, which are in accordance with SEM images of pores with similar dimensions to the ones used for some of our studies (the example pore in this case is an NP200) done in conjunction with Dr Robert Vogel. The electrophoretic mobility is the derivative of \( 1/T \) (with \( T \) being the signal duration) and voltage, \( V \), multiplied by the square of the sensing zone length, \( l \). \( l \) is a fitting parameter that is included in a calibration constant, which is calculated using a calibration particle with known zeta potential. Convection and electroosmosis have been neglected for being much smaller than the electrophoretic contribution to particle motion. Finally, Henry’s equation is used to relate the particle zeta potentials with the measured electrophoretic mobility of single particles.

The method in conjunction with TRPS technology follows a related approach and is described in Blundell et al., where the effects of electroosmosis and convection (through an applied pressure) are considered in addition to electrophoresis when determining the zeta potential of single particles. An external applied pressure, additional to the inherent pressure on the system, may be required for samples of low concentration or those containing particles with a wide range of zeta potentials in order to capture the whole spectrum of particle zeta potentials in a potentially complex sample. Also, without any net pressure, most neutral particles may not translocate the pore and hence may not be measured that may result in a skewed set of final results from a given sample population.

The average velocities and electric fields are recorded at multiple points through the sensing zone (as opposed to the entrance, exit, or middle of the sensing zone) as shown in figure 3.1b.
Figure 3.1 – a) Blockade events produced as particles traverse the pore and the resulting blockade shape that depicts $\Delta I_p$ and FWHM. b) Examples of blockade reference points within each blockade that indicate a particle’s position within the pore at any given time. $1/T_{1.0}$ represents the time at which the blockade is 100% magnitude of $dR_{\text{max}}$. $T_{0.30}$ and $T_{0.60}$ are the times at which the blockade is at 30% and 60% of $dR_{\text{max}}$, and are equivalent to positions $I_{0.30}$ and $I_{0.60}$. c) A plot of $1/T$ (ms) vs voltage (V) used within the calibration method to calculate each particle’s translocation time and thus zeta potential amongst a given sample population.

This method identifies the point of greatest resistance in the signal trace, otherwise known as maximum blockade magnitude. For each blockade/pulse recorded, the time at which the peak occurs is at 100% magnitude, $dR_{\text{max}}$, (relative to the baseline current), can be defined as $T_{1.0}$. Figure 3.1b shows an example of 4 sections (out of the 14 recorded from $T_{0.05}$ to $T_{0.90}$) that represent the blockade magnitudes of 60%, 50%, 40%, and 30% of $dR_{\text{max}}$ and are relative to $T_{0.60}$, $T_{0.50}$, $T_{0.40}$, and $T_{0.30}$, respectively. These values also indicate various positions of particles within the pore at given times. When the proportional blockade magnitude is equal for any given particle (small or large), these particles are at equivalent positions within the pore, the respective positions are denoted as $I_{0.60}$, $I_{0.50}$, $I_{0.40}$, and $I_{0.30}$, respectively and are shown in figure 3.1b.
The calibration process and zeta potential calculation can be summarised as follows; each pore is calibrated using calibration particles (carboxylated polystyrene particles) with a known average zeta potential. Using this calibration standard, the pore is calibrated by measuring the linear dependence of $1/T_x$ vs voltage, $V$. $1/T_x$ is averaged over at least 100 particles for $x= 0.30, 0.35, 0.40, 0.45, 0.50, \text{ and } 0.60$. $1/T_x$ is proportional to the time averaged total particle velocity, $v_{x \text{tot}}^i$, of particle $i$, with $l_x$ being the position within the pore reached after $t = T_x^i$.

$$v_{x \text{tot}}^i = \frac{I_x}{T_x^i} = \frac{\int_0^T v(t)dt}{T_x^i}$$

(3.2)

$$\frac{1}{T_x} = \sum_{i=1}^N \frac{1}{I_x^i}$$

(3.3)

In the same way, $v_{x \text{tot}}$ is the average over $N$ calibration particles:

$$v_{x \text{tot}} = \sum_{i=1}^N v_{x \text{tot}}^i / N$$

(3.4)

For the purpose of simplicity, the total velocity averaged over time and particle velocity, $v_{x \text{tot}}$, is set equal to $1/T_x$. The total particle velocity is the sum of electrokinetic and convective velocities:

$$v_{x \text{tot}} = v_{x e l} + v_{x con}$$

(3.5)

Equation 3.4 applies for both single particle velocities and velocities averaged over many particles. The electrokinetic (electroosmotic and electrophoretic) velocity, $v_{x e l}$, of the calibration particles is determined from the slope $v_x^V$ of the linear $1/T_x$ vs voltage curves.

$$v_x^V = v_{x e l} / V$$

(3.6)

The electrokinetic velocity per unit voltage is equivalent to the electrokinetic mobility, which is the sum of electroosmotic and electrophoretic mobilities. The convective velocity of the calibration particles for each $T_x$ is determined from the $y$-intercept of the linear $1/T_x (\triangleq v_{x \text{tot}})$ vs voltage curves. The convective velocity per unit pressure is defined as $v_x^P$ and is calculated as follows;
\[ v_{x,\text{Cal}}^P = v_{x,\text{con}} / P \] (3.7)

A further iteration of the above calibration method includes the average convective velocity per unit pressure of the calibration particles, \( v_{x,\text{Cal}}^P \) (equation 3.7) being calculated by measuring the slope of the linear \( 1/T_x \) (\( \triangleq v_{x,\text{tot}} \)) vs pressure, \( P \), curves. In these cases, the calibration particle \( 1/T_x \) and thus particle velocity values are measured at a minimum of two externally applied pressures. This method is applied to at least the highest voltage used in each study.

From this the electrokinetic particle velocities of sample, \( (v^i)_x \) \( \text{el \ Sample} \), and calibration, \( (v_x)_{\text{el \ Cal}} \), are related with their zeta potentials, \( \xi_{\text{net \ Sample}} \) and \( \xi_{\text{net \ Cal}} \) (equation 3.8), assuming a linear relationship between velocity (mobility) and zeta potential as given in the Smoluchowski approximation\(^{5,6}\).

\[
\frac{(v^i)_x \text{el \ Sample}}{(v_x)_{\text{el \ Cal}}} = \frac{\xi_{\text{net \ Sample}}}{\xi_{\text{net \ Cal}}} \quad (3.8)
\]

The net zeta potentials for both sample and calibration particles are the differences in the respective particle zeta potentials and the membrane zeta potential, \( \xi_m \) (equation 3.9).

\[
\xi_{p,\text{Sample}} = \xi_{\text{net \ Sample}} + \xi_m \quad (3.9)
\]

The zeta potential of each sample particle \( i \), \( \xi_{\text{Sample}}^i \), is given by averaging respective zeta potential values, calculated at various locations within the pore (equation 3.10), with \( l_x \) being the position within the pore reached after time, \( t=T_x \). Please note that \( l_x \) is set to equal 0 right at the narrow pore entrance of the conical pore, where the signal magnitude reaches its maximum, as shown in figure 3.2.1b. Zeta potentials are evaluated by taking the average at several discrete points, \( l_x \).
\[ \xi_i^{\text{Sample}} = \frac{\sum x^i x^{\text{Sample}}}{\sum x} - \frac{\sum x (v_i^{\text{Sample}} - v_i^{P})/(v_i^{P})}{\sum x} \times \xi_{\text{net Cal}} + \xi_m \quad (3.10) \]

\( v_{x i}^{\text{Sample}} \) is the sum of the time averaged electrokinetic (electroosmotic and electrophoretic) and convection velocity components of sample particulates at position \( l_x \) within the pore (equation 3.11).

\[ v_{x i}^{\text{Sample}} = \frac{\int_{0}^{l_x} v_i(t) dt}{T_i} = \frac{\int_{0}^{l_x} v_i(t) dt}{T_i} \quad (3.11) \]

\( v_{x i}^{P} \), \( v_{x i}^{V} \), \( P \), and \( V \) are electrokinetic velocity per unit voltage, convective velocity per unit pressure, applied pressure and voltage for the sample runs respectively. The electrokinetic velocity per unit voltage is equivalent to the electrokinetic mobility, which is the sum of electroosmotic and electrophoretic mobility. \( v_{x i}^{P} \) and \( v_{x i}^{V} \) are calculated by averaging typically over more than 500 calibration particles. \( \xi_{\text{net Cal}} \) and \( \xi_m \) are the zeta potentials of polystyrene standard particles and the membrane respectively. The zeta potentials of polystyrene standards and the thermoplastic polyurethane membrane can be verified using phase analysis light scattering (PALS) and streaming potential techniques, respectively.

### 3.4 Summary

This method describes how zeta potential is calculated using TRPS technology via particle electrokinetic and convective velocities as a function of applied voltage and pressure. A calibration based method is used here to establish the surface charge of the sample particles via their translocation velocities relative to a position in the pore at any given time. Calibration particles of known zeta potential are used to achieve the difference in particle zeta potential from that of the pore membrane to establish the charge effects of the pore walls itself. The determined zeta potential values are a key indication of particle surface charge that will be of great use to differentiate samples of a similar, if not the same size in a complex sample such as whole blood, plasma/serum, or samples in biological media. The theory was developed and tested using several rigorous experimental processes and verified using the tunable resistive pulse sensing technique.
3.5 Acknowledgements

I would like to thank Dr Robert Vogel, Izon Science Ltd, for his input and support with the final interpretation and derivation of the zeta potential calculations.

3.6 References


4 Charge Analysis of DNA-Modified Nanoparticles

4.1 Abstract
Modifying a nanoparticle’s surface and altering their functionality is advantageous for protein, DNA, peptide, and small molecule detection, as well as other medical and environmental samples. Tunable resistive pulse sensing (TRPS) allows for the characterisation of the transport mechanism of both functionalised and unmodified nanoparticles as they traverse small pores. RPS systems are already proven to be useful in measuring size, concentration and zeta potential of analytes. This chapter focuses on utilising TRPS technology to monitor the translocation effects and thus zeta potential of nanoparticles with DNA-modified surfaces. Inferring zeta potential from the translocation durations of particles is detailed in chapter 2. This method can be applied to measure the change in zeta potential upon DNA functionalization of nanoparticles and the detection of a range of DNA targets. The parameters investigated as a function of zeta potential were as follows; packing density, length, structure, and hybridisation time. Each parameter attributed a difference in measured zeta potential for both mean values and population distributions as a function of DNA structure and relevant DNA binding mechanisms. The signal resolution was encouraging for ssDNA, dsDNA, and changes in base length as small as 15 nucleotides. A change in signal was also observed for what may appear to be small changes in DNA structure with respect to the range of DNA targets studied, including discrimination between partial and fully complementary target sequences. The results from the assays studied show great potential for the charge method and for application in diagnostic and environmental fields.

4.2 Introduction
TRPS is a recent adaptation of RPS, a Coulter counter based technology described in chapter 1 and 2. Immobilising DNA onto nanoparticle surfaces is important to consider when designing nanoparticle-based assays and are becoming increasingly popular, in particular for application in DNA-protein interactions\(^1\)\(^–\)\(^3\) and targeted drug delivery\(^4\)\(^–\)\(^6\). It is now common practice to functionalise nanoparticles with DNA in the biosensing field\(^7\)\(^–\)\(^9\) and we are choosing to functionalise DNA onto the surface of
superparamagnetic particles, allowing for rapid and simple purification steps to enable the successful removal of the target analyte. Charge analysis and zeta potential measurements can be used to infer the stability of a sample in solution. When DNA is immobilised onto the nanoparticle surface, the DNA can take on one of two roles, the first being the more natural as the immobilised DNA is treated as a capture probe designed to hybridise to target DNA. The second is the more passive role where the charge on the DNA due to the phosphate backbone acts as a stabiliser for the particles in solution due to the high charge density now on the particle’s surface, thus helping suspend them in solution.

The charge effects on the particle surface and thus the zeta potential can also differ depending on the DNA structure (i.e. single-stranded or double-stranded) based on the persistence length of the respective strand. As mentioned previously in chapter 2, the persistence length of double-stranded DNA is a 50-fold magnitude higher than that of single-stranded DNA, making it far more rigid and less flexible. The mean zeta potential does not allow for a true measure of the polyelectrolyte distribution across each individual particle to be interpreted so to allow this the spread of a given sample population can be used. Investigating the distribution of zeta potential values amongst a given sample population means each individual zeta potential value has to be measured and a true insight into each sample. Measuring the zeta potential of each individual particle is challenging, however other electrophoretic and electrochemical techniques have allowed an insight into these measurements as previously discussed in chapter 1. TRPS can monitor particle translocation effects and carry out zeta potential measurements utilising its particle-by-particle nature. Particle-by-particle analysis allows for every sub population within a sample to be resolved, an advantage over ensemble techniques.

Zeta potential is measured using a similar concept shown by Arjmandi et al. using pyramidal shaped pores. It is a calibration based method that has been discussed in chapter 2, the zeta potential is measured based on signal durations of each blockade event as a function of applied voltage. The electrophoretic mobility is calculated from the particle velocity and applied electric field that can then be used to infer zeta potential using the Smoluchowski approach. The zeta potential is independent of the blockade magnitude, making TRPS' simultaneous size and charge measurements possible.
The Arjmandi theory\textsuperscript{14} is adapted applied to a calibration particle standard of known zeta potential and then move on to measuring relative changes in zeta potential as a function of immobilised DNA. This chapter describes how measured zeta potential is correlated to the DNA concentration and thus packing density. The higher the DNA concentration immobilised to the nanoparticle surface, the more uniform the zeta potential distribution showing uniform polyelectrolyte binding around the surface. The method has also been applied to investigating the effects of DNA length and the effect of dsDNA. Controlling the packing density on the particle surfaces and the mechanism to which the targets bind to the capture probe allows for the sensitivity of the technique to be investigated. The sensitivity of the TRPS instrument can allow for target DNA to be detected with hybridisation times under 30 minutes. Key to the assay design is the DNA length and positioning of the target DNA strand (via complementary sequences) to improve and optimise the signal produced. The method is impactful on designing nanoparticle based assays and shows the potential in zeta potential measurements on biological analytes, showing great advantages in bioassay fields.

4.3 Aims and Objectives

The main objective from this work is to determine the sensitivity and selectivity of DNA aptamers when functionalised to a nanoparticle’s surface. This is determined from the particle translocation velocities that can be translated into zeta potential values, as described in chapter 2. Further aims include investigating the effect of a range of DNA-modifications on a nanoparticle surface; including the effect of packing density, DNA length, DNA structure, and hybridisation time. This is particularly important when designing aptamer-based particle assays for future research.

4.4 Materials and Methods

4.4.1 Chemicals and Reagents

4.4.1.1 Buffers

The buffer used was PBST, phosphate buffered saline (PBS, 0.01 M phosphate buffer, 0.0027 M potassium chloride, 0.137 M sodium chloride, pH 7.4) with Tween-20 as a surfactant (T, 0.05 (v/v) %) in 200 mL deionised water (18.2 MΩ cm). PBS tablets (P4417) and Tween-20 (P1379) were purchased from Sigma Aldrich, UK.
4.4.1.2 Particles and Particle Standards

Streptavidin coated superparamagnetic particles (120 nm, 4352 pmol/mg binding capacity, product 03121, as detailed by the supplier) were purchased from Ademtech, France. Carboxylated polystyrene particles with a mean nominal diameter of 220 nm (CPC200s) were purchased from Bangs Laboratories, US. The specific surface charge as determined by the manufacturer was 86 µeq/g and equivalent to a surface charge density of $3.2 \times 10^{-19} \text{C/nm}^2$. The CPC200s were measured at a concentration of $1 \times 10^{10}$ particles/mL for this study and were used as a particle standard for zeta potential measurements. Zeta potentials of CPC200 particles were determined using PALS analysis, as detailed in the appendices.

4.4.1.3 Custom DNA Oligonucleotides

All oligonucleotides used in this study were purchased as lyophilised powders (100 pmol/µL) from Sigma Aldrich, UK with customised DNA sequences fit for purpose as detailed in table 4.1. Please note the abbreviation [Btn] is relative to a biotin modification that was added to the 3’ end of the oligonucleotide, when required. The CP abbreviation is to describe the ‘capture probe’, further explained in section 4.5, and is the same 25 base DNA sequence used for the varied length study described in section 4.5.2.
<table>
<thead>
<tr>
<th>Sequence Name</th>
<th>Base Sequence</th>
<th>Number of Bases</th>
</tr>
</thead>
<tbody>
<tr>
<td>VL10mer</td>
<td>5’NNNNNNNNNN[Btn]3’</td>
<td>10</td>
</tr>
<tr>
<td>VL36mer</td>
<td>5’TGGGAGTAGGTGTGTTGGGGCTCCCCTTTTT[Btn]3’</td>
<td>36</td>
</tr>
<tr>
<td>VL50mer</td>
<td>5’ATACCAGTCTATTCAATGGGCCCCGTCGTATTGGTGGGTGTGCTGGCCAG[Btn]3’</td>
<td>50</td>
</tr>
<tr>
<td>VL25mer/CP</td>
<td>5’ATGGTTAACCTCACTACGCGTGCGG[Btn]3’</td>
<td>25</td>
</tr>
<tr>
<td>cDNA</td>
<td>5’GCCACGCGTAGTGGAGGT[Btn]3’</td>
<td>25</td>
</tr>
<tr>
<td>MidT</td>
<td>5’GTAGTGAGGT3’</td>
<td>10</td>
</tr>
<tr>
<td>EndT</td>
<td>5’GTGTAACCAT3’</td>
<td>10</td>
</tr>
<tr>
<td>OverT</td>
<td>5’GTGAGGTTTACCATTTTTTTTTTTTTTTTTT3’</td>
<td>30</td>
</tr>
</tbody>
</table>

Table 4.1 - Summary of DNA oligonucleotides used in this study including details of base sequence and base lengths. [Btn] notation indicates a biotin tag attached to the 3’ end of the DNA sequence. The cDNA is a complementary DNA strand to VL25mer/CP (capture probe) DNA. cDNA, MidT, EndT, and OverT are all examples of target DNA used in this study.

4.4.2 Methods

4.4.2.1 Hybridising DNA to Streptavidin Coated Particles

120 nm diameter streptavidin coated particles were diluted to a concentration of 1 x 10^{10} particles/mL. The diluted particle solutions were then vortexed for 30 s and sonicated for 2 mins to ensure monodispersity.

The biotinylated DNA capture probe was added to the streptavidin coated particles (4352 pmol/mg binding capacity as determined by the supplier) at the required concentration (concentrations of 10, 20, 30, 47, 95, 140, and 210 nM were investigated for CP (capture probe) DNA, and concentrations of 75 and 210 nM were investigated for the varied lengths of DNA, VL10, 25 and 50). Once the biotinylated DNA had been added, the samples were placed on a rotary wheel at room temperature for 30 minutes. Any unbound DNA remaining in solution was then removed via magnetic separation.
by placing the samples onto a Magrack (GE Healthcare, UK) for 30 minutes. The supernatant was then removed and replaced with new PBST buffer.

4.4.2.2 Addition of Complementary Target DNA
Target DNA was added in excess (500 nM) to ensure the maximum possible target binding was reached. The samples were then placed on a rotary wheel at room temperature to investigate the effect of DNA hybridisation time (30 minutes and 16 hours were investigated for each respective DNA target). It was important to note the temperature of the lab.

4.4.2.3 TRPS Setup and Zeta Potential Calibration
The data capture and analysis software used alongside the qNano instrument (Izon Science Ltd, NZ) was Izon Control Suite v.2.2.2.117. Details of the instrument setup can be found in chapter 4. The nanopores used for this study were all capable of detecting particles in the size range of 85-300 nm (an NP150, as determined by the manufacturer, Izon Science Ltd, NZ). This is the most appropriate pore size for these experiments based on particles being analysed (120 nm and 220 nm in diameter). The calibration method for nanopores used in this study relative to zeta potential measurements is detailed fully in chapter 2, section 2.2.2.

4.5 Results and Discussion

4.5.1 Effects of Packing Density on Zeta Potential
The zeta potential method was first applied to DNA-modified nanoparticles as a function of DNA concentration. ssDNA (25 bases in length, termed capture probe, VL25mer/CP) was immobilised onto streptavidin coated particles and the DNA concentration ranged from 10-210 nM, whilst the particle concentration remained unchanged. The theoretical binding capacity of the streptavidin coated particles (as provided by the supplier) was detailed as 188 nM. The highest DNA concentration investigated (210 nM) was therefore when the DNA was in excess and therefore should result in 100% coverage of the particles. For comparison, table 4.2 shows the mean size and zeta potential values for streptavidin coated particles and figure 4.1 shows the size (a) and charge (b) distributions of unmodified streptavidin coated particles as a base comparison for the samples with DNA functionalisation.
<table>
<thead>
<tr>
<th>Mean</th>
<th>Median</th>
<th>Mode</th>
<th>Minimum</th>
<th>Maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Particle Diameter (nm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>116 ± 1.68</td>
<td>111 ± 1.25</td>
<td>111</td>
<td>94</td>
<td>232</td>
</tr>
<tr>
<td>Zeta Potential (mV)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-8.94 ± 0.34</td>
<td>-2.88 ± 0.50</td>
<td>-2.37</td>
<td>-25.71</td>
<td>6.09</td>
</tr>
</tbody>
</table>

Table 4.2 - Particle size (nm) and zeta potential (mV) values for samples where a minimum of 300 particles were measured and a standard deviation where n=3 independent replicates.

Figure 4.1 - a) size and b) zeta potential distributions amongst a given sample population of unmodified streptavidin coated particles. A minimum of 300 particles were measured in each sample.

The highest concentration of DNA equated to approximately 12648 pieces of DNA per particle (in a sample containing 1 x 10^{10} particles/mL) and, if each of these strands were attached to the surface, would result in approximately 1 DNA strand every 2 nm^2 across the particle surface. Table 4.3 shows the total pieces of DNA per particle for each of the DNA concentrations studied.
<table>
<thead>
<tr>
<th>DNA concentration (nM)</th>
<th>Number of DNA strands /particle</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>602</td>
</tr>
<tr>
<td>20</td>
<td>1204</td>
</tr>
<tr>
<td>30</td>
<td>1806</td>
</tr>
<tr>
<td>47</td>
<td>2829</td>
</tr>
<tr>
<td>75</td>
<td>4517</td>
</tr>
<tr>
<td>95</td>
<td>5719</td>
</tr>
<tr>
<td>140</td>
<td>8428</td>
</tr>
<tr>
<td>210</td>
<td>12648</td>
</tr>
</tbody>
</table>

Table 4.3 - The relative number of DNA strands per particle for each DNA concentration studied, for samples containing $1 \times 10^{10}$ particles/mL.

TRPS can complete simultaneous size and charge measurements, an example of which can be seen in figure 4.2. The green and red bars/data points show the distributions at the lowest and the middle concentration (10 and 47 nM respectively) and the blue bars/data points show the results from the samples with the highest CP concentration of 210 nM. The particle-by-particle nature of the technology is depicted here as each data point is representative of single particle amongst a sample population.
These data show the advantages of being able to complete rapid size and charge measurements simultaneously because although there are no size changes seen between the samples (regardless of the CP concentration added), there are changes observed for zeta potential between each of the samples. The shift in zeta potential values from the base particle (figure 4.1, no hybridised DNA, purple dataset) to those hybridised with 10 nM (green dataset) and 210 nM (blue dataset) is shown in figure 4.3b. The figure shows that even a small amount of DNA as low as 10 nM, equivalent to approximately 5% of the particle’s binding capacity, shows a change in zeta potential distribution and a change in mean zeta potential of a given sample (see figure 4.4a), but has no influence on the measured size of the particles (figure 4.3a).
Figure 4.3 - Comparison of a) size and b) zeta potential distributions for particles with no hybridised DNA (purple bars), 10 nM DNA (green bars), and 210 nM hybridised DNA (blue bars). Each distribution is representative of a sample where a minimum of 300 particles were measured.

The mean measured zeta potential values (a) and particle velocities (b) are shown in figure 4.4. The particle velocities are determined from the inverse of the translocation time it takes for the particle to become 50% of the way through the pore ($1/T_{0.50}$), which can also be defined as an estimate of the particle speeds (see chapter 3).
The results show that an increase in DNA concentration results in a larger measured zeta potential and correlate with an increase in DNA concentration resulting in an increased particle velocity (figure 4.4b). The larger zeta potential is attributed to the negative phosphate backbone of DNA, each phosphate group will contribute to a negative point charge, which in turn will increase the charge density close to the particle surface.

Figure 4.5 shows the frequency (as a percentage) of each particle amongst the sample population versus the measured zeta potential at each DNA concentration studied. When there is a low packing density (and thus a low concentration of DNA immobilised to the particle surface), the distribution is relatively narrow with a long skewed tail. When more DNA is added to the sample, the distribution appears to widen and is seen to have a more symmetrical nature around the mean zeta potential value. The histograms again portray the particle-by-particle nature of TRPS, providing a deeper insight and a more detailed analysis of the sample.
Figure 4.5 - Zeta potential distributions vs frequency as a percentage for samples containing capture probe DNA (VL25mer/CP) concentrations of 10, 20, 30, 47, 95, 140, and 210 nM.
4.5.2 Effects of DNA Base Length

The results from section 4.4.1 indicate that a similar relationship should be seen for an increase in DNA length as there was for the increase in DNA concentration as more bases will mean a longer negative phosphate backbone to affect the particle surface charge. The effect of additional charges to the system such as long dsDNA strands (4-6 kilo base pairs) hybridised to colloids has previously been investigated by Steinbock et al. using microparticles and a microcapillary based Coulter counter technology\textsuperscript{17}. TRPS is a sensitive method and to test this, the focus of this study is measure changes in zeta potential using much smaller ssDNA strands. The lengths of ssDNA chosen were of 10, 25, 36, and 50 base, equivalent to 7.0, 17.5, 25.2, and 35.0 nm in length respectively if the DNA is fully extended\textsuperscript{18,19} and two concentrations were investigated.

![Figure 4.6](image)

Figure 4.6 - a) Mean zeta potential (mV) vs capture probe DNA base length and b) $1/T_{0.50}$ (ms) vs capture probe DNA base length where the turquoise bars represent a DNA concentration of 75 nM and the red bars represent a DNA concentration of 210 nM. A minimum of 200 particles were measured per sample and error bars represent standard deviation where n=2 independent replicates. Figure is adapted from Blundell et al\textsuperscript{16}.

The two concentrations studied were 75 and 210 nM, shown by the turquoise and red bars, respectively, and both concentrations showed the largest zeta potential value for a longer length of DNA (figure 4.6). As expected, a larger zeta potential value was observed for the particles hybridised with the longer ssDNA strands that also displayed an increase in particle velocity.
Figure 4.7 - Overlaid repeat datasets of zeta potential distributions of particles hybridised with a) 75 nM and b) 210 nM DNA of varying base lengths. In each sample, a minimum of 200 particles were measured.

At a 75 nM DNA concentration (figure 4.7a), the packing density will be much lower and therefore the flexible ssDNA has more space between each strand and can exist in what is known as its ‘condensed mushroom’ form\textsuperscript{20}. More space between each strand allows for a larger gyration effect\textsuperscript{20,21} that causes an increase in skewness amongst the zeta potential distribution. When the DNA strands are closely packed and of a high packing density, the skew is expected to be reduced. Some minor skewness may be present in the samples but this may be an inherent property of the streptavidin coated particles themselves not having a completely uniform protein coating, data of which can be seen in figure 4.7. For each concentration of DNA studied, the width of the zeta potential distribution increases as the DNA length is increased. This is due to the steric hindrance present with the longer pieces of DNA that may in fact prevent the high packing density around the particle’s surface based on their gyration radius that can block the binding of the DNA onto the particle surface. From this, it can be said that the shorter DNA strands are much more likely to produce a high packing density surrounding the particle, and thus these particles will have a more uniform distribution.
of DNA on their surfaces, resulting in a narrower distribution of zeta potential values. The symmetry of the distributions improved when a higher concentration of DNA was used and the spreads were seen to be more uniform. This is due to the high packing density surrounding the particle for each length of DNA at 210 nM, in comparison to the 75 nM DNA samples. The data was reproducible and this is demonstrated from overlaid charge distributions for two datasets using 75 and 210 nM DNA.

4.5.3 Detecting Target DNA Hybridisation

The technology effectively detected subtle differences in both DNA length and concentration, the next investigation entailed detecting a range of target DNA using the initial CP DNA and discriminating between ssDNA and dsDNA. The capture probe (CP) DNA was the same throughout and was always 25 bases in length. When added in excess of the particle binding capacity, any unbound CP DNA was removed before any target DNA was added in excess. It is important to note the melting temperatures \((T_m)\) of the DNA strands used in this study and to make sure the temperature of the lab did not exceed these temperatures whilst these experiments were being completed. Table 4.4 shows the melting temperatures of all of the DNA sequences used in this study.

<table>
<thead>
<tr>
<th>Name</th>
<th>Base Sequence</th>
<th>Melting Temperature, (T_m) (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Capture probe (CP)</td>
<td>5'ATGGTTAAACCTCACTACGCGTGGCG[Btn]3'</td>
<td>74.7</td>
</tr>
<tr>
<td>cDNA</td>
<td>5'GCCACGCGTAGTGAGGTTAACCAT3'</td>
<td>74.7</td>
</tr>
<tr>
<td>MidT</td>
<td>5'GTAGTGAGGT3'</td>
<td>37.3</td>
</tr>
<tr>
<td>EndT</td>
<td>5'GTTTAACCAT3'</td>
<td>33.3</td>
</tr>
<tr>
<td>OverT</td>
<td>5'GTGAGGTTAACCATTTTTTTTTTTTTTTTTTTTTTT3'</td>
<td>67.0</td>
</tr>
</tbody>
</table>

Table 4.4 - Summary of melting temperatures \((T_m)\) for each of the DNA sequences used in this study. All of the DNA hybridisations were completed in PBS (137 mM) at room temperature \((≤ 25° C)\).

The formation of double stranded DNA can be inferred as it produces a larger zeta potential than single stranded DNA in each target case, shown in figure 4.8.
Hybridising the target DNA with CP functionalised particles for 16 hours (zeta potential values represented by the green triangles) showed the largest relative change in zeta potential but these differences in zeta could be detected using hybridisation times of 30 minutes (red squares).

The relative changes in zeta potential values can be attributed to the difference in persistence length (a 50-fold increase from ssDNA to dsDNA)\textsuperscript{11,12}. The higher the persistence length, the larger the hydrodynamic radius of the particle, where two competing factors will then affect the surface charge density. One factor is the lengthening of the DNA when dsDNA is formed which will result in the phosphate groups being more spaced out and further away from the particle surface. Although this would result in a smaller charge density, this is countered by the addition of the second strand to make it double stranded that doubles the number of negative point charges. The more negative point charges, the faster the electrophoretic mobility resulting in increased particle velocities and thus larger zeta potential values.

\textbf{Figure 4.8 - Relative change in zeta potential (mV) from DNA capture probe, CP, at 100 \% capacity on the particle’s surface to when a variety of DNA targets are hybridised in excess for 30 minutes (red squares) and 16 hours (green triangles). Error bars represent standard deviation where n=3 independent replicates where a minimum of 400 particles were measured per sample. Figure adapted from Blundell et al\textsuperscript{16}.}
Previous TRPS work details the detection of target-probe DNA hybridisation that successfully discriminated between ‘probe’ and ‘target-probe’ hybridised particles completed by Booth et al.\textsuperscript{22}. However, in these experiments, the example DNA was a capture probe consisting of 23 bases with a target strand of 50 bases, extending far enough out into solution to be considered as predominantly ssDNA. Our examples include a target that does extend out into solution (overhanging target) as well as those that don’t and can be considered as predominantly dsDNA. A range of DNA targets were investigated that bound to various positions of the capture probe to demonstrate the sensitivity and reliability of a TRPS zeta potential measurement. The targets studied are defined in table 4.5.

<table>
<thead>
<tr>
<th>Target Name</th>
<th>Fully/Partially complementary</th>
<th>Binding Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>cDNA</td>
<td>Fully complementary</td>
<td>Binds entirety of CP</td>
</tr>
<tr>
<td>MidT</td>
<td>Partially complementary</td>
<td>Binds to middle 10 bases of CP</td>
</tr>
<tr>
<td>EndT</td>
<td>Partially complementary</td>
<td>Binds to end 10 bases of CP</td>
</tr>
<tr>
<td>OverT</td>
<td>Partially complementary</td>
<td>Binds to end 15 bases of CP and overhangs 15 bases into solution</td>
</tr>
</tbody>
</table>

Table 4.5 - Summary of DNA targets investigated in this study, all target DNA was at least partially complementary to the 25mer capture probe, CP.

The measured zeta potential values for each target are illustrated in figure 4.8. Any target DNA being added to the original capture probe resulted in a larger zeta potential value, be it middle binding, end binding, fully complementary, or an overhanging target. The relative change in the zeta potential values recorded were all larger when the target DNA was hybridised with the particles for an increased hybridisation time of 16 hours. Figure 4.9 again demonstrates simultaneous size and charge measurements that can be completed by TRPS to discriminate between ssDNA (capture probe 25mer, orange bars/data points) and dsDNA (capture probe 25mer + cDNA target, green bars/data points). The results showed similarities to the effects of
DNA concentration, there were no differences observed between the size of the particles, but there was a shift in the zeta potential distribution to a larger value when dsDNA was present on the particle’s surface. The effect was not as prominent as it was for the change in DNA concentration but still showed the detection of subtle differences in DNA-nanoparticle system based on DNA structure (ssDNA vs dsDNA).

![Figure 4.9 - Particle size and zeta potential distributions for streptavidin coated particles modified with ssDNA (25mer capture probe) and dsDNA (25mer capture probe + cDNA 25mer). The orange bars/data points and green bars/data points are representative of single stranded and double stranded DNA samples, respectively. A minimum of 200 particles were measured for each sample.](image)

The overhanging DNA target (OverT) always gave the largest negative zeta potential of all of the targets studied and was the largest in length. Although a larger length of DNA is expected to give a more negative zeta potential value, you may also encounter drag effects when the DNA extends further out into solution that may slow the particles down and be interpreted as exhibiting a smaller zeta potential. However, the results shown in figure 4.8 indicate that the increase in number of bases (an additional 30 to the original capture probe) contributes to the overall increased negative charge density surrounding the particle is a more dominant effect than the possible drag effects that
may be observed. Another suggestion that may attribute toward this result is the lower persistence length of the ssDNA portion extending out into the solution. The lower persistence length means a more flexible strand that may in fact coil back and around the capture probe, vastly increasing the charge density around the particle surface. The coiling effect causing the increase in surface charge density would result in an increase in electrophoretic velocity through a ‘hairy layer mechanism’ being created\textsuperscript{23}. Seeing as the overhanging DNA will be further from the particle surface, the distance between each DNA will increase, spatially allowing for more movement and more space to fold, although this effect could also be seen for the partially complementary middle binding target (MidT) it will be enhanced for the overhanging target as this results in the longest DNA length from the particle’s surface.

Of great interest is the ability of the measurement to distinguish between the same sized target (10 bases) being added but to a different position of the capture probe DNA. The end-binding target (EndT) recorded a smaller zeta potential value to the middle-binding target (MidT) even though they were both 10 bases in length and both forming the same proportion of double stranded DNA. This effect can be attributed again to the persistence lengths of single stranded DNA. The MidT binds to the middle of the capture probe and thus leaves some ssDNA extending out into solution; this is again more flexible than the dsDNA and could fold back on itself to increase the charge density around the particle’s surface\textsuperscript{23}, resulting in a larger zeta potential value. When the dsDNA is formed at the end of the capture probe sequence (EndT), it is hypothesised that the ability for the DNA to fold or coil back to the surface is restricted and the oligomer is more rigid across the entire length of the CP DNA, this would in effect move the dsDNA charge away from the particle’s surface and lower the surface charge density.

The ability to distinguish between such subtle differences in DNA binding will be of great use to the key design of future assays to be analysed on TRPS systems. The zeta potential distribution analysis was again useful to analyse the distribution of charge across the entire sample population. The charge distribution histograms gather insight and give valuable information on each individual particle’s zeta potential. The distribution shape changing is an indication of a difference in DNA binding mechanisms or the target analyte itself. Histograms from the range of target DNA are illustrated in figure 4.10.
The various target DNA was then hybridised to a CP DNA hybridised particle with different concentrations of CP DNA used on the particle surface, as well as investigating hybridisation times. The mean zeta potential values for a low (blue diamonds) and high (red squares) CP DNA concentration are illustrated in figure 4.11.

As well as the binding position of the partially complementary target DNA and the effect this has on particle transport, DNA binding kinetics is also interesting to study,
in particular target DNA hybridisation kinetics. There has been various work that has shown that the capture of target DNA is influenced by the CP DNA probe density at a surface\textsuperscript{24–27}. When the surface is densely packed with single stranded DNA, a dense packed polymer brush-like structure is formed. In this case the DNA forms a rigid polymer coating that has a measured thickness equivalent to the extended DNA length based on its sequence, \(H\textsuperscript{28}\). The electrostatic potential and shear plane position are thus determined by the packing density of the DNA, and thus the target DNA hybridisation kinetics. Polyelectrolyte layers introduce complications and the significance of zeta potential becomes more confounded. For example, when the Debye length, \(\kappa^{-1}\), is significantly lower than the polyelectrolyte thickness, \(H\), (when \(\kappa^{-1}/H<<1\)), the zeta potential may no longer be a direct reflection of the stern potential, as the plane of shear will be positioned much further away from the particle’s surface\textsuperscript{29}. The interest arises in the fact that when the surface is less densely packed with DNA, the plane of shear may in fact enter the DNA layer\textsuperscript{29} where the original definition of zeta potential may be lost.

The hybridisation time for target DNA was investigated at both 30 minutes and 16 hours, to which the samples containing particle surfaces densely packed with CP DNA
displayed a widening in zeta potential distribution as the hybridisation time increased. This is attributed to the target having more time to reach its desired orientation to successfully bind to the capture probe. More time is required for complete complementary target binding and when this is available there will be an increased proportion of double stranded DNA on the particle’s surface. An increase in the amount of dsDNA on the surface will result in a higher surface charge density, resulting in an increase in particle velocity and thus a larger zeta potential value recorded.

When a lower concentration of CP DNA was used with a hybridisation time of just 30 minutes, the charge distributions were shaped much narrower (figure 4.12a) than the target hybridised, for 30 minutes, to a sample with hybridised to a high concentration of CP DNA (figure 4.12b). There was also a significant reduction in the tailing effect, as illustrated in figure 4.12.

Previous work by Halperin et al. has shown that a lower CP DNA density results in a faster rate of reaction for the target DNA to bind\textsuperscript{30}, explaining the more narrow

---

**Figure 4.12** - Zeta potential distributions for a sample population at a) low CP concentrations (75 nM) and a hybridisation time of 30 minutes, b) high CP DNA concentration (250 nM) and a hybridisation time of 30 minutes, and c) high CP DNA concentration (250 nM) and a hybridisation time of 16 hours for the cDNA and OverT respective targets. Figure reproduced from Blundell et al\textsuperscript{16}. Each sample consisted of at least 400 measured particles.
distribution observed here. When the particle surface is densely populated with the capture probe, it is more difficult, and a smaller change is seen in zeta potential for low hybridisation reaction times (such as 30 minutes). Therefore, to optimise the assay, when using quicker assay times, a lower concentration of CP DNA should be used to create a less densely packed surface surrounding the particles.

The shape of the charge distribution was interesting between samples with a different concentration of CP DNA on the surface and for those with varied target DNA hybridisation times. The distributions displayed in figure 4.12a appear much more Gaussian that that of figures 4.12b and 4.12c based on the increased ease of ability to form dsDNA as there is less steric hindrance from other ssDNA that would be present on a highly packed surface of ssDNA, therefore the formation of this dsDNA is easily detected. The formation kinetics of dsDNA are increased when there the target DNA has more space or time to reach the desired orientation for successful complementary binding at a faster rate. The larger change in zeta potential seen for this sample may also be due to the lower net charge of the particle with less ssDNA on the surface (as described in section 4.4.1), in comparison to the particle hybridised to a much higher concentration of ssDNA. The target binding still occurs for the capture probe samples with a high CP DNA concentration using a hybridisation time of 16 hours. This example showed the biggest increase in zeta potential values recorded based on the increased amount of dsDNA now present on the particle surfaces in comparison to the limited amount of dsDNA that can be formed around the particles with a lower concentration of ssDNA to start with (its saturation point).

4.6 Conclusions

TRPS has demonstrated its ability to successfully detect and characterise both unmodified and DNA-modified particles in a real-time measurement simultaneously completing size and charge analysis of each sample. The data extracted from particle-by-particle analysis allows a new level of detail, using the charge distributions to analyse each component of a given sample population. This study has emphasised the sensitivity of TRPS technology being able to discriminate between varied DNA concentrations on a particle surface, different lengths of ssDNA, and a range of DNA targets to the same capture probe. The level of detail obtained from a TRPS measurement to discriminate between each DNA target will be of great use to future
nucleotide and colloid research as well as having implications on future aptamer assays. Immobilising DNA onto a particle surface alters the particle’s behaviour in an electrolyte solution that can be monitored using nanopore systems, the results from this study could have implications on future aptamer-based assays for biosensing medical applications. Target DNA can be captured in assay times of 30 minutes once the capture probe DNA concentration and hybridisation times had been optimised. Being able to capture target analytes in such a short incubation time will be incredibly useful within diagnostic and medical fields, particularly for point of care assays.

4.7 Acknowledgements

I would like to thank Dr Robert Vogel, Izon Science Ltd, for his collaboration in this project and his input and support with the interpretation and derivation of the zeta potential calculations.

4.8 References


5 Characterisation of Modified Nanoparticles, Particle Conjugates, and Bacteriophage using TRPS

5.1 Abstract

Finding a common sensor technology that does not require or rely upon PCR or lateral flow for the detection and characterisation of small particles, molecules and analytes is challenging. Nanoparticles can be functionalised with a biological component, be it DNA or a protein for example, to capture a target analyte and can aid in this challenge.

The work in this chapter develops two methodologies; The first involved using small particle conjugates, the concept was to use a larger particle that gives a large signal as a “tag” for a smaller particle/molecule inherently difficult to measure individually. Recording the changes in signal upon particle conjugation could overcome current challenges. In this concept an assay was developed to confirm the presence of proteins on a particle’s surface, and is different from the individual protein detection studies carried out prior to this. Here we aim to confirm the presence and then quantify their abundance. The detection, quantification and phenotyping of small biological particles would be of use to the microvesicle and exosome fields. By measuring the relative electrophoretic mobility of individual aptamer-modified nanoparticles, a range of protein targets can be selectively detected and quantified on a nanoparticle’s surface.

In the second methodology we look to further optimise the TRPS’ ability to characterise small particles directly. We test the system to study the detection of bacteriophages in buffer and culture medium. A rapid measurement of phage could be extremely beneficial for therapeutic fields, as well as targeted drug delivery systems.

5.2 Introduction

There are numerous applications to sub 150 nm particle analysis in fields including colloidal science, nanotoxicology, bionanotechnology, drug delivery. Altering a surface or nanoparticle’s surface chemistry, with proteins and/or DNA aptamers for example, is proving favourable for monitoring DNA-protein, DNA-DNA or DNA-small
molecule interactions providing insight into biological activity, as well as providing a powerful diagnostic platform\textsuperscript{4,7–15}. DNA aptamers are highly affinitive and specific to a target analyte that have been used in several particle-based assays in recent years\textsuperscript{12,16}. Functionalised nanoparticles are becoming more popular as analytical tools in biosensing technologies due to their versatility and scope for a range of target analytes. One emerging technology platform to incorporate such particles is the nanopore-based method, resistive pulse sensing, RPS\textsuperscript{17,18}.

Part of the study is to investigate controlled aggregation of two particles (one functionalised with a capture probe and one with a target) and observe a change in size and more specifically, charge when the aggregation process takes place. Preliminary results of this are detailed in this chapter (section 5.4.2).

One example, aside from exosomes, where this might be of use is for virus applications as therapeutics. We have chosen to use bacteriophage to do this because they have shown huge potential in the fight for antimicrobial resistance\textsuperscript{19,20}. They also offer an unusual “rod” shape, and are used in a range of media thus making their characterisation more challenging. A bacteriophage is a compilation of proteins that encapsulate a genome within a bacterium and are known to act like viruses in their infection and thus replication properties, and are also known for their antimicrobial properties that could be particularly useful in the medical industry. Most bacteriophage may need to be suspended in a complex biological medium that they need to be purified from for analysis and characterisation. TRPS is a beneficial technique that can overcome this difficulty by analysing samples in a range of biological media. TRPS has previously been used to complete successful measurements of nanoparticles suspended in plasma and serum\textsuperscript{21}, see chapter 7. Monitoring the behaviour of nanoparticles in these complex solutions has been widely investigated, in particular for study of protein corona formation\textsuperscript{22–25}. TRPS may therefore be deemed suitable to analyse bacteriophage in their respective biological environments. Quantifying and characterising phage samples in this way will exhibit valuable information surrounding their behaviours in solution. Quantifying phages in a sample that infect all hosts can be completed using flow cytometry\textsuperscript{26} and transmission electron microscopy (TEM)\textsuperscript{27,28} and phage concentration can be determined using plaque assays\textsuperscript{29}, for example. To count the number of phage that infect a specific host, an isolation approach is required\textsuperscript{30} although this may not give a true representation of the proportion of phages
present being isolated, as they may infect the host but not the model strain if the appropriate phage receptors are not present1.

5.3 Aims and Objectives

This chapter presents the use of TRPS to measure protein and DNA-modified nanoparticles, aggregation properties of particle conjugates, and a range of bacteriophage samples. The experiments detailed in this chapter were conducted to determine the versatility of TRPS measurements by analysing a range of samples in a variety of solutions/matrices. An aggregation assay was carried out to investigate the properties of a dual particle sample that could result in multiple aggregates types, each analysed using TRPS technology.

5.4 Materials and Methods

5.4.1 Chemicals and Reagents

5.4.1.1 Chemicals and Buffers

Phosphate buffered saline (PBS, 0.01 M phosphate buffer, 0.0027 M potassium chloride, 0.137 M sodium chloride, pH 7.4) and 2-(N-Morpholino)ethanesulfonic acid hydrate (MES hydrate, 0.10 M, pH 6, ≥99.5 %) were dissolved in deionised water (18 MΩ cm, TKA, Smart2Pure). PBS tablets (P4417) and MES hydrate (M2933) were purchased from Sigma Aldrich, UK. SM buffer (5.8 g NaCl, 2.0 g MgSO4.6H2O, 50 mL 1 M Tris-Cl (pH 7.5), 5 mL 2 % Gelatin) was made up to 1 L with deionised water and autoclaved.

EDC hydrochloride (N-(3-Dimethylaminopopyl)-N-ethylcarbodiimide hydrochloride, E6383) was also purchased from Sigma Aldrich, UK. EZ-Link™ Pentylamine-biotin (50 mM, 21345) coupling agent was purchased from Life Technologies, UK.

5.4.1.2 Particles and Particle Standards

Streptavidin coated superparamagnetic particles (120 nm, 4352 pmol/mg binding capacity, product 03121) and carboxylated superparamagnetic particles (120 nm, product 02120) were purchased from Ademtech, France. Carboxylated polystyrene particles with mean nominal diameters of 115 nm and 70 nm, CPC100 and CPC70 respectively, were purchased from Bangs Laboratories, US. CPC100s were used as calibration particles for sample zeta potential measurements, the zeta potentials of
CPC100s were determined using PALS analysis, a technique described in the appendices.

5.4.1.3 Proteins, Oligonucleotides, and Bacteriophage
Recombinant human vascular endothelial cell growth factor, VEGF (lyophilised, >95 %, PHC9394) was purchased from Thermo Scientific, UK. The custom DNA oligonucleotide, V7t1 biotin31 (5'TGTGGGGGTGGACGGGCCGGGTAGATTTTT[biotin]3') was purchased as a lyophilised powder (100 pmol/μL) from Sigma Aldrich, UK. The DNA was initially suspended in deionised water (18 MΩ cm resistivity, TKA, Smart2Pure) to a concentration of 100 μM. Bacteriophage samples were sourced by University of Leicester and were suspended in SM and BIH buffers.

5.4.2 Methods

5.4.2.1 Biotinylation of CPC70 Particles
Pentylamine biotin (25 mM), CPC70s (1 x 10^10 particles/mL), and EDC (1 mg/mL) were suspended in MES buffer (0.10 M, pH 6.0) and incubated at room temperature for 2 hours. The sample was then centrifuged at 13000 rpm for 5 minutes before resuspending the new formed pellet in PBS buffer (137 mM, pH 7.4). Prior to TRPS analysis, the biotinylated particles were vortexted for 30 seconds and sonicated for 1 minute.

5.4.2.2 Conjugation of Small Particles
Depending on the particle ratio of interest, the required amount of biotinylated CPC70 particles were added to the required amount of streptavidin coated particles and the sample was hybridised on a rotary wheel at room temperature for 10 minutes. The biotinylated CPC70s will be further termed as ‘bioCPCs’ and the streptavidin coated particles as ‘strept particles’. Table 5.1 describes the volumes and concentration of each particle required for the ratios studied.
### Table 5.1 - Required concentration and volume of each particle type involved in the conjugation assay.

<table>
<thead>
<tr>
<th>Ratio of bioCPC:strept particles</th>
<th>Concentration of bioCPC particles added (particles/mL)</th>
<th>Volume of bioCPC particles added (μL)</th>
<th>Concentration of strept particles added (particles/mL)</th>
<th>Volume of strept particles added (μL)</th>
<th>Total sample volume (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:0</td>
<td>1 x 10^{10}</td>
<td>500</td>
<td>N/A</td>
<td>0</td>
<td>500</td>
</tr>
<tr>
<td>0:1</td>
<td>N/A</td>
<td>0</td>
<td>1.1 x 10^{10}</td>
<td>500</td>
<td>500</td>
</tr>
<tr>
<td>1:1</td>
<td>1 x 10^{10}</td>
<td>250</td>
<td>1.1 x 10^{10}</td>
<td>250</td>
<td>500</td>
</tr>
<tr>
<td>10:1</td>
<td>1 x 10^{10}</td>
<td>250</td>
<td>1.1 x 10^{9}</td>
<td>250</td>
<td>500</td>
</tr>
</tbody>
</table>

**5.4.2.3 Protein and DNA Functionalisation of Base Particles**

Streptavidin coated superparamagnetic particles were incubated with the relevant biotinylated DNA aptamer via a 30 minute incubation (at room temperature) of 1 x 10^{10} particles/mL with the required amount of DNA for 100 % surface coverage on a rotary wheel. Carboxylated superparamagnetic particles were functionalised with VEGF protein via standard EDC chemistry. VEGF protein (50 nM), Carboxylated particles (1 x 10^{10} particles/mL), and EDC (1 mg/mL) were suspended in MES buffer (0.10 M, pH 6.0) and incubated at room temperature for 2 hours.

Excess DNA and protein was removed from the solutions via magnetic separation by placing the samples on a Magrack for approximately 30 minutes, forming a cluster of functionalised particles. The supernatant was then removed and replaced with fresh PBS buffer, taking care not to disturb the newly formed particle cluster. Each sample was vortexed for 30 seconds and sonicated for at least 1 minute prior to TRPS analysis.

Two assays were investigated in this study; assay 1 involves the aptamer functionalised particles with the addition of target protein into the sample solution, assay 2 used the protein functionalised particles with the addition of loose DNA aptamer added to the samples. The amount of protein and DNA added in the
respective assay was equivalent to what would result in 100 % surface coverage of the particles.

5.4.2.4 TRPS Analysis
All measurements were conducted using the qNano instrument (Izon Science Ltd, NZ) combining tunable nanopores (NP100, analysis size range of 40-320 nm as determined by the supplier) with proprietary data capture and analysis software, Izon Control Suite V3.1.2.53. A detailed method for the TRPS setup can be found in chapter 2.

Prior to TRPS analysis, all samples were vortexed for 30 seconds and sonicated for 1 minute. During each sample run, the system was washed by placing PBS (40 µL) into the upper fluid cell several times whilst applying various pressures to ensure there were no residual particles remaining and therefore no cross contamination between samples.

5.4.2.5 Zeta Potential Calibration using TRPS
Based on the size of the sample particles and molecules being analysed and calibration CPC100s and CPC200s (115 nm and 200 nm, respectively), the most ideal pore to use was an NP100 (optimal size range 40-320 nm). The calibration particles are measured at 3 applied voltages that are dependent on the applied stretch and consequent baseline current observed. Each applied voltage was run alongside an applied pressure (V1P1, V2P1, and V3P1) and the highest voltage of the calibration was run at an additional applied pressure also (V1P2), to assess the pressure stability of the samples. Each sample measurement was completed at a current of 100 ± 10 nA in accordance with the calibration runs for a particular NP100 pore. For the purpose of zeta potential, it was imperative that the stretch of the nanopore and the applied potential were not changed during a sample or calibration measurement of a particular dataset. The sample measurements were all completed at the highest or second highest voltage that the calibration measurements were carried out at. Calibration measurements were completed on each new day analysis was completed and when a new nanopore was introduced.
5.5 Results and Discussion

5.5.1 Streptavidin-biotin Control Conjugation Assay

A streptavidin-biotin interaction is known to be of high affinity\textsuperscript{32} and thus this interaction was chosen as a test case for controlled aggregation between two differently sized particles. In this case mean nominal diameters, given by the suppliers, were 120 nm (streptavidin coated particles) and 70 nm (biotinylated CPC70s). The ratios of each particle were closely monitored in attempts to control the number of biotinylated particles interacting with each streptavidin coated particle. Three cases were studied: a) biotinylated CPC70s in excess (10:1, bioCPC:strept), b) an even ratio of both particle types (1:1), and c) streptavidin coated particles only; the results of each ratio based on size and zeta potential were investigated. We initially chose to study the bioCPCs in excess relative to the strept particles (see figure 5.1c) as this might reflect a bioassay more closely, showing much smaller changes in volume that also helps study the sensitivity of TRPS technology.

Figure 5.1 is a schematic for the possible binding effects of the bioCPCs (blue) and streptavidin coated particles (red) as a result of incubation with each other at the ratios detailed in table 5.1.

![Figure 5.1](image)

The size and zeta potential results are summarised in figures 5.2 and 5.3, respectively, the streptavidin coated particles recorded a measured zeta potential value of -15 mV, which was reduced upon addition of biotinylated CPC70s. The largest effect on zeta potential was observed when the biotinylated CPC70s were added in excess, which
is expected as these samples contained the highest volume of biotinylated particles available to shield the negative charge of the streptavidin coated particles.

The streptavidin coated particles recorded a mean particle diameter of 118 nm amongst a given sample population when measured via TRPS. When equal concentrations of each particle type were added, the mean particle diameter increase to 307 nm, with a maximum recored particle size of 525 nm, a much larger change in size than when the biotinylated CPC70s were added in excess to the streptavidin coated particles (a recorded mean particle diameter of 160 nm). Although this change was not as large as the 1:1 ratio, there was still a change in the mean particle size by 42 nm, confirming the presence of the biotinylated CPC70s saturating the surface of the streptavidin coated particles.
Figure 5.3 - Mean zeta potential values for varied ratios of biotinylated and streptavidin coated particles. Measurements were completed using an NP100 pore and a minimum of 50 particles were measured for each sample.

When equal concentrations of each particle type were added i.e. 1:1, a reduction in zeta potential was again observed due to the gradual shielding of some of the negatively charged streptavidin coated particles. The magnitude of change in zeta potential was much smaller but this is due to the majority of the streptavidin coated particles negative surface charge still being exposed. Figure 5.4 shows a size vs zeta potential scatterplot illustrating the effects on size and zeta potential simultaneously for each of the particle ratios studied.
The red outlined squares indicate a sample solely containing streptavidin coated particles (strept), these particles recorded a tight size distribution amongst the given sample population. When the bioCPCs were added in excess (blue diamonds) the mean particle size shifted from 118 nm to circa 150 nm. This size change was typically expected when excess bioCPCs were present in the sample as upon aggregation there will be a change in electrolyte volume displaced as the aggregated particles pass through the pore and thus a different blockade signal observed. Both the strept particles and bioCPC particles are spherical in shape so the change in volume expected per aggregation of a single bioCPC particle is 22.8 % (table 5.2). Based on the surface area of the strept particles, it can be estimated that the surface will become saturated when directly aggregated to 6 bioCPC particles, an increase in volume of 137.1 %, equivalent to a particle diameter change to 153 nm.
No. conjugated biotinylated particles to a streptavidin coated particle | Expected percentage increase in volume (%)  
--- | ---  
1 | 22.8  
2 | 45.7  
3 | 68.5  
4 | 91.4  
5 | 114.2  
6 (maximum capacity expected when biotinylated particles are in excess) | 137.1  

Table 5.2 - Percentage volume change expected for each aggregated biotinylated CPC70 particle.

Of interest, is the result obtained from the equal ratio of both particles in a sample mixture; when the amount of each particle is the same in one sample, the particle size range is extended to between 150 and 500 nm. This change may be due to the clustering of the streptavidin coated particles around the limited number of biotinylated particles in the sample, causing much larger aggregates, described schematically in figure 5.1. Out of the ratios studied, the largest change in zeta potential was observed when the biotinylated, 70 nm particles were added in excess to saturate the streptavidin coated particle surfaces as it allowed for the complete shielding of the negative surface charge of the 115 nm particles. Although this ratio (10:1, bioCPC:Strept particles) was seen to give the largest change in zeta potential, the findings from the 1:1 ratio are still significant because even when the particles are seen to cluster and there are less biotinylated particles present, there is still a small change in zeta potential observed (circa 2 mV). After investigating the aggregation effects induced by a streptavidin-biotin interaction, the assay can be further developed to detect and monitor protein-DNA interactions for biologically relevant particles, for example.

5.5.2 Particle ‘tagging’ Experiment via Protein-Aptamer Interactions

Having shown the aggregation concept could work using the streptavidin and biotin interaction, the next stage was to functionalise small particles with proteins. The first
experiment carried out was to test the protein-aptamer interaction for VEGF protein. The 120 nm particles in this study were functionalised with the anti-VEGF aptamer, V7t1 to 100 % surface coverage (green dataset). Upon addition of excess VEGF target protein, a reduction in particle velocity and thus smaller zeta potential (purple dataset), was recorded. Figure 5.5 illustrates that change in zeta potential distribution amongst given sample population.

To confirm the specificity of the V7t1 aptamer, a random aptamer of similar base length was also functionalised to a sample of streptavidin coated particles and the VEGF target added in excess. Table 5.3 details the particle translocation velocities and zeta potentials for particles functionalised both with the random and V7t1 aptamer, both particles exhibiting similar translocation and zeta potential properties. Table 5.3, also shows the particle velocities of both aptamer functionalised particles when incubated with the VEGF protein target. The particles functionalised with the random aptamer showed little change in particle velocity when VEGF was added (0.68 /ms), in comparison to the V7t1 aptamer functionalised particles that showed a larger change in particle velocity when VEGF was added (3.60 /ms).
<table>
<thead>
<tr>
<th>Particle Functionalisation</th>
<th>Average Particle Velocity, $1/T_{0.50}$ (/ms) positioned halfway through the pore</th>
<th>Mean Measured Zeta Potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Random Aptamer</td>
<td>7.58 ± 0.15</td>
<td>-18.27 ± 0.48</td>
</tr>
<tr>
<td>Random Aptamer + VEGF</td>
<td>6.90 ± 0.23</td>
<td>-17.17 ± 0.61</td>
</tr>
<tr>
<td>V7t1 Aptamer</td>
<td>7.41 ± 0.09</td>
<td>-19.27 ± 0.15</td>
</tr>
<tr>
<td>V7t1 Aptamer + VEGF</td>
<td>3.81 ± 0.39</td>
<td>-13.50 ± 0.64</td>
</tr>
</tbody>
</table>

Table 5.3 – Particle velocity (/ms) and mean zeta potential (mV) values for particles functionalised with a random aptamer, specific V7t1 aptamer and their responses with the addition of VEGF protein. Standard deviations were taken of a minimum of 500 particles per sample where $n=3$.

The velocities can be transferred to zeta potentials and the particles functionalised to the non-specific aptamer showed negligible change in the mean zeta potential (1.10 mV) when VEGF was added, the small change could be attributed to non-specific binding to the particle surface. The particles functionalised with the specific V7t1 aptamer however, showed a smaller mean zeta potential value when the VEGF target was added (-13.50 mV, a change of 5.77 mV from the V7t1 aptamer functionalised particle). This indicates successful protein binding to the aptamer that creates shielding of the negative surface charge exhibited from the DNA aptamers on the particle surface. Table 5.4 includes the mean particle diameters of those functionalised to the random and VEGF specific aptamers with/without the addition of VEGF protein.

<table>
<thead>
<tr>
<th>V7t1 Aptamer</th>
<th>V7t1 Aptamer (1)</th>
<th>V7t1 Aptamer (2)</th>
<th>V7t1 Aptamer + VEGF</th>
<th>V7t1 Aptamer + VEGF (1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>163</td>
<td>168</td>
<td>159</td>
<td>190</td>
<td>200</td>
</tr>
<tr>
<td>Random Aptamer</td>
<td>Random Aptamer (1)</td>
<td>Random Aptamer (2)</td>
<td>Random Aptamer + VEGF</td>
<td>Random Aptamer + VEGF (1)</td>
</tr>
<tr>
<td>177</td>
<td>176</td>
<td>179</td>
<td>177</td>
<td>176</td>
</tr>
</tbody>
</table>

Table 5.4 - Summary of mean particle diameters (nm) for particles functionalised with both the specific and non-specific DNA aptamers as well as those samples with the addition of VEGF protein. Each sample contained a minimum of 500 particles.
The results showed that a mean size change (an increase of approximately 32 nm) was only observed for the V7t1 aptamer particles, and not those functionalised with a non-specific aptamer.

Upon addition of the target protein to the VEGF aptamer-modified particles, an inherent size change is expected. Although a change in the mean particle diameter is evident (see table 5.4), the changes in size amongst the sample population may not always be the case, see figure 5.6.

![Figure 5.6 – Zeta potential (mV) vs particle diameter (nm) for particles functionalised with the specific DNA aptamer (green squares) and those upon addition of VEGF protein (purple circles). It should be noted that each data point represents a single particle amongst a given sample population and a minimum of 250 particles were measured in each sample.](image)

Next the assay was inverted, i.e. the protein placed on the particles surface and DNA/aptamer was present in solution. To investigate this, the 115 nm carboxylated particles were functionalised with VEGF protein and loose V7t1 aptamer was then added to the samples to monitor successful protein-DNA binding.

Table 5.5 shows the values recorded for VEGF functionalised onto the 115 nm carboxylated particles, the functionalisation was confirmed as successful and was shown by a reduction in particle velocity and thus smaller zeta potential value. This is
due to the negative carboxyl groups on the particle surface being shielded by the positive VEGF protein at pH 7.4 (due to its pI of 8.1). Once the protein was present on the particle’s surface, loose DNA aptamer (V7t1) was added to the sample.

<table>
<thead>
<tr>
<th>Particle Functionalisation</th>
<th>Particle Velocity halfway through the pore (/ms)</th>
<th>Mean Measured Zeta Potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carboxyl</td>
<td>11.55 ± 0.22</td>
<td>-22.63 ± 0.91</td>
</tr>
<tr>
<td>VEGF</td>
<td>8.78 ± 0.23</td>
<td>-11.87 ± 0.48</td>
</tr>
<tr>
<td>VEGF + V7t1 Aptamer</td>
<td>9.47 ± 0.04</td>
<td>-14.85 ± 0.85</td>
</tr>
</tbody>
</table>

Table 5.5 - Particle translocation velocity when the particle is positioned 50% of the way through the pore (/ms) and Mean zeta potential (mV) for carboxylated base particle, those functionalised with VEGF protein, and VEGF functionalised particles upon addition of loose V7t1 aptamer. Standard deviation was taken of a minimum of 500 particles per sample where n=3 independent replicates.

After the aptamer was added to the sample, the mean particle zeta potential became more negative that was indicated by the increase in particle velocity. Table 5.6 shows a summary of mean zeta potential values from table 5.3 and 5.5

<table>
<thead>
<tr>
<th>Assay 1</th>
<th>Assay 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Streptavidin Coated Base Particle (Particle 1)</td>
<td>Particle 1 functionalised with V7t1 DNA Aptamer (Particle 1A)</td>
</tr>
<tr>
<td>-13.48 ± 0.72</td>
<td>-19.27 ± 0.15</td>
</tr>
</tbody>
</table>

Table 5.6 - Summary of mean zeta potential values (mV) for both assays investigated in this study. Standard deviation was taken of a minimum of 500 particles per sample where n=3 independent replicates.

Table 5.6 shows that the addition of loose DNA strands (V7t1 aptamer) to a VEGF-modified particle has a smaller effect on the mean measured zeta potential (2.98 mV) in comparison to the addition of VEGF protein to the aptamer-modified particle (5.77 mV). The change in zeta potential was larger when the protein target was added to the aptamer functionalised particles rather than the addition of loose DNA aptamer to
protein functionalised particles. This may be due to the addition of counterions surrounding each particle upon the aptamer binding to the protein on the particle’s surface. More counterions may be present to stabilise the single-stranded DNA bound to the protein target, which would reduce the particle velocities and thus zeta potential values and not give a true representation of the addition of negative DNA strands.

Another parameter that needs to be investigated as further work is the variation of the aptamer and protein surface coverage on the base particle. Varying the packing density below 100 % of a particle’s surface may give an indication on the binding mechanisms of the protein/DNA target to the relative particle modification. The next stage would be to mimic the aggregation assay and use aptamer modified particles to bind to protein covered beads. The combination of a change in size and velocity would hopefully allow the particles carrying the VEGF protein to be identified, and then the magnitude of the size and velocity change could be used to quantify the number of proteins on the surface.

5.5.3 Phage Analysis

Research of small molecules including biologically relevant particles/molecules has become increasingly popular in recent decades, including those in their respective mediums. A prime example of this is the analysis of bacteriophages. Bacteriophages are viruses that infect bacteria and analysis of such is advantageous in understanding microbial systems, figure 5.7 illustrates the shape and structure of bacteriophage molecules.

![Figure 5.7 - Schematic representation of a bacteriophage molecule, showing its shape and capsid/tail components.](image)

The majority of bacteriophages are within the size range of 24 to 200 nm in length including a range of phage types. Some of the phages investigated in this study had
similar structures of icosahedral shaped heads with a subsequent tail. Those studied include coliphage which infect *E. coli*, *Salmonella* phage, and *Clostridium difficile* (*C. difficile*) phage. *C. difficile* phages have gained interest in recent decades for their uses in therapeutics based on their highly specific interactions with their biological hosts. In these experiments, a range of bacteriophages were analysed, table 5.7 details the size parameters expected of those studied.

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>Buffer</th>
<th>Tail Length, $P_t$ (nm)</th>
<th>Capsid Diameter, $P_c$ (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salmonella</td>
<td>SM</td>
<td>180</td>
<td>100</td>
</tr>
<tr>
<td>Coliphage</td>
<td>SM</td>
<td>100 (15 nm diameter)</td>
<td>70</td>
</tr>
<tr>
<td>CDHM1</td>
<td>BIH</td>
<td>110 (20 nm diameter)</td>
<td>60</td>
</tr>
<tr>
<td>CDHM6</td>
<td>BIH</td>
<td>230 (20 nm diameter)</td>
<td>50</td>
</tr>
</tbody>
</table>

Table 5.7 - Provided details of bacteriophage samples (based on their type) used in this study including capsid diameter, tail length and buffer information.

Each sample was run in its respective buffer, the buffer in which the phage samples were prepared, as well as the calibration particles required for the zeta potential measurement calibration. The zeta potential was measured for the samples in SM buffer, as the molarity and composition of this buffer was similar to that of PBS that would allow for the zeta potential to be calculated; BIH buffer however, was not compatible so only size and particle velocity data was gathered.

Figure 5.7 shows the mean (blue) and modal (red) particle diameters for two different types of the *C. difficile* phage (CDHM1 and CDHM6). The modal diameters were more similar (142.75 nm and 132 nm for CDHM1 and 6, respectively) than the mean diameters (176.75 nm and 151.25 nm for CDHM1 and 6, respectively), suggesting there are larger phage molecules or a higher number of aggregates present in the CDHM1 sample, more information on the range of measured particle/molecule sizes are detailed in table 5.8 and figure 5.8.
Table 5.8 - Summary of measured phage sizes for CDHM1 and CDHM6 (mean and mode) amongst a sample population. Standard deviation is representative of n=4 independent replicates all containing a minimum of 200 measured particles.

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>Buffer</th>
<th>Mean Measured Size (nm)</th>
<th>Mode Measured Size (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDHM1</td>
<td>BIH</td>
<td>176.75 ± 3.70</td>
<td>140.75 ± 6.90</td>
</tr>
<tr>
<td>CDHM6</td>
<td>BIH</td>
<td>151.25 ± 4.65</td>
<td>132 ± 3.31</td>
</tr>
</tbody>
</table>

Figure 5.8 - Mean (blue dataset) and mode (red dataset) particle diameters (nm) for CDHM1 and CDHM6 phage samples. Error bar are representative of the standard deviation of a minimum of 200 measured particles where n=4 independent replicates.

Although both phages were of *Clostridium difficile* domain, the two different types exhibited different properties when analysed using TRPS. The size distributions in figure 5.9 show that although the size range of both phage samples were a similar spread, there were the presence of larger molecules or possible aggregates in the CDHM1 sample (purple dataset).
Salmonella and coliphage samples were also analysed and compared as both were suspended in SM buffer. The mean and modal diameters of the phage, as well as zeta potentials were measured by TRPS, results of which are detailed in table 5.9.

Table 5.9 – Summary of size and zeta potential data for salmonella phage samples and coliphage samples. Standard deviation is representative of n=3 independent replicates. All samples contained a minimum of 200 particles.

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>Buffer</th>
<th>Mean Measured Size (nm)</th>
<th>Mode Measured Size (nm)</th>
<th>Measured Zeta Potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salmonella phage</td>
<td>SM</td>
<td>101.5 ± 0.5</td>
<td>96 ± 4</td>
<td>4.3</td>
</tr>
<tr>
<td>Coliphage</td>
<td>SM</td>
<td>88 ± 2.94</td>
<td>87 ± 5.72</td>
<td>8.25 ± 0.85</td>
</tr>
</tbody>
</table>

Figure 5.10 shows the Salmonella based phage to be 13 nm larger than the coliphage, smaller than the expected difference in tail length and capsid diameter (table 5.7).
Size and zeta potential distributions amongst a sample population allows for more detailed analysis of a given sample. TRPS analyses particles and molecules individually and each blockade event represents a single particle as it traverses the pore that are translated into each data point shown in figure 5.11. Both bacteriophage studied varied in size and zeta potential distribution shape. The vast majority of entities in each sample were encompassed in a similar size range except for some tailing for the *Salmonella* phage indicating the presence of larger particulates or aggregates in this sample.
Figure 5.11 - Zeta potential (mV) vs particle size (nm) for Salmonella and coliphage samples represented by the green triangles and pink squares, respectively. It should be noted that each data point indicates a single particle amongst a given sample population. All samples contained a minimum of 250 particles.

One of the advantages of TRPS analysis is the ability to complete simultaneous size, concentration, and charge measurements. Techniques that only complete size measurements may struggle to differentiate between these two particular phage samples based on them both containing phage in an overall similar size range. Figure 5.11 however, shows the difference in zeta potential distribution shape between the samples. The Salmonella sample contained phage with a much narrower zeta potential range but the size distribution was wider in shape. This may be attributed to this particular bacteriophage having a lower tendency to agglomerate in solution.

Size and charge properties are important in bacteriophage analysis to gather an understanding on their behaviour in their respective environments. Further to this, additional information such as pore translocation effects including orientation and shape will also impact on their behaviour in solution. TRPS is useful for this development as the translocation effects are monitored by the blockade event produced as particles traverse the pore. The blockade shape may indicate the orientation, direction and transport mechanism of particles as they pass through the pore. Figures 5.12a and 5.12b show the peak shape of blockades produced from calibration particles (200 nm carboxylated polystyrene) and Salmonella phage, respectively.
Figure 5.12 - Examples of blockade size and shape with respective enlarged sections for a) carboxylated polystyrene calibration particles and b) a Salmonella phage sample.

The calibration particles produce a single, narrow blockade conventional to TRPS measurements. The initial drop in current is at the point at which the particle enters the pore and the tailing effect back to the baseline current is observed as the particle traverses and exits the pore. The blockade produced from a phage sample showed different characteristics and resulted in what can be considered a non-conventional peak. It should also be noted of the size variation of various particulates present in the phage sample (see figure 5.12b), also shown by the green triangles in figure 5.11. When the phage enters the pore, the reduction in current is observed as the phage displaces the electrolyte; however, this is not a ‘clean’ entry as observed with the calibration particles. This blockade shape suggests the phage is translocating the pore via a possible ‘tumbling’ mechanism pore, a result of the range of zeta potentials amongst the sample, which could be caused from variable head and tail components of the phage. Figure 5.11 has shown there are a range of positive, negative and neutral
particulates in the phage samples; a positive or neutral particle will traverse the pore in the opposite direction and produce a peak opposite to that of the negative calibration particle, attributes of which observed in figure 5.12b. The blockade produced by the phage can also be interpreted as containing multiple peaks around the pore entry that can be interpreted as determining the orientation of the phage as they traverse the pore. As mentioned previously, a phage molecule consists of a capsid head and a tail so the difference in charge of both parts could result in the phage traversing the pore in a more complicated way than a straight forward spherical particle. Previous work has been carried out on gold nanorods that show a similar blockade shape to figure 5.12b being exhibited as the rods traverse the pore, the preceding small peak before the full blockade event (see enlarged section of figure 5.12b) could be an indication of the bacteriophage entering the pore ‘side-on’ and then tumbling to a ‘head/tail-on’ orientation.

Further work is required to monitor the behaviour of the phage as they traverse small pores to possibly monitor whether the blockade output is influenced by whether the phage enters the pore head or tail first. It is apparent that the mode of transport of the phage molecules is more complicated than that of a spherical particle (such as the calibration particles used in this study). The shape and direction of the blockade produced can thus be used to reflect the orientation and direction of the phage molecules as they enter and pass through the pore, however further work is required to further analyse the transport mechanism of bacteriophages through pores.

5.6 Conclusions and Further Work

Tunable resistive pulse sensing (TRPS) has proven useful in detecting particle conjugates in complex sample mixtures as well as bacteriophage samples in their respective media. The technique can simultaneously complete size and charge measurements, allowing for particle agglomeration to be monitored with ease, even to the extent of aggregates of 4 times the size of the original particles. Interestingly, when one particle is incubated in excess of another, a notable saturation of a particle surface is detected by the vast change in zeta potential as the change in particle translocation velocity is largely affected by a change in particle surface charge.

As well as detection and characterisation of particle-particle conjugates via protein-protein interactions, the assay has been developed to monitor DNA aptamer-protein
interactions within a particle conjugation setting. Preliminary results have found that particle velocities as they traverse a pore and thus zeta potential are affected by both DNA and protein particle surface modifications. Of interest was the level of sensitivity of TRPS that could also monitor the addition of loose protein and DNA to their respective target-functionalised particles. The effect was larger for the addition of protein to aptamer modified particles and can be attributed to the binding mechanism of the protein to the aptamers on the particle surface. This may be different to the aptamers binding to proteins already functionalised onto a particle’s surface. Further work is required to fully interpret and characterise these differences in binding. A further development of this study is to monitor agglomeration effects of both DNA aptamer and protein modified nanoparticles in a sample mixture. The aptamer-protein based aggregation assay will give valuable insight into the behaviour of biologically relevant particles in solution as well as further information on DNA-protein interactions. Further work also includes using a ‘scrambled’ anti-VEGF aptamer as well as the random aptamer example used in this study to test the protein-aptamer binding. This will determine the sequence specificity of the anti-VEGF aptamer.

Bacteriophage are complex in composition as they are nanoscale entities made up of a capsid ‘head’ and subsequent ‘tail’. TRPS is a pore-based technique where the zeta potential and translocation events have been monitored by the relative blockade event produced as the phage traverse the pore. A ‘double-peak’ is observed as some bacteriophage samples translocate the pore and is attributed to a possible tumbling effect caused from the oppositely charged head/tail of each phage. Further work is required to prove this is the case by looking into controlling the direction the phage passing through the pore system. This will enable individual analysis of both the capsid heads and subsequent tails of each phage that will allow for more information on bacteriophage samples and their behaviour in their respective media.

5.7 Acknowledgements

I would like to thank Dr Martha Clokie for the phage samples analysed in this study.
5.8 References


6 Protein Detection using Tunable Pores

6.1 Abstract

This chapter focuses on developing and comparing a tunable pore platform to detect and analyse protein-based assays using resistive pulses and rectification ratios. Both methods will be investigated via the quantification of a biomarker, Vascular Endothelial Growth Factor (VEGF), a protein of interest in the cancer therapeutics field. The first of the two assays incorporates aptamer-modified nanoparticles, measuring their translocation speeds through a conical pore. By controlling the aptamer packing density on a particle’s surface and measuring each translocation event, a change in velocity can be observed for protein concentrations as low as 18 pM, equating to circa 10 proteins per particle. The second exploits current rectification properties of conical pores abolishing the need for particles or other “tagging” elements to the respective target. This assay approach encompasses the first reported Layer-by-Layer (LbL) assembly of polyelectrolytes directly onto the surface of a polyurethane pore. The current rectification ratios confirm the presence of polymers that each produce pH and ionic strength dependent currents. A Layer-by-Layer assembly allows for the simple immobilisation of DNA aptamers onto the pore surface that demonstrate a specific response to the VEGF target. The detection limits for the DNA-modified pore assay are 5 pM for VEGF and both this setup and the nanoparticle-assay offer individual advantages in their ease in preparation and purpose but differ in their levels of sensitivity. Despite the comparable sensitivity, the particle assay offers a larger dynamic range. The scope and ease of each assay format allows for a versatile technology that can be tailored to suit target analytes.

6.2 Introduction

Nanoscale elements within synthetic materials have grown in interest over the last two decades\textsuperscript{1–3} having applications in biosensing\textsuperscript{4,5}, material characterisation\textsuperscript{6,7}, quantification of target-ligand interactions\textsuperscript{8–10}, and drug delivery\textsuperscript{11}. Nanoscale channels have also been used to mimic biological systems that enables the study of ionic transport within confined geometries\textsuperscript{12–15}. Many synthetic nanopores have been created in numerous materials including graphene\textsuperscript{16–19}, polymers\textsuperscript{20}, silicon nitride\textsuperscript{21}, and glass\textsuperscript{12,13,22,23}. Ion transport and analyte translocation through the channels can
be controlled by tuning the applied potential, pore wall charge, pore size, as well as the supporting electrolyte concentration and composition. A further degree of selectivity can be implemented by modifying the pore walls with selective materials and ligands\textsuperscript{24–26}.

The translocation speed and frequency of materials, such as small molecules, proteins, or nanoparticles through pores are also governed by size and charge\textsuperscript{5,27–30}. Nanopore sensing systems can be separated into two different categories: Resistive Pulse Sensing (RPS) where an analyte translocation event creates a characteristic change in resistivity within the pore, and Current Rectification studies that monitor current-voltage, \textit{I-V}, responses dominated by the charges on the pore wall. The flux of material through the pore is a function of the small and large pore geometries, \(D_s\) and \(D_l\), respectively, pore length, \(L\), and analyte charge. RPS has been used to measure a range of analytes from single molecules, DNA, proteins and cellular vesicles, to cells, bacteria and viruses by controlling the aspect ratio of the pore\textsuperscript{5,27,30}.

Tunable pores, such as synthetic polyurethane pores, allow for manipulation (stretching) in real time to suit a given sample\textsuperscript{31} and have been incorporated into RPS systems for the creation of tunable resistive pulse sensing (TRPS)\textsuperscript{31,32}. The pores are conical in shape and in this study, using an NP200, have a typical opening diameter, \(D_s\) of > 700 nm. The flexibility and versatility of the pores allows for both particle translocation effects and current rectification ratios to be monitored, widening the scope of the technology.

### 6.3 Aims and Objectives

The main objective from this study was to determine the detection limits for both a particle-based assay and current rectification pore-based approach for the same target. Both approaches were focused on aptamer-protein interactions and sensitivities investigated for each. Experiments were also completed to investigate the effects of pore modification with selected polymers and the effect this has on the pore as a current rectifier. These current rectification studies will allow for the development of label-free assays for analyte detection.
6.4 Materials and Methods

6.4.1 Chemicals and Reagents

6.4.1.1 Chemicals, Buffers, and Polymer Coatings
Poly(ethyleneimine), PEI, low molecular weight (LMW, Mw ~ 2000 g mol⁻¹, 50 % wt., 408700) and analytical standard high molecular weight (HMW, Mw ~ 750 000 g mol⁻¹, 50 % wt., P3143); Poly(acrylic acid-co-maleic acid), PAAMA (Mw ~ 3000 g mol⁻¹, 50% wt., 416053); Phosphate buffered saline, PBS (0.01 M phosphate buffer, 0.0027 M potassium chloride, 0.137 M sodium chloride, pH 7.4), and 2-(N-Morpholino)ethanesulfonic acid hydrate, MES hydrate (≥99.5 %, M2933) were purchased from Sigma Aldrich, UK. Potassium chloride, KCl (>99 %, P/4240/60) and potassium hydroxide, KOH (0.1 M, >85 %, P/5600/60) were purchased from Fisher Scientific, UK. Hydrochloric acid, HCl (0.5 M, 37 %) was purchased from VWR, UK. 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, EDC (22980) was purchased from Thermo Scientific, UK. HCl and KOH were used to alter the pH of solutions that were measured using a Mettler Toledo easy five pH meter with a Mettler Toledo InLab micro electrode.

6.4.1.2 Particles and Nanopores
Tunable conical polyurethane pores (NP200) were purchased from Izon Science Ltd, NZ. Carboxylated polystyrene particles with a mean nominal diameter of 220 nm (CPC200) were purchased from Bangs Laboratories, US. Streptavidin coated superparamagnetic particles (120 nm, 4352 pmol/mg binding capacity, 03121) were purchased from Ademtech, France. All stock particles were vortexed for 30 seconds followed by a 2-minute sonication before dilution or further analysis to ensure monodispersity.

6.4.1.3 Proteins, DNA, and Biomarkers
Fibrinogen from human plasma (≥80 %, F3879), albumin from human serum (lyophilised powder, ≥97 %, A9511), γ-globulin from human blood (≥99 %, G4386), and bovine serum albumin (BSA, lyophilised powder, ≥96 %, A2153) were purchased from Sigma Aldrich, UK. Recombinant human vascular endothelial cell growth factor, VEGF (lyophilised, >95 %, PHC9394) was purchased from Thermo Scientific, UK. The custom DNA oligonucleotide, V7t1 amine
(5’TGTGGGGGTGGACGGGCCGGGTAGATTTTT, the sequence was synthesised with a biotin or amine functional group at the 3’ end) was purchased as a lyophilised powder (100 pmol/µL) from Sigma Aldrich, UK.

All reagents were used without further purification and all solutions were prepared using purified water with a resistance of 18 MΩ cm (TKA, Smart2Pure).

6.4.2 Methods

6.4.2.1 Particle Assay

Streptavidin coated particles (120 nm diameter) were diluted to a final concentration of approximately 5 x 10⁹ particles/mL in PBS. The diluted solutions were then vortexed for 30 seconds and sonicated for 2 minutes to ensure they are well dispersed. The biotinylated DNA aptamer was added to the streptavidin coated particles (4352 pmol/mg binding capacity, as determined by the supplier) at 113 and 226 nM for 50 and 100 % DNA coverage per particle, respectively. The samples were then placed on a rotary wheel to incubate at room temperature for 30 minutes. Any unbound DNA remaining in solution was then removed via magnetic separation by placing the samples onto a Magrack (GE Healthcare, UK) for 30 minutes. The supernatant was then removed and replaced with new PBS buffer. The VEGF was added to the respective samples at the required concentrations and then placed on a rotary wheel at room temperature for 30 minutes before being analysed.

6.4.2.2 Pore Modification

The conical polyurethane pores were modified by incubating the pore in the polymer (PEI and PAAMA) solutions (5 % wt. in deionised water, 18 MΩ cm) at a stretch of 45 mm for 2 hours, ensuring the intended pore side was covered with the polymer solutions. Once incubated, the pores were rinsed with deionised water. The pores were then incubated with the second polymer layer at 45 mm stretch for 2 hours before again being rinsed with deionised water. This process was repeated until the required number of layers was present.

To immobilise DNA onto the PAAMA modified pore surface, the aptamer was added to solution of 1 mg mL⁻¹ EDC in 100 mM MES buffer (pH 5.9). The final concentration of the DNA was 220 nM. The pores were incubated with the DNA/EDC solution for 2 hours.
6.4.2.3 Current-Voltage (I-V) Measurements

The pores, both modified and unmodified, were mounted laterally between two fluid cells (upper and lower, see chapter 2) containing an electrolyte solution. Current-Voltage (I-V) curves were recorded using the Izon Control Suite proprietary software v.3.2. The potential was stepped in 100 mV increments from +1.6 to -1.6 V and the resulting current measured at each potential. The current rectification properties were measured in a range of electrolyte solutions, including KCl solutions ranging from 5 to 50 mM. When a BSA control was used, 50 nM BSA was incubated with the DNA-modified pore for 30 minutes, with the current rectification properties being measured in equivalent KCl solutions as with the VEGF samples.

6.4.2.4 VEGF I-V Assay

VEGF was suspended in PBS buffer to give the desired final concentration. DNA-modified pores were incubated with the VEGF solution where the solution was placed solely on the side of the pore with the small pore opening, $D_s$ for 30 minutes. When multiple VEGF solutions of varied concentrations were used, the lowest VEGF concentrations were measured first, followed by the subsequent higher concentrations. The pore was rinsed with water in triplicate and with PBS in triplicate after each protein concentration was incubated but prior to the I-V measurements using TRPS in the previously mentioned range of electrolyte solutions. A BSA control was used at a 50 nM concentration and incubated with the aptamer-modified pore for 30 minutes prior to I-V measurements (using KCl electrolyte) and was completed before adding VEGF to the pore.

6.4.2.5 TRPS Analysis

All TRPS measurements were carried out on the qNano instrument (Izon Science Ltd, NZ) using an NP200 nanopore (analysis size range of 80-640 nm) alongside Izon Control Suite data capture and analysis software v.3.2. A detailed method for the TRPS setup can be found in chapter 2. The pore diameters were measured using scanning electron microscopy, SEM, and were calculated from the current at a 45 mm stretch. Most experiments in this study were run at 45 mm and the applied stretch is quantified by the distance between the jaws, also described in chapter 2.

The velocity of the particles was calculated using the properties of each blockade event, or pulse, including the pulse width and duration. Blockade duration events are
recorded from the peak \((dR_{\text{max}})\) of the blockade back to the baseline current; the total time this takes gives the blockade duration. A full description of the particle velocity calculations can be found in chapter 3 and explains the use of several blockade reference points, including \(T_{0.50}\), where \(1/T_{0.50}\) is used to represent the relative particle velocity.

### 6.5 Results and Discussion

#### 6.5.1 Particle-Based Assay using Resistive Pulses

In various resistive pulse sensing strategies, sample handling and assay speed can be facilitated using nanomaterials through either; the immobilisation of a target analyte onto nanomaterial surfaces to encourage analyte induced aggregation\(^{34,35}\), or measuring and monitoring nanoparticle translocation speeds/frequencies upon successful binding of a target analyte\(^{10,30,36-39}\), as shown in chapter 5. The charge of particles is a major contributing factor to pore translocation speed and frequency, and the use of DNA-modified materials/pores is vastly becoming increasingly popular\(^{10,30,40-42}\).

The particle-based assay utilised 120 nm streptavidin coated particles that were modified with a biotinylated VEGF aptamer\(^{33}\) at two different percentages of surface coverage. The first of the two experiments consisted of the streptavidin binding sites being saturated with the VEGF aptamer (termed FC, full coverage, in figures 6.1 and 6.2); and the second consists of half the binding sites per particle, as per the supplier’s specification, being used (50 % aptamer coverage, termed HC, half coverage, in figures 6.1 and 6.2). The translocation speeds can also be used to confirm the aptamer surface coverage of the streptavidin coated particles, i.e. a particle with a lower percentage aptamer coverage will show a slower translocation speed than one with 100 % aptamer coverage\(^{28}\). Figure 6.1 shows the particle velocities for streptavidin coated particles functionalised with 50 % (blue dataset) and 100 % (red dataset) aptamer coverage, the trend correlating with data described in chapter 5. The average particle velocities are denoted as \(1/T_{0.50}\) were measured relative to when the particle was positioned 50 % of the way through the pore (see chapter 3 for more details).
Figure 6.1 - Particle velocity when positioned halfway through the pore \((1/T_{0.50})\) for particles functionalised with VEGF aptamer for half coverage (HC) and full coverage (FC). Error bars represent standard deviation when \(n=3\) independent replicates. A minimum of 500 particles were measured per sample.

The change in translocation velocity is relative to a blank of an aptamer-modified particle that was not incubated with the target protein, results of which are depicted in figure 6.2. Previous studies have described how translocation velocity can be measured from each blockade event, or pulse, and converted into zeta potentials using Henry’s law$^{28}$. This technique was able to distinguish between various DNA coverages and base lengths, as well as a range of DNA structures (i.e. ssDNA/dsDNA) on the particle’s surface$^{28}$, as shown in chapter 5. In this particular study, rather than a conversion into zeta potentials, it is the relative changes in translocation velocity values that are used to reveal the presence of the DNA and proteins.
It can be hypothesised that following an aptamer binding to its target, the translocation velocities of the particles will change. Figure 6.2 shows the relative changes in translocation velocity (from the blank) observed as a function of VEGF concentration, with respect to both 50 and 100 % DNA coverages. By tuning the aptamer concentration on the particle’s surface, the dynamic range of the assay can be extended.

The aptamer-modified particles with HC plateaued at VEGF concentrations above 2 nM, which can be attributed to the protein binding capacity being reached and thus the maximum change in translocation effects to also be attained. Any additional proteins in the sample in this case will not change the numbers on the particle’s surface and will therefore not be measured via this technique. The aptamer-modified particles with FC exhibited a continuous decrease in particle velocity upon addition of VEGF until the protein concentration reached 18 nM, equivalent to circa 1000 proteins per particle.

For both aptamer coverages, a decrease in translocation velocity was seen as the VEGF concentration increased, the addition of VEGF is expected to slow the particle translocation velocity as seen in chapter 6.
shape is subjected to changing from a ssDNA structure to a folded tertiary structure, bringing the DNA closer to the particle surface, which could lead to a higher surface charge density on the particle itself. An increased surface charge density would result in an increase in particle velocity, however we see an opposite effect; the aptamer conformational change to a tertiary structure as it binds the protein target requires an increased amount of counter ions to stabilise that tertiary structure, decreasing the particle velocity. A decrease in particle velocity may be due to the charge of the protein target with a pI of 8.5 that is therefore positively charged at the pH used in this experiment (pH 7.4). Other parameters alluding to counter ion condensation include the shielding of the polyanionic DNA backbone. Shielding the negative charge on the phosphate backbone can evoke an increase in counter ion condensation onto the DNA\textsuperscript{42} and thus resulting in a decrease in particle velocity. Both particle assays (HC and FC) see this decrease with respect to their blanks, at a concentration of 18 pM as the lower limit of detection.

Aptamer-modified nanomaterials have been used in previous works in conjunction with the TRPS platform to measure a change in particle translocation frequency as the transduction signal\textsuperscript{10,37}. The method presented in this study is more beneficial as it capitalises on the ability of TRPS technology to use data from each individual particle as they traverse the pore, and doesn’t rely on averaging the data across hundreds of particles per minute. The advantages of this include reduced run times a reduction in bias for multimodal samples containing different particle concentrations.

6.5.2 Pore Modifications and Current Rectification Measurements

An alternative to a particle based assay is to use the change in ionic current through the pore, which can be controlled through electrostatic interactions via the pore wall. Conical shaped pores show this effect to a greater extent and is typically recorded for pore openings where the diameter, $D_s$, is equal to the electrical double layer thickness\textsuperscript{22,43}, although the double layer does not need to fully extend across the pore opening\textsuperscript{22}. Larger pores have been seen to exhibit current rectification effects\textsuperscript{23,44}. Detailed descriptions of rectification properties of conical pores can be found elsewhere\textsuperscript{43}, where the degree of rectification is defined as the ratio of absolute currents recorded at a given negative potential and the identical absolute positive potential. At lower electrolyte concentrations, conical pores with charged surfaces do not exhibit ohmic behaviour; the magnitude of the current through the nanopore at
negative potentials is greater or smaller than the current at the corresponding positive potentials, see figure 6.3. The current ratios can be tuned through variation of the electrolyte, pH, ionic strength, and applied voltage. In each case, the current-voltage, I-V, curves will record a preferred direction of current flow\textsuperscript{22,43} as shown in figure 6.3.

![Figure 6.3 - Schematic of the ion condensation present in the pore system and the respective current-voltage response for a positive(PEI) and negative (PAAMA) pore coatings.](image)

Tunable polyurethane pores have a small negative surface charge at pH >5\textsuperscript{28,44}. Unmodified pores used in this study had a pore diameter, $D_s$, of approximately 800 nm at a stretch of 45 mm, which can be calculated using the measured current in 50 mM KCl. The dimensions are likely to be averages as in some instances, the pore opening will not be spherical in shape, see figure 6.4.

![Figure 6.4 - SEM image of the topside of the NP200 pore.](image)
Table 6.1 shows the rectification ratios for unmodified pores in 5, 10, 50 mM KCl, and PBS (137 mM). For 5 and 50 mM, the current rectification ratios were 1.48 and 1.04 respectively, illustrating the return of the ohmic response at higher ionic strengths.

<table>
<thead>
<tr>
<th>Ionic Strength (mM)</th>
<th>Rectification Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>1.48</td>
</tr>
<tr>
<td>10</td>
<td>1.46</td>
</tr>
<tr>
<td>50</td>
<td>1.04</td>
</tr>
<tr>
<td>PBS (137 mM)</td>
<td>1.09</td>
</tr>
</tbody>
</table>

Table 6.1 - Rectification ratios for an unmodified pore at each ionic strength studied.

This study focused on seeing if the surface chemistry of the pore could be modified simply using a layer-by-layer, LbL, assembly and in this case, PEI and PAAMA coatings were investigated. This system is well studied having been previously used to modify a range of materials\(^{45-50}\). A LbL assembly was preferred over other techniques with polyurethane pores, such as plasma treatment or grafting polymers into the matrix via swelling\(^{51-53}\), as it allows for a facile and rapid dip coating strategy. A LbL approach also allows for the thickness and porosity of the PEI/PAAMA monolayer/bilayer to be controlled in the future as shown by Fu \textit{et al}\(^{46}\) who used a system similar to that by Yang \textit{et al}\(^{48}\). They both showed the control of the bilayers to ensure they do not extend across the pore opening and that the thickness remains restricted to a few nanometres, we have adapted this approach to also ensure the PEI and PAAMA bilayers did not extend across the pore opening.

Figure 6.5a illustrates the bilayer construction with alternate PEI and PAAMA layers and 6.5b shows the current-voltage curves for unmodified pores, single layer HMW PEI modified pores and HMW PEI-PAAMA modified pores, and double alternate layer HMW PEI-PAAMA modified pores in 5mM KCl.
Coating the pore surface with HMW PEI resulted in a reduced and enhanced current flow through the pore under negative and positive applied potentials, respectively, indicating a positive surface charge now on the pore. The sequential PAAMA coating switches the surface charge to negative, resulting in the preferred direction of current flow being inverted, also shown in figure 6.5b. All rectification ratios using HMW PEI are shown in table 6.2 and were measured at potentials of ±1.6 V. The addition of each polymer layer in turn caused a favoured direction of current flow, and can be described as an ‘on state’. The magnitude of the rectification ratio is important as it can be used to assess the presence and quality of the pore coating.
ratio values were seen to improve as the number of coating layers increased, indicating a greater quality of the coating as more layers were present.

<table>
<thead>
<tr>
<th>[KCl] (mM)</th>
<th>Unmodified Pore</th>
<th>PEI Layer 1</th>
<th>PAAMA Layer 1</th>
<th>PEI Layer 2</th>
<th>PAAMA Layer 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>1.38</td>
<td>0.45</td>
<td>2.42</td>
<td>0.37</td>
<td>2.86</td>
</tr>
<tr>
<td>10</td>
<td>1.09</td>
<td>0.67</td>
<td>1.68</td>
<td>0.46</td>
<td>2.44</td>
</tr>
<tr>
<td>50</td>
<td>1.09</td>
<td>0.92</td>
<td>1.10</td>
<td>0.73</td>
<td>2.09</td>
</tr>
<tr>
<td>PBS (137 mM, pH 7.4)</td>
<td>1.15</td>
<td>0.81</td>
<td>1.10</td>
<td>0.93</td>
<td>1.29</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>[KCl] (mM)</th>
<th>Unmodified Pore</th>
<th>PEI Layer 1</th>
<th>PAAMA Layer 1</th>
<th>PEI Layer 2</th>
<th>PAAMA Layer 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>1.06</td>
<td>0.87</td>
<td>2.89</td>
<td>0.45</td>
<td>3.07</td>
</tr>
<tr>
<td>10</td>
<td>1.01</td>
<td>0.96</td>
<td>1.21</td>
<td>0.73</td>
<td>1.35</td>
</tr>
<tr>
<td>50</td>
<td>0.98</td>
<td>0.96</td>
<td>1.03</td>
<td>0.95</td>
<td>0.96</td>
</tr>
<tr>
<td>PBS (137 mM, pH 7.4)</td>
<td>0.98</td>
<td>1.01</td>
<td>1.00</td>
<td>1.02</td>
<td>0.97</td>
</tr>
</tbody>
</table>

Table 6.2 - Rectification ratios for unmodified pores and pores modified with each polymer layer using HMW PEI (top data) and LMW PEI (bottom data) for each ionic strength studied.

A similar effect was seen for LMW PEI, however the rectification ratios for each subsequent PEI and PAAMA layer were smaller than for HMW PEI as shown in figure 6.6. This is most likely due to the nature of the PEI layer coating the pore surfaces; for example, the mechanism for LbL assembly goes through island formation before forming a complete layer and it is hypothesised that LMW PEI takes longer to form that fully coated surface layer. Interestingly, even with the two bilayers added onto the pore walls, the pore opening remained unobstructed in both PEI cases.
Figure 6.6 - A plot describing the current-voltage response for each layer as it was coated onto the pore using LMW PEI. The black represents the response from an unmodified pore, the filled blue squares and white squares represent the 1st and 2nd PEI coatings, respectively. The filled red triangles and white triangles represent the 1st and 2nd PAAMA coatings, respectively. The figures are adapted from Blundell et al\textsuperscript{54}.

Figure 6.7 shows the effect of increasing the ionic strength on the current-voltage curves, with rectification ratios detailed in table 6.2. Increasing the ionic strength caused the rectification ratios for modified pores to tend to 1.0, which indicates a reduction in rectification effects. However, it is interesting to note that even at ionic strengths surpassing 100 mM KCl and a pore diameter circa 800 nm, some rectification is still observed. The current rectification may have been enhanced by the nature of the pore modification used here\textsuperscript{54}. 
The polyurethane pores have a large top surface area, $T_S$, (*circa* 2.5 mm diameter$^{54}$). During the pore modification LbL process, the top surface is also coated as well as the inner pore walls. Modifying both surfaces of the pore has been previously reported as
having a larger effect on the rectification ratio\textsuperscript{22}. The LbL approach was reproducible on multiple pores and showed little hysteresis through multiple cycles and scan directions investigated\textsuperscript{54}. It was also important to investigate the longevity of the pore coatings and the LbL approach resulted in the coatings being stable over short periods of time, even after multiple uses, washes, and being dried and stored overnight (figure 6.8a/b). Once the coatings had diminished over time (and the rectification effects had returned to that of an unmodified pore) the same pore could be recoated and reused to show comparable results.
Figure 6.8 - Current-voltage responses for the pores coated with PEI/PAAMA bilayer and left for 2 hours (green dataset) and 4 days (purple dataset). a) and b) represent the longevity experiment on two different pores in 5 mM KCl. b) shows the coating starting to become diminished after 7 days (orange dataset) as the I-V response begins to return to that of an unmodified pore (black dataset). Figure is modified from that in Blundell et al.

The polyurethane pores in this study are flexible and altering the pore stretch should have inherent effects on the rectification ratio, a smaller stretch produces a higher rectification ratio. Figure 6.9 details the current-voltage curves as the stretch is decreased from 5 mm to 2 mm. As the pore opening size, $D_s$, and pore thickness is reduced, the observed current at positive potentials decreases.
As previously mentioned, changes in pH can also affect the current rectification properties of the pores. To test the effect of altering pH, the pore rectification effects were investigated at pH 3 and pH 7. Altering the pH will invoke a different charge density on the PEI-modified surface, for example a lowered pH of 3 will increase the charge density as more amine groups become protonated; which results in an increased current flow at positive potentials. Conversely, an increase in pH to a value of 7 will reduce the positive charge on the PEI. Changes in the current-voltage curves are also observed for the PAAMA-modified surfaces when the electrolyte pH is altered, opposite charge density effects to PEI\textsuperscript{54}. At low pH, the charge density across the carboxyl groups is reduced and an I-V response similar to that of an unmodified pore is observed. A higher pH value of 7, for example will increase the negative charge density across the PAAMA surface resulting in a decrease in current flow at positive potentials\textsuperscript{54}.

The current flow through the pore has two contributing factors; the electroosmotic flow across the pore surface and migration of ions through the centre. Whilst others have shown this to be a small contributing factor to current rectification in smaller pores\textsuperscript{22}, and that combined with the effect of the charge on the pore wall and top surface, $T_s$, has the largest contribution to the current rectification\textsuperscript{22}. The observation of
rectification behaviour does illustrate that the enriched ion zone at the pore mouth used to describe the rectification properties of smaller pores\textsuperscript{22,40} sufficiently exerts an influence across the larger opening, even when the electrical double layer is much shorter than the opening, $D_s$. This effect has also been attributed to biphasic pulse behaviour in TRPS\textsuperscript{44}. The reported setup allows for the easy modification of the pore wall, and by using different polymers or different thickness it may be possible to further tune these larger pores to be ion selective.

The positioning of the pore coating on the pore was therefore investigated to see whether a full pore wall coating ($T_s$ and inner pore walls) is required to cause the observed current-voltage responses, or if the PEI/PAAMA bilayer on the top surface is sufficient for the response, illustrated in figure 6.10.

![Figure 6.10 - Schematic of sole modification of the top surface, $T_s$ of the pore using a double Layer-by-Layer assembly of PEI (blue) and PAAMA (red).](image)

The current-voltage curves are illustrated in figure 6.11 for each ionic strength studied when the PEI/PAAMA bilayer was just added to the topside of the pore via the same incubation process.
The results showed there was no change in the $I-V$ response upon sole topside addition of HMW PEI and PAAMA. The rectification ratios are described in table 6.3 for each of the electrolytes studied.

<table>
<thead>
<tr>
<th>[KCl] (mM)</th>
<th>Unmodified Pore</th>
<th>HMW PEI Layer</th>
<th>PAAMA Layer</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>1.64</td>
<td>1.38</td>
<td>1.65</td>
</tr>
<tr>
<td>10</td>
<td>1.12</td>
<td>1.00</td>
<td>1.10</td>
</tr>
<tr>
<td>50</td>
<td>1.05</td>
<td>0.93</td>
<td>1.04</td>
</tr>
<tr>
<td>PBS (pH 7.4)</td>
<td>0.97</td>
<td>0.94</td>
<td>1.09</td>
</tr>
</tbody>
</table>

Table 6.3 - Table of rectification ratio values for each electrolyte studied for an unmodified pore and a top surface-modified pore with PEI and PAAMA.
6.5.3 Modifying Pore Walls with ssDNA and Subsequent Protein Detection

The main motivation for modifying pores with polymers using the LbL approach was the ability to introduce functionalities on the pore wall that could be easily coupled with biomolecules. Gold-modified pores, for example allow thiol terminated ligands to be functionalised to the pore wall or for the use of the carboxyl groups\(^{42,56}\). The ssDNA in this study is immobilised onto the pore surfaces via EDC chemistry. Figure 6.12 shows the full current-voltage curves for a pore modified with PEI/PAAMA via LbL assembly, followed by DNA attachment in 50 mM KCl. A single bilayer of PEI and PAAMA was used as it was seen to be a sufficient coating for pore rectification effects to be observed (verified using I-V curves) and reduced the number of preparation stages. As shown in figure 6.12, the DNA-modified pore surface showed smaller rectification effects than the PAAMA-modified surfaces. This may be due to the increase in counterion condensation that occurs to stabilise the high number of ssDNA strands, resulting in inevitable shielding of the high negative charge densities, more ions present in the pore could also lead to a higher measured current.

![Figure 6.12 - Current-voltage response for a DNA-modified pore (orange dataset) in comparison to an unmodified (black dataset), PEI-modified (blue dataset), and PAAMA-modified (red dataset) completed in 50 mM KCl. Figure is adapted from work by Blundell et al\(^{54}\).](image)

Once the DNA had been successfully crosslinked onto the pore surfaces, the pore was incubated with the protein target, VEGF and a strong rectification effect was observed (figure 6.13 for 50 mM KCl).
The DNA aptamer-protein interaction caused a decrease in current at positive potentials and was specific to VEGF (in the presence of BSA, figure 6.14a-d). The rectification properties observed here were similar to those observed for a lysozyme system in cylindrically shaped pores\textsuperscript{56}. The reason for such behaviour was attributed to the proteins high pI value as well as the protein-DNA binding causing an inverted surface charge. The inversion thus resulted in a positive surface charge on one side of the pore and overall an asymmetric surface charge resulting in the current rectification. Our experiment may produce a similar effect as the introduction of the protein on one side of the pore produces a different surface charge density from the top to the bottom. Introducing the protein to one side could have resulted in a positively charged narrow pore opening (top surface), which would have suggested rectification properties to be opposite to what was observed (figures 6.13 and 6.14), i.e. an increase in current flow at positive potentials\textsuperscript{57}, and thus was not the case in this study.
The decrease in current at positive potentials, caused by the VEGF binding, is hypothesised to be a rectification effect that relies on VEGF’s high pI value (8.2) and positive charge. When the protein is bound to the DNA at the pore opening and $T_s$, the cation cloud density is increased at the pore opening. This cation increase is additional to the increase in counter ions required to stabilise the aptamer tertiary structure formed as it binds to VEGF and these two ionic factors contribute to the decrease in conductivity at the pore opening at positive potentials, as described elsewhere. The current-voltage response was also monitored for a DNA-modified pore as a function of protein concentration (Figures 6.15 for 50 mM KCl).
As the concentration of VEGF increased, the current measured at positive potentials decreased until concentrations beyond 5 nM. The rectification ratios for experiments in 50 mM are detailed in table 6.4.
### Table 6.4 - Current rectification ratios for a range of VEGF concentrations, including a BSA control.

<table>
<thead>
<tr>
<th>VEGF Concentration (nM)</th>
<th>Rectification Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 pM</td>
<td>1.36</td>
</tr>
<tr>
<td>50 pM</td>
<td>1.93</td>
</tr>
<tr>
<td>0.5 nM</td>
<td>3.64</td>
</tr>
<tr>
<td>5 nM</td>
<td>11.60</td>
</tr>
<tr>
<td>50 nM</td>
<td>10.12</td>
</tr>
<tr>
<td>50 nM BSA</td>
<td>1.53</td>
</tr>
</tbody>
</table>

6.6 Conclusions and Further Work

This study has presented a comparison between resistive pulse and current rectification aptamer assays using the same technology platform that could each detect VEGF to concentrations of 18 pM and 5 pM, respectively. The resistive pulse particle assay may offer a format that can be easily prepared and multiplexed by pairing each target with a unique particle size or shape. Although this widens the dynamic range for analysis, this assay's sensitivity is limited by the number of proteins required on each particle surface to sufficiently alter the surface charge to cause a measurable difference in translocation velocity. Further optimisation and assay design steps may be required to improve the sensitivity of this technique. This can be achieved through specifically designing the particle size and shape to allow for a controlled and limited number of aptamers per particle, although this can complicate the assay time as the particle flux can be reduced when smaller particles/pores are used. The number of aptamers could also be controlled through the use of Janus particles to localise the biorecognition sites. The second assay incorporated a LbL polymer assembly onto the polyurethane pore surface allowing for pH and ionic strength controlled current flow. The LbL method reported here offers a simple and reusable technique in modifying the surface chemistry of the pores. The tunable pores used in this study are unique in their abilities and versatility to both assay types investigated.

The LbL pore modification approach is the first reported method that offers pH and ionic strength dependent current rectification behaviour on a TRPS system. It is also the first reports of the strong rectification on pores of the investigated dimensions, circa 800 nm. The strength of the rectification properties indicates that the electrical double
layer does not need to extend significantly across the pore opening. The pore surfaces can be modified easily using the LbL assembly of polymers, such as polyethyleneimine (PEI) and polyacrylic acid co-maleic acid (PAAMA). The use of PEI and PAAMA allowed for easy modification and reversible changes in pore surface charge, resulting in a pH and ionic strength controlled current flow. The LbL assembly is stable for a number of days that allows for DNA-modification of the pore wall via standard carbodiimide chemistry. However, further work is required to investigate the effects of pore modifications on the top surface of the pore and the effects this has on the current rectification mechanism, to assess the total electroosmotic flow contribution in these systems. Further work is also required using aptamers to other targets, in this example an aptamer to BSA would also be useful in the control experiments.

A potential limitation for future assays lies within complex biological solutions, where there is a tendency of proteins to foul the pore wall or particle surfaces. This limits the assay because if non-specific protein fouling occurs to the particle surface or pore wall between or throughout experiments, this will impact on the assay’s reproducibility. This is because the particle translocation velocity is a contribution of the electrophoretic speed of the particle and the electroosmotic contribution of the pore wall, which should remain constant throughout experiments to ensure good reproducibility. This limitation could be abolished if the particles and/ or pore wall can incorporate a non-fouling coating that would prevent the non-specific adsorption of proteins that could be investigated as further work. Another potential solution would be to modify the pore walls directly to facilitate the detection of a specifically chosen analyte. There are other nanopore systems that have used the natural surface functionality of the nanomaterial or utilised a gold-modified pore to allow for simple attachment of materials. At present there have been no reported modifications to the tunable polyurethane, PU, pores for RPS studies, although there are strategies available for the modification of the PU material.

This alternative will reduce the need for external nanomaterial ‘labels’ as the pore can be modified to detect a particular analyte itself.
6.7 Acknowledgements

I would like to thank Laura Mayne, a fellow PhD student in the research group who gave continued support with this study and helped with some of the optimisation aspects including longevity, pH, pore stretch, and pore to pore reproducibility as well as running the protein experiments with respect to a BSA control.

6.8 References


7 Behaviour of Nanoparticles in Biological Solutions: Study of Protein Corona

7.1 Abstract

When introduced to a biological fluid such as blood, nanoparticles interact with the variety of biological components, particularly with proteins that result in the formation of a ‘protein corona’. The formation of a protein corona can significantly modify the behaviour and dynamics of nanoparticles in biological fluids. The use of TRPS technology provides a flexible sensing platform allowing for rapid detection and characterisation of nanoparticles in a range of environments. This study determines interactions between nanoparticles and various components of human blood including whole plasma, serum, albumin, immunoglobulin, and fibrinogen through zeta potential measurements using TRPS.

Normal human plasma, serum and physiological concentrations of purified human albumin, immunoglobulin, and fibrinogen were studied. The three purified proteins have previously been found to be present in all nanoparticle-protein corona studies to date and have been validated using TRPS. Preliminary data has shown that nanoparticle interactions with human plasma are more prominent than those with serum; this has been demonstrated by the changes in zeta potential when nanoparticles are immersed in the respective sample. The change in zeta potential was monitored as a function of biological constituent, protein concentration, and temperature.

Understanding the impacts on nanoparticle dynamics from each of the ‘protein corona’ components studied is important to monitor the nanoparticle-protein binding in biological fluids such as human plasma. Here we are making zeta potential measurements of the protein corona in solutions that mimic a solution of high ionic strength and high protein composition, similar to that of the natural environment. Investigating the effect of temperature also allows physiological temperature to be taken into account to again mimic the natural environment. Not only can TRPS be used to characterise the protein corona in terms of charge, it can also monitor the change in ‘hard’ and ‘soft’ corona elements through the introduction of components of
higher affinities to the nanoparticle surface (in this case spiking a sample containing particles immersed in serum with human plasma. Indication and information about the binding kinetics and mechanisms of the protein corona is profoundly useful to diagnostic\textsuperscript{1,2} and drug delivery fields\textsuperscript{3–5}.

7.2 Introduction

Analysis of nanoparticles in natural environments is completed less than nanoparticles in purified samples with select targets. Purifying samples can sometimes give a misrepresentation of how the particles would actually behave in a biologically relevant or natural environment. There are several factors that are influential to nanoparticle dynamics and behaviour in solution including pH, temperature, and ionic strength. When nanoparticles are introduced to biological fluids, an almost immediate response is the coverage of the particle surfaces with various proteins and peptides found in these solutions. This coverage can otherwise be known as a ‘protein corona’ layer\textsuperscript{6–8}. The protein corona composition is the main contributor to changes in the nanoparticle’s surface chemistry and thus its behaviour in solution. Changes of this magnitude will alter the properties of the particles and have even been reported as affecting pathophysiological effects of the particles themselves\textsuperscript{9}. Particles of similar compositions but difference surface chemistries will entail different protein corona structures\textsuperscript{6,10} that can be complex and difficult to characterise.

Previous techniques used to characterise protein corona structures include dynamic light scattering\textsuperscript{11}, fluorescence quenching\textsuperscript{11}, differential centrifugal sedimentation\textsuperscript{12,13}, mass spectrometry\textsuperscript{10,14}, circular dichroism (CD)\textsuperscript{11} and electron microscopy\textsuperscript{11} that have also been used to interpret the layer thickness of the corona. The layer thickness and density are one of five main components defining a protein corona composition; identity and quantity, orientation, conformation, and affinities constitute the other four\textsuperscript{15}. As well as studies on the conformation of the protein corona, the affinity is also a popular and interesting characteristic, previously measured by size exclusion chromatography, surface plasmon resonance and isothermal calorimetry\textsuperscript{12,13,16}. Zeta potential, the electrostatic potential at the plane of shear, is one of the more recurrent values used to characterise a corona\textsuperscript{6,8,10,17,18} as an indication of surface charge.

The structure of a protein corona is detailed and complex and is formed of a series of layers that are defined as the ‘hard’ and ‘soft’ corona. The proteins creating the hard
corona layer are those with a higher affinity to the nanoparticle surface than those in the soft corona. The soft corona is made up of weaker protein-protein interactions with those in the hard corona, whereas the hard corona involves much stronger interactions with the nanoparticle surface\textsuperscript{13,19}. Apolipoproteins found in physiological fluids are prone to binding to a range of particle surfaces and are present in almost every protein corona formation\textsuperscript{14}.

The initial formation of the corona may not be the structural entirety of the corona itself as this is dependent on protein adsorption kinetics and the binding mechanisms that will occur in a natural environment. The adsorption, and desorption rate constants of the proteins, $k_{on}$ and $k_{off}$, are key to understanding the binding mechanisms as well as the process being naturally time dependent. The adsorption rate is dependent on the number of times the protein interacts with the nanoparticle surface alongside the probability of successful protein-particle binding\textsuperscript{20}. Desorption of bound proteins will depend heavily on the binding strength between the two materials\textsuperscript{20}. If an interaction is of low energy and is weak, the $k_{off}$ value will be high and if the interaction is strong and of high energy, the $k_{off}$ value will be low. Characterising and validating nanoparticle-protein interactions and protein binding from each protein studied will develop a much needed further understanding on how nanoparticle exposure to a biological matrix can impact on nanomedical fields.

### 7.3 Aims and Objectives

The main aim of this study was the investigation of protein binding kinetics within biological solutions and kinetics of formation of a protein corona. Validation of nanoparticle-protein interactions in such solutions is important for the future design of particle-based assays that can be completed \textit{in vivo}.

### 7.4 Materials and Methods

#### 7.4.1 Chemicals and Reagents

##### 7.4.1.1 Buffers

Phosphate buffered saline (1 x 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)) was used as the initial electrolyte for analysis. PBS tablets (P4417) were purchased from Sigma Aldrich, UK.

**7.4.1.2 Particles and Particle Standards**
Carboxylated polystyrene particles with a nominal diameter of 210 nm, CPC200s, were purchased from Bangs Laboratories, US and were used as a calibrant for zeta potential analysis, as well as sample particles. The particles were vortexed for 30 seconds followed by a 2-minute sonication prior to any TRPS analysis or sample incubation to ensure monodispersity.

**7.4.1.3 Isolated Proteins and Blood Samples**
The isolated proteins (fibrinogen from human plasma (≥80 %, F3879), albumin from human serum (lyophilised powder, ≥97 %, A9511), and γ-globulin from human blood (≥99 %, G4386)) were purchased from Sigma Aldrich, UK, without modification of purification unless otherwise stated.

Blood samples were collected and prepared at Peterborough City Hospital Pathology Laboratory, UK. Plasma collection was completed using blood from a healthy volunteer donor that was collected in citrate medium (Sarstedt, UK) and centrifuged at 3000 rpm for 8 minutes. Serum was gathered using blood from a healthy volunteer donor that was collected into a Sarstedt monovette/collection tube, and was centrifuged at 3000 rpm for 6 minutes. The supernatants from each sample were transferred into separate sample vials and stored at room temperature prior to use.

**7.4.2 Methods**

**7.4.2.1 Isolated Protein Studies**
Using PBS as an electrolyte, isolated fibrinogen, γ-globulin, and albumin samples were prepared to the following concentrations; 3.2 g/L, 20 g/L, and 43 g/L respectively, as to mimic protein concentrations found in human blood. The concentrations of proteins were measured from human plasma and serum samples. The samples used in this study were analysed by an Instrument Laboratory ACL TOP CTS500 coagulation analyser (Werfen, Spain) to obtain the fibrinogen concentration. Albumin and immunoglobulin levels were taken from test serum samples that were analysed by a Roche Cobas biochemistry analyser (Roche Diagnostics, Switzerland). CPC200s were added resulting in a final concentration of $1 \times 10^{10}$ particles/mL. Each sample
was vortexed for 30 s and sonicated for 1 minute before incubation. Samples were then incubated at 25°C and 37°C in a mini dry bath (Benchmark Scientific, US) for 10 minutes prior to TRPS analysis.

### 7.4.2.2 Serum and Plasma Studies

Human plasma and serum were prepared immediately before the experiments to minimise *ex-vivo* artefactual changes. The prepared plasma and serum were separately diluted 10-fold with PBS before CPC200s were added to both samples resulting in a final particle concentration of 1 x 10^{10} particles/mL. Samples were vortexed for 30 s and sonicated for 1 minute, followed by incubation in a mini dry bath (Benchmark Scientific, US) at 25°C and 37°C for 10 minutes before being removed for TRPS analysis. It should be noted that it is possible for some proteins in human plasma and serum to interact and adsorb onto the pore walls; therefore, a control measurement of CPC200s in PBS (of known zeta potential) was completed before and after each protein/plasma/serum sample to establish if any changes had occurred to the pore itself.

### 7.4.2.3 Plasma Spiking Assay

Human serum was 10x diluted in PBS before CPC200s were added to a final concentration of 1 x 10^{10} particles/mL. Samples were vortexed for 30 s and sonicated for 1 minute before being incubated for 10 minutes at 25°C and 37°C in a mini dry bath (Benchmark Scientific, US). At 10 minutes, 5% (v/v) human plasma was added to the serum samples and the samples were vortexed for 30 s. TRPS measurements were completed once the plasma had incubated with the serum sample for 5, 10, 15, 20, 30 and 60 minutes.

### 7.4.2.4 TRPS Analysis ad Zeta Potential Calibration

All TRPS measurements were carried out on the qNano (Izon Science Ltd, NZ) using Izon Control Suite data capture and analysis software v.3.1.2.53. The nanopores used throughout all experiments were capable of detecting particles in the size range of 100-300 nm (as stated by the manufacturer, Izon Science Ltd, NZ), denoted as an NP200. Full details of TRPS setup and calibration of the nanopores for zeta potential measurements using pressure can be found in chapter 2, subsection 2.2.2.
7.5 Results and Discussion

Zeta potential measurements were made using particle velocities as they traversed a nanopore membrane. The theory is fully detailed in chapter 3 and can also be found elsewhere\textsuperscript{21,22}. In summary, particle translocation effects are measured as a function of an applied voltage, where the average particle velocities are taken over the sensing zone, in this case a regular conical pore. Figure 7.1 is a summarised representation of the components required to complete a zeta potential measurement based on the particle electrophoretic mobility through the pore.

![Diagram showing a conical pore with various positions labeled as I_0.2, I_0.4, I_0.6, I_0.8, I_1.0, and T_0.2, T_0.4, T_0.6, T_0.8, T_1.0.](image)

Figure 7.1 - Example of a TRPS measurement using a conical pore and the signal trace indicating the blockade duration times, T with respect to the relevant position within the pore, I. Figure adapted from Blundell et al\textsuperscript{23}.

The figure shows the regular conical sensing zone and examples of the blockade duration times, T, that are equivalent to the times at which the particle is at a certain position within the nanopore, I. For example, T0.4 is the time equivalent to when the blockade was at 40% magnitude and is relative to position I0.4 when the particle is 60% of the way through the pore; T1.0 is the time equivalent to the blockade being at dR_{\text{max}}, at its maximum magnitude, and relative to I1.0 the pore entrance. Each T is referred to as a ‘blockade reference point’ and the average particle velocities are determined across several of these reference points to reduce any errors in the zeta
The calibration of the pore membrane has been previously described in detail in chapter 2.

The isolated proteins of choice for this study are human albumin, γ-globulin (4 and 2 % relative abundances in both blood plasma and serum, respectively), and fibrinogen (0.4 % relative abundance in blood plasma)\(^{23}\). Figure 7.2 demonstrates the zeta potential values for each of the isolated proteins incubated with the nanoparticles at room temperature (purple bars) and 37°C (orange bars). The green bars indicate a control experiment of the nanoparticles in PBS buffer showing that the protein samples did not have a direct influence on the zeta potential of the pore itself. These controls were important between each protein sample to ensure the zeta potential of the pore membrane did not change for each sample as it is a key factor in calculating the particle zeta potential, further described in chapter 3.

The concentrations of the proteins varied to replicate a natural environment and compositions of human blood in the human body. The albumin solution was of a 40 g/L concentration, the highest of all the isolated proteins studied (fibrinogen and γ-globulin were of concentrations of 4 and 20 g/L, respectively), but it should be noted that each protein was added to the particles in excess so it is expected that the particles will each be saturated by the proteins. Full coverage of the particles by the
proteins suggests the proteins will adsorb onto the particle surfaces and a protein corona will have formed. This assumption is confirmed by the change in zeta potential shown in figure 7.2 as the formation of a protein corona results in a change to the particle’s surface chemistry, causing a change in the particle’s velocity and thus zeta potential.

At room temperature, the largest change in zeta potential from nanoparticles in buffer was seen for the albumin protein (9.2 mV), with both the fibrinogen and γ-globulin showing smaller changes in zeta (3.2 and 3.6 mV, respectively)\textsuperscript{23}. The magnitude of change in zeta potential may be due to the isoelectric points of the proteins. Albumin has an isoelectric point of 4.7, much lower than fibrinogen (5.8) and γ-globulin (6.6)\textsuperscript{24}. Tengvall \textit{et al.} have found that adsorbed molecules will occupy a larger area of a surface when the adsorption pH is further from that of protein itself, due to the increased structural instabilities caused by electrostatic repulsions\textsuperscript{25}. The pH of each solution (using PBS as an electrolyte) was around 7.4 and it is therefore expected that the albumin protein would occupy the largest area of the nanoparticle surface at room temperature.

The albumin protein was of the highest concentration at 40 g/L. To demonstrate that the impact on nanoparticle translocation effects was protein dependent and not concentration, the nanoparticles were incubated with the 3 isolated proteins of constant concentration, 5 g/L at 25°C. The data is summarised in figure 7.3 and shows that the largest shift in zeta potential (compared to the particles in PBS buffer control, green lines) was still observed for the albumin protein (8.9 mV) followed by γ-globulin (4.9 mV) and the smallest change from fibrinogen (4.3 mV)\textsuperscript{23}. This confirms the effects on the nanoparticle surface are protein dependent and not concentration dependent in this study.
The effect of each protein on particle zeta potential was also investigated as a function of temperature and it was found that increasing the incubation temperature had a significant effect on the protein-nanoparticle interactions. This was shown by the significantly larger changes in zeta potential observed for both fibrinogen and γ-globulin, particularly γ-globulin (11.3 mV, figure 7.2, from particles in PBS) when the incubation temperature was elevated. There was also a larger change in zeta potential for albumin, but this wasn’t as significant as it was for the other two proteins.

A lot more information about the given samples can be extracted due to the particle-by-particle nature of TRPS. Each peak observed in the signal trace (figure 7.1) is representative of a single particle as it traverses the nanopore showing the analysis of every individual particle amongst a sample population. From this, the zeta potential distribution can be used to gather additional information about the nanoparticle behaviour in the sample solutions. Figure 7.4 is an example of the differences in zeta potential distribution between the samples containing each of the isolated proteins.
Zeta potential distributions for samples incubated with the particles at room temperature (25°C) are shown by the orange (fibrinogen), blue (γ-globulin) and purple (albumin) filled datasets and samples incubated with the particles for 10 minutes at 37°C are shown by the outlined data plots for fibrinogen (orange), γ-globulin (blue) and albumin (purple). The shape of the spread of the zeta potential data doesn’t particularly change between each of the proteins incubated with the particles at room temperature but does widen when the incubation temperature is increased to 37°C. This effect was most prominently seen for the fibrinogen data and least so for the albumin sample. Between the samples incubated with the particles at 25°C and those incubated at 37°C, it is γ-globulin that demonstrated the largest change in zeta potential (5.0 mV) as a function of temperature. The smallest change was demonstrated by the albumin protein with a zeta potential difference of 1.3 mV between a lower and higher incubation temperature. From a control sample of particles in buffer, it is the shift in the zeta potential spread to smaller zeta potential values when the incubation temperature is increased that is significant. The change in zeta potential may be due to the protein affinities being influenced by the incubation temperature as previous work has found that negative particles have a maximum protein adsorption at 15, 35, and 37°C, explaining why the particles incubated at 37°C showed a larger change than those incubated at 25°C.
The spread of data concerning particle size and zeta potential is illustrated in figure 7.5 compared to the particles incubated in PBS buffer (black triangles). Figure 7.5a shows the spread of data when the proteins are incubated with the particles at 25°C and figure 7.5b shows the data spread when the incubation has occurred at 37°C.

Figure 7.5 - Zeta potential (mV) vs particle size (nm). The orange, blue and purple data points are zeta potential and size distributions for particles incubated for 10 minutes with fibrinogen, γ-globulin, and albumin, respectively at a) 25°C and b) 37°C. The black data points represent the carboxylated particles in PBS buffer. It should be noted that each data point represents a single particle amongst a given sample population where a minimum of 350 particles was measured per sample. Figure adapted from Blundell et al.²³.
Of interest, the size distribution of the γ-globulin sample and particularly the fibrinogen dataset (blue and orange, respectively) are wider when incubated with particles at 25°C (182-391 nm for γ-globulin and 183-385 nm for fibrinogen). At 37°C the population distributions are narrower and this attributes to the protein binding kinetics differing as a function of temperature. As mentioned previously the protein affinities can be affected by temperature26, the proteins that have a higher affinity to the nanoparticle surface will either form a strengthened, robust hard corona layer or induce a slower release or desorption of the proteins from the surface. A robust hard corona layer will significantly alter the surface chemistry and thus shielding the original negative charge of the particle resulting in a slower velocity through the pore and thus a smaller measured zeta potential. A lower incubation temperature may not have formed as robust of a hard corona layer and may still have a larger soft corona layer surrounding the particle surface based on the findings that maximum protein adsorption may not have been reached at 25°C26, which also supports the small changes in zeta potential shown in figure 7.2.

Although there is a lot of interest surrounding isolated protein-nanoparticle interactions, the more challenging aspect is when there is a medium containing a complex mixture of proteins, such as plasma or serum. Although both similarly extracted from whole blood samples, the compositions of both are different. For example, the protein composition of serum includes albumin, γ-globulin, and apolipoproteins. Plasma however, contains the same proteins as serum but with the addition of clotting factors such as fibrinogen (relevant to this particular study). Incubating the particles with plasma and serum for 10 minutes will provide valuable information on how nanoparticles behave in biological solutions in a complex medium. The plasma and serum were both investigated under the same conditions as the isolated protein samples, incubated with the nanoparticles for 10 minutes at 25 (blue bars) and 37°C (red bars), results summarised in figure 7.6.
Figure 7.6 - Mean zeta potential (mV) of particles in PBS buffer (green), particles incubated in plasma and serum for 10 minutes at 25°C (blue bars) and 37°C (red bars). Figure adapted from Blundell et al. Error bars represent a standard deviation of n=3 independent replicates. A minimum of 300 particles were measured per sample.

There were only very small changes in zeta potential (1.5 and 1.3 mV for plasma and serum, respectively) for the samples incubated with the particles at 25°C, as was seen for two of the isolated proteins (fibrinogen and γ-globulin) at this incubation temperature. However, when incubated with the particles at the higher temperature of 37°C, only the serum sample showed a significant change in measured zeta potential totalling 5.9 mV. This is interesting as the plasma still only showed a small change in mean zeta potential, even once incubated with the particles at an elevated temperature (1.9 mV). Figure 7.7 shows examples of zeta potential distributions for both plasma (red dataset) and serum (blue dataset) incubated with the particles at 37°C, in comparison to particles suspended in PBS buffer (green dataset).
Figure 7.7 - Zeta potential distributions (mV) of particles incubated for 10 minutes at 37°C. The green, red, and blue datasets represent the particles incubated with PBS buffer, plasma, and serum, respectively. Each sample measured a minimum of 300 particles.

A comparison of the zeta potential distributions for plasma and serum samples incubated at the two temperatures is demonstrated in figure 8.8. The effect the biological fluids had on the nanoparticles may have been due to their composition. The main difference in composition between plasma and serum is the clotting factors present in plasma, which will impact on the protein corona structure and therefore the protein-nanoparticle interactions. The difference in protein composition for the plasma and serum is what can make protein corona formation challenging in physiological conditions as protein-nanoparticle alongside protein-protein interactions are occurring simultaneously and sometimes competitively binding to the nanoparticle surface. The competitive binding to the nanoparticle surface is natural between the proteins in plasma and serum so the proteins of higher affinity and/or concentration will bind to the nanoparticle surface at a faster rate. The protein-protein interactions will impact on a protein corona formation as some proteins may have a higher affinity to other proteins in the medium over the particle surface.
Both the plasma (figure 7.8a) and serum (figure 7.8b) showed a change in zeta potential distribution shape when the incubation temperature was changed. The distributions are much wider when the incubation temperature was lower and much narrower on the contrary. The advantages of distribution studies are the subtle differences that can be determined outside of the mean values. Although the mean zeta potential value for plasma didn’t change much with the incubation temperature, the distribution histogram showed significant shape changes. For example, in figure 8.6, the particles incubated with plasma showed a mean zeta potential value difference of a mere 0.4 mV but a change in distribution shape of nearly half the size for 37°C (outlined datasets) than for 25°C (filled datasets) as shown in figure 7.8a.
Another definition of the distribution shape is median skewness, values that can be calculated to indicate the level of skew amongst a given sample population. The median skewness values for the plasma and serum experiments are detailed in table 7.1, where the same effect was seen for both plasma and serum, the higher the incubation temperature, the more negative the median skewness value:

<table>
<thead>
<tr>
<th>Biological Sample</th>
<th>Incubation Temperature (°C)</th>
<th>Incubation Time (min)</th>
<th>Median Skewness of Sample Distribution</th>
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<tr>
<td>Plasma</td>
<td>25</td>
<td>10</td>
<td>0.111</td>
</tr>
<tr>
<td>Plasma</td>
<td>37</td>
<td>10</td>
<td>-0.065</td>
</tr>
<tr>
<td>Serum</td>
<td>25</td>
<td>10</td>
<td>-0.105</td>
</tr>
<tr>
<td>Serum</td>
<td>37</td>
<td>10</td>
<td>-0.343</td>
</tr>
</tbody>
</table>

Table 7.1 - Summary of median skewness values for sample population distributions of zeta potential for particles in both plasma and serum in PBS at the two investigated incubation temperatures.

It was intriguing to find the impact a small difference in protein composition could have on the nanoparticle-protein interactions. To investigate this further, an experiment was designed to encourage possible competitive and displacement reactions of proteins within the sample. This will interpret the nature of the hard corona and the possible definition of proteins having prominent effects as well as the composition and formation of the soft corona. This part of the study was relevant to divulge further into protein adsorption onto a particle’s surface and in turn, the Vroman effect. The Vroman effect is defined as the constant change in protein composition whilst undergoing continuous adsorption and desorption at an interface or surface27. The rate constants of adsorption, desorption, and dissociation are dependent on the interface itself as well as the proteins present in the sample.

To put this into context, the three isolated proteins studied here (albumin, γ-globulin, and fibrinogen) will each adsorb rapidly onto a particle’s surface as they are all of high abundance in their respective samples, but it should be known that each protein also dissociate from the surface quickly and are generally replaced by apolipoproteins almost immediately28. The apolipoproteins have the advantage of a slow dissociation constant so will remain on a surface for longer29, even though they are of lower abundances and bind to the surface after other proteins in the solution. This leads to
the assumption that there are several protein-based competition elements occurring when nanoparticles are immersed into biological fluids. Not only is there competition for the nanoparticle surface, but also for other proteins in solution that some proteins may have a higher affinity for over the nanoparticle surface, which will directly influence the protein corona structure and composition. Over time the competitive assays occurring within the solution will encourage displacement and exchange reactions to the nanoparticle surfaces and the hard/soft coronas. As explained previously in the chapter, the hard corona will involve the proteins of higher affinities to the particle’s surface and should remain on that surface during and after any biophysical occurrences have taken place. The soft corona, however, involves the weaker protein interactions that are expected to dissociate much more rapidly, thus encouraging protein exchange much more readily.

The formation of hard and soft coronas is dependent on the relative concentrations of proteins in a given sample, such as plasma and serum, as it is well known that proteins of a higher concentration will tend to gain coverage over the nanoparticle surfaces at the first instance at a faster rate than those of lower concentrations. If the proteins of lower concentrations have a higher affinity to the nanoparticle surface, they will eventually exchange with the less affinitive proteins that initially occupied the particle surfaces. Based on the nature of the study, the process is also surface dependent and it should be noted that there have been cases where proteins of the highest concentration may also have the highest affinitive properties to a given nanomaterial and will therefore adsorb first and have the longest residence time.
Figure 7.9 - a) Mean zeta potential (mV) vs time after plasma introduction to the sample (min). The samples were initially incubated with serum for 10 minutes at 37°C and plasma was subsequently added and the zeta potential measured at several time intervals. b) A schematic representation of the formation kinetics of a hard and soft corona dependent on the plasma/serum proteins present in solution, including protein displacement and exchange reactions. b1) particles incubated in serum, followed by introducing plasma to the sample for i) 5 minutes, ii) 10 minutes, iii) 15 minutes, and iv) 20-60 minutes. Figure is adapted from Blundell et al\textsuperscript{23}. Error bars represent standard deviation where n=3 independent replicates.

Figure 7.9i shows the corona coverage of the particles when they were immersed in serum for 10 minutes and plasma for 5 minutes where the serum proteins from the serum (as they have been incubated with the particles for longer) or proteins of higher concentration/affinity have formed the hard corona (pink) and the others have formed the soft corona (orange). After the plasma has been introduced for 5-10 minutes (7.9ii) the proteins present in the plasma, but not the serum (purple), become part of the soft corona layer. The introduction of additional proteins will shield the particle’s original surface charge and thus result in a smaller zeta potential. After 15 minutes of the plasma being present (7.9iii), some of the plasma proteins (purple) may have had an eventual higher affinity to the nanoparticle surface causing the original serum protein hard corona (pink) to be displaced and exchanged into the soft corona layer\textsuperscript{13,19}. The different proteins that have formed the new hard corona layer will have a different effect on the nanoparticle itself, which may be responsible for a reduction in the particle
velocities and thus zeta potential value. After 20 minutes and tailing off to 60 minutes (7.9iv), the zeta potential value started to become larger again suggesting there is less of a protein corona surrounding the particle surface. The low density of proteins now surrounding the particle may be due to the depletion of the soft corona that only incorporates weak protein-protein interactions. The most weakly bound proteins may dissipate into solution over time\textsuperscript{31} and may have been the proteins that were displaced from the original hard corona after being reversibly bound to the particle surface. As the proteins dissociate away from the nanoparticles, there is less hindrance on the particle’s transport and thus a larger zeta potential value is expected to be reported. These results are of interest as it shows how the behaviour of nanoparticles can change dependent on whether they come into contact with isolated or a complex mixture of proteins.

### 7.6 Conclusions and Further Work

This study has investigated the effects the main constituents of a protein corona have on a carboxyl nanoparticle surface. The effects on the nanoparticle behaviour varied dependent on the medium and complexity of the protein solution, whether the particles were immersed with isolated proteins diluted with PBS or in whole plasma/serum samples with a range of proteins. This is important for future bioassays regarding the protein corona as although the isolated proteins studied each affected the nanoparticle surface, particle transport needs to be investigated for samples containing composite protein mixtures that may replicate the composition in whole blood, in the human body.

Formation of the protein corona has been found to be protein dependent at 25°C as well as temperature dependent with an increased incubation temperature encouraging protein binding to the particle surface. Each isolated protein (albumin, γ-globulin, and fibrinogen) and the serum sample showed a large change in zeta potential when incubated with the nanoparticles at 37°C, whereas the plasma sample showed little change. The plasma did however, have a substantial effect on the zeta potential of particles that had previously been incubated with serum (at 37°C), demonstrating some of the protein exchange processes that are completed when competition between the protein compositions is introduced. Further work on this aspect would be to investigate more isolated proteins from plasma/serum including the apolipoproteins and other clotting factors, for example. Investigating these proteins individually as well
as in mixtures with each other will allow for further information and characterisation of
the proteins that are more likely to displace/exchange with others in the protein corona
formation process. It would also be interesting to analyse mixtures of the 3 most
common proteins found in the corona (albumin, γ-globulin and fibrinogen) to
investigate their interactions with each other in a less complex medium.

TRPS has easily identified the formation of a protein corona on a nanoparticle surface,
both the formation of stable hard/soft corona layers and monitoring weaker protein-
protein interaction and dissociation effects involved. The single particle analysis
approach has been advantageous to this study as the distribution analysis of a number
of samples has given a more detailed picture on the binding kinetics and affinities of
the proteins to the nanoparticle surfaces. Being able to monitor protein-nanoparticle
interactions in complex media and in physiological conditions is of great use to
therapeutic and diagnostic industries, as well as future bioassay/biosensor research.

7.7 Acknowledgements

I would like to thank Matthew Healey, a fellow PhD student in the research group who
helped with the plasma spiking experiment and helped support the study. I would also
like to thank Dr Muttuswamy Sivakumaran for sourcing and preparing the plasma and
serum samples from human blood.
7.8 References


Chapter 3 details a derived method for calculating zeta potential using TRPS analysis and has been optimised based on signal interpretation and calculating zeta potential relative to particle velocity as they traverse small pores. Measuring Zeta potentials has then been investigated with respect to DNA/protein packing density, DNA base length and structure, target analyte hybridisation time, influence of protein rich solutions, and impact of phage samples. Incorporation of a tunable pore into Coulter counter-based systems such as RPS allows for the pore stretch to be altered in real time to suit each sample during its analysis for optimised measurements to be carried out easily in real time.

It was seen that small differences in DNA base length, concentration, and structure could be discriminated in assay times as short as 30 minutes, see chapter 4. The level of sensitivity of the measurements were as low as differentiating between 15 bases in length and between two different dsDNA structures that in fact have the same DNA density (the mid-binding and end-binding partially complementary targets to the capture probe DNA). A method and technology that encompassed this level of sensitivity and short assay times is encouraging for medical and diagnostic fields. Another challenging aspect for these fields is the quantification and characterisation of small particles/molecules and chapter 5 summarises experiments designed to overcome these challenges. A controlled aggregation assay was devised and first demonstrated using a streptavidin-biotin interaction where 115 nm streptavidin coated particles were incubated with an excess of 70 nm biotinylated particles, a change in size and zeta potential was seen when the smaller particles saturated the 115 nm particle surfaces. The aggregation profile when the particle concentrations were equal was of interest as a small change in zeta potential was observed as well as a mean particle size increase to around 307 nm. This assay design was then applied to an aptamer-protein interaction where VEGF and an anti-VEGF aptamer were detected using zeta potential measurements with the relevant particle ‘tag’. The aptamer and protein particle modifications detailed in chapter 5 have proven successful for small analytes such as VEGF protein and results are encouraging for future bacteriophage analysis. The prospect of a small particle ‘tagging’ concept drives toward the possibility
of detecting and characterising several small molecules/entities that are a challenge to detect and characterise individually.

Applications are focused largely toward biomolecules in their respective media and natural environments. TRPS has the versatility to carry out measurements in a range of biological solutions. Currently bacteriophage, proteins, DNA, and surface-modified nanoparticles have been primarily addressed with avenues leading toward viruses, bacteria, and blood particulates, for example. Characterisation of the charge effects of bacteriophage and their behaviour in solution is limited so introducing experiments controlling the orientation of the bacteriophage will be an asset to these fields. Applications within biomedical and diagnostic fields as the developed technique can detect, quantify and characterise complex samples in real time without the need for a fluorescent label, isolation or purification. The technique can be further applied to environmental fields. Chapter 5 shows the size analysis of Salmonella phage, coliphage, and Clostridium difficile phage samples in their respective media. The analysis was extended to zeta potential measurements for the Salmonella and coliphage samples.

Chapter 6 describes other means of protein detection was to modify the pores themselves with a DNA aptamer and use the current rectification effects to detect and quantify VEGF, this method was compared with a particle assay using aptamer-modified particles and detection limits were both found to be in the picomolar region (18 pM and 5 pM for the pore and particle-based assays, respectively). There is a large scope for the analysis of rod-shaped particles to gain further information on how particles and molecules of this design may behave in solution with respect to the TRPS technique, and may provide more information on bacteriophage transport mechanisms in solution. The effect of counterions and the electrostatic forces within the nanopores is not yet fully understood and future experiments may allow for more information to be gathered on this particular subject to help expand the current rectification experiments and to continue the work on aptamer/protein modified pores for a label-free pore based assay negating the need for particles, possibly into a multiplexing context.

Further work is required to optimise multiplexed and multimodal particle analysis of samples containing three, four and beyond different sizes of particle. The multiplexing
aspect could also be expanded in versatility into the separation of particles with differing charges amongst a complex sample population. This would then lead to the thorough analysis of whole blood samples without the need for sample purification or sample clean up. The analysis of real blood is a profound experiment for the future of this technology in hand held point of care devices, for example. Further development of a method for multimodal sample analysis includes using numerous particles, each specific to a particular protein in a complex sample mixture. By optimising particle-based assays with aptamer capture probes, the dynamic range of analyte detection and characterisation will be expanded that could eventually lead to reduced non-specific binding effects in complex mixtures.

Chapter 7 shows examples of particle analysis in complex mixtures as carboxyl polystyrene particles were immersed in protein rich solutions, including plasma and serum leading to protein ‘corona’ formation around the particles. Size and zeta potential measurements were completed as a function of temperature and protein composition and it was found that a higher, physiological temperature (37°C) showed larger changes in zeta potential from particles in PBS in comparison to incubations completed at 25°C. The plasma samples also had larger effects on the particle zeta potentials that may have been due to the protein composition of plasma, compared with serum, and thus the differences in each respective protein corona formation. Of interest to this study were the results seen when particles immersed in serum were spiked with plasma and this showed a potential shift in the protein corona structure (dependent on higher/lower affinity proteins) and a displacement reaction was seen to take place and was indicated by the reduction in zeta potential upon addition of plasma to the samples. Further work would include further protein mixtures to be investigated to try and characterise fully the protein composition within a corona structure and report on which specific proteins have which inherent effects on the particle surfaces.

Inferring zeta potential from particle translocation velocities via TRPS measurements is a now a primarily developed method that has been applied to samples in biological media, plasma/serum samples, and protein and DNA modified particles. Tunable polymer-based nanopores have been used as a biosensor for a range of target analytes throughout this project, not only for particle-based assays, but also for target biological analytes through pore modification experiments with limits of detection in the picomolar region. The nanopores have been easily modified using a firstly reported
Layer-by-Layer assembly and can be functionalised with DNA aptamers to detect target analytes using current rectification measurements.

Aptamer development, the ability to analyse complex biological samples, understanding nanoparticle transport through pore systems and nanopore rectification properties are all important in biosensing toward the accurate and rapid quantification of biological analytes. TRPS technology can detect, quantify and characterise various analytes in a range of solutions in real time using pore and particle-based assays. The completion of zeta potential measurements from particle velocities in addition to this is particularly useful for the advancement of TRPS as a versatile analytical platform.
9 Appendix

A summary of PALS analysis and streaming potential measurements used to determine the size and zeta potentials of the particle standards, and the zeta potential of the polyurethane tunable pores, respectively.

9.1 Phase Analysis Light Scattering (PALS)

Phase analysis light scattering is used to determine size and zeta potential values of carboxylated polystyrene nanoparticles that are used as standards for zeta potential measurements and charge analysis using tunable resistive pulse sensing (TRPS).

Zeta potentials of CPC100 and CPC200 particles were measured on a Malvern Zetasizer Nano ZS. PALS analysis was completed to determine the average zeta potential of carboxylated polystyrene standards dispersed in PBS electrolyte. For these studies, the measured mean zeta potential of CPC200s, for example, was -20 mV. Please note that the PALS analysis for the calibration particles used in the following studies was completed by Robert Vogel (Izon Science Ltd, NZ, and University of Queensland, Australia).

9.2 Streaming Potential Measurements

Streaming potential measurements are carried out to determine the zeta potential of the regular conical nanopores used for each of the studies described in chapters 5-8. Streaming potential and current measurements of the thermoplastic polyurethane (TPU) pore membrane were made using a Surpass instrument (Anton Paar GMBH, USA). TPU membranes were cut to form a cylindrical cell with an adjustable gap and the streaming potential was measured for a range of applied pressures within a cyclic pressure sweep. The zeta potential was evaluated with the Surpass Visiolab software, applying the Helmholtz-Smoluchowski equation\(^1\). The zeta potentials were recorded as -11 mV.

9.3 References

## 10 Attended Conferences

<table>
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<td>Poster</td>
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<td>Faraday Discussion 175: Physical Chemistry of Functionalised Biomedical Nanoparticles</td>
<td>Bristol University, Bristol</td>
<td>Poster</td>
<td>17/09/2014-19/09/2014</td>
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<td>Loughborough University, Loughborough</td>
<td>Oral</td>
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<td>Salve Regina University, Rhode Island</td>
<td>Poster</td>
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Table 10.1 - Summary of conferences attended throughout PhD
11 Publications

Emergence of Tunable Resistive Pulse Sensing as a Biosensor
Emma L. C. J. Blundell, Laura J. Mayne, Emily R. Billinge, and Mark Platt
*Analytical Methods* 2015 7, 7055-7066
DOI: 10.1039/C4AY03023K

Particle-by-Particle Charge Analysis of DNA-Modified Nanoparticles Using Tunable Resistive Pulse Sensing
Emma L. C. J. Blundell, Robert Vogel, and Mark Platt
*Langmuir* 2016 32 (4), 1082-1090
DOI: 10.1021/acs.langmuir.5b03024

Characterisation of the Protein Corona using Tunable Resistive Pulse Sensing: Determining the Change and Distribution of a Particle’s Surface Charge
Emma L. C. J. Blundell, Mathew J. Healey, Elizabeth Holton, Muttuswamy Sivakumaran, Sarabjit Mastana, and Mark Platt
*Analytical Bioanalytical Chemistry* 2016 408 (21), 5757-5768
DOI:10.1007/s00216-D16-9678-6

Protein Detection using Tunable Pores: Resistive Pulses and Current Rectification
Emma L. C. J. Blundell, Laura, J. Mayne, Michael Lickorish, Steven D. R. Christie, and Mark Platt
*Faraday Discussions* 2016 193, 487-505
DOI:10.1039/C6FD00072J

Determination of Zeta Potential via Nanoparticle Translocation Velocities through a Tunable Nanopore: Using DNA-Modified Particles as an Example
Emma L. C. J. Blundell, Robert Vogel, and Mark Platt
*Journal of Visualised Experiments* 2016 116 (e54577)
DOI:10.3791/54577
Emergence of tunable resistive pulse sensing as a biosensor

Emma L. C. J. Blundell,† Laura J. Mayne,† Emily R. Billinge† and Mark Platt*

The article is written as a guide and tutorial that focuses on the use of Tunable Resistive Pulse Sensing, TRPS, as a platform for the detection of biological analytes. Within the field of biosensors there is a continuous emergence of new technologies or adaptations to platforms that push the limits of detection or expand dynamic ranges. TRPS is both unique and powerful in its ability to detect a wide range of biological analytes; including metabolites, proteins, cellular vesicles, viruses and whole cells. Each analyte can be analysed on the same platform without modification by changing the pore size, and is simple enough to follow to allow users from a range of backgrounds to start developing their own assays. The instrument can provide information regarding analyte concentration, size, and charge. Here we hope to give an overview of where this technology is being used and provide some guidance to new users, in the hope it will inspire and enable future experiments.

1. Introduction

In this tutorial review we highlight some of the current research and advances within the field of Resistive Pulse Sensing, RPS, focusing on an emerging variant of RPS using tunable pores, known as Tunable Resistive Pulse Sensing, TRPS. The review aims to provide an overview of its use of within the field of biosensors and provide hints and tips to encourage new users as they develop their own TRPS methods.

Modern day resistive pulse sensors trace their origins back to the Coulter counter, created in the 1940s to count and size biological cells and microorganisms. The principle is remarkably simple; two reservoirs are filled with conductive solutions, each containing an electrode, which are then separated by an aperture “the pore”. The sample is added to one of the reservoirs and an ionic current is passed between the electrodes and through the pore. If an analyte passes through the pore it occludes the ionic current causing a transient current decrease known as a “blockade event”. The magnitude of the blockade event provides the information needed to determine the size of the analyte, and the number of blockades per unit time provides information on the analyte concentration, Fig. 1. The size of the pore ultimately determines the sensitivity of the technique and thus the analyte that can be analysed, as a significant occlusion event is only observed when the analyte is comparable in size to the pore.

In the 1990s the Coulter counter was revived in the form of a biological nanopore sensor with the use of the z-haemolysin

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Fig. 1  (A) Sectional schematic of a pore. The sample is typically placed into the upper fluid cell. (B) Example of baseline current and ‘blockade’ events (current dips) that are each caused by an analyte traversing the pore. Each event is analysed for full width half maximum (FWHM) duration and $\Delta I_p$. (C) The Izon qNano instrument, showing the fluid cell, teeth and crucifix plastic membrane with aperture.

protein for the detection of ssDNA.\textsuperscript{4,5} The diameter of the haemolysin-pores is around 1.4 nm at its narrowest point and as the ssDNA passes through the pore, each of the 4 different bases produces a unique signal allowing the sequence of the DNA to be determined.\textsuperscript{5} With improvements in manufacturing, characterisation and nanofabrication techniques it became possible to reproducibly produce pores from the microscale down to the nanoscale in a range of materials. Solid-state nanopores often support more chemical versatility than biological equivalents, with carbon nanotubes,\textsuperscript{6} PDMS,\textsuperscript{7} glass,\textsuperscript{8} silicon,\textsuperscript{9} polycarbonate,\textsuperscript{10} and graphene\textsuperscript{11} having been used as substrates. Some of these materials have also been incorporated into fluidic devices.\textsuperscript{8} Here we don’t wish to review the synthetic procedures for preparing biological and solid state pores and we would direct the reader to reviews found elsewhere.\textsuperscript{2–4,9,12}

The ability to tune the pore size to the analyte of interest has allowed the RPS technique to detect analytes that range from single molecules, DNA, proteins, cellular vesicles to whole cells including viruses and bacteria, and again detailed reviews on the types of analytes and applications can be found elsewhere.\textsuperscript{13,14} One property that all solid state and biological pores share is the fixed nature of the pore size. Once prepared and assembled the pore size cannot be changed, thus pores which can be reversibly manipulated in real time offer great advantages in this field.

An elegant and novel adaptation to RPS incorporates a tunable elastomeric pore, which allows for further versatility as the pore can be stretched in real time to suit the sample.\textsuperscript{15} Tunable pores are fabricated by mechanically puncturing a thermoplastic polyurethane membrane. The membrane is mounted onto “teeth” in the instrument and then stretched in a controlled bi-axial, reversible manner to change the pore geometry, see Fig. 1.

The technique is currently referred to as Tunable Resistive Pulse Sensing, TRPS, but has been previously known as Scanning Ion Occlusion Spectroscopy, SIOS,\textsuperscript{16} and variants such as size-tunable pore sensors, or tunable elastomeric pore sensors are found in the literature. We will use the term TRPS in this review as an umbrella term to cover all of these variants unless a specific quote from a publication is used. TRPS has been developed to accurately determine the concentration, size and surface charge of dispersed inorganic particles and whilst we concentrate on biological analytes within this review, we direct the reader to key papers for the characterisation and identification of inorganic particles.\textsuperscript{17–20}

TRPS is much more versatile than solid state pore equivalents, but there are limitations to how much each pore can be stretched, thus users typically match a membrane with a pore size to the sample of interest. The company Izon Science Ltd supplies tunable pores, TPs, in a range of sizes, each with a wide optimal size range. This enables the user to match a pore to a specific sample. The pore used for a TRPS measurement

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Mark Platt graduated with a degree in Chemistry and Analytical Chemistry from the University of Salford in 2001. He moved into the area of liquid/liquid electrochemistry with Prof. R. A. W. Dryfe and obtained his PhD in 2004 from the University of Manchester. He spent time as a postdoc in Penn State (USA), Cambridge then Manchester (UK) and finally Dublin (Ire) before taking up an academic position at Loughborough University. His research interests sit at the interface between materials, electrochemistry and analytical chemistry developing sensors for health and wellbeing.
determines the size of the particle it can measure, the smallest pore commercially available is the NP100 that has a size limitation of 70–200 nm. This lower limit is determined by the smallest analyte size that can produce detectable blockades in relation to the background current noise. As these TPs are relatively inexpensive (tens of euros per pore) it has enabled laboratories from different disciplines to develop methods of analysis using RPS without the usual concerns of synthesis/breaking or blocking the pores. Passing complex biological samples through a small aperture often leads to blockages, and if the blockade is not removed the pore is unable to perform any further analysis; this is often the end of life for many solid-state pores. Along with its versatility to change the pore size to match the analyte the tunable pore, TP, also allows users to pause data capture if a blockade occurs, stretch and open the pore dislodging the trapped analyte, and then reduce the pore size back to its original size before continuing with the experiment. Alternatively the TPs are robust enough that trapped analytes can also be dislodged by tapping the instrument or by applying a pressure to the upper fluid cell, \( P_1 > P_2 \) (Fig. 1), effectively forcing the buffer and blockade through.

Given the appearance of simplicity, reduced cost and versatility, new users could be understood for thinking that the data analysis is simple. In fact the translocation of a particle through the pore is complex and some assumptions are made to simplify the analysis. Current methodologies of analysis and interpretation owe their thanks to Willmott, Vogel, Kozak and Trau who have led the way in modelling and understanding the TRPS technology.\(^{15,20–23}\) Models and methodologies exist within the literature for studying particle shape, charge, orientation and direction of transport.\(^{17,20,21–25}\)

Each translocation event reveals a large amount of information on the analyte, such as its size, zeta potential and shape. Whilst the data is capable of being extracted to interrogate it with third party software, the supplier of the instrument provides an interface (Izon Control Suite) for new users and where possible here in the tutorial we try to use the basic features of the software available to everyone.

For a new technology to be seen as enabling and to be adopted by the scientific community several key features are highly desirable; cost effectiveness, ease of use and accurate, reproducible data. In reality, detailed and accurate data can sometimes come with a cost, not always in price, but in effort required to extract the data. Setting up a TRPS measurement is simple, but getting reproducible data on consecutive runs requires the analyst to carry out the measurement carefully, attentively, and with a detailed level of understanding of the system. However, time and effort exerted during data collection is rewarded with the high quality of information. As can be found in cytometry technologies, particle-by-particle analysis of the sample leads to a much more accurate and sensitive assay.\(^26\) Since the first bioassay publications using TRPS in 2007 the number of applications and publications has doubled each year, herein we provide an introduction on how to set up the system, troubleshooting ideas and a review of the applications of the TRPS system.

## 2. Theory

The instrument most widely associated with TRPS is produced by the company Izon Science referred to as the qNano or qViro, and as with standard RPS equipment two fluid reservoirs are to be filled with a conducting electrolyte solution. In this setup the pore is mounted horizontally and reservoirs oriented above and below the pore membrane, with the sample typically placed into the top reservoir, see Fig. 1. The qNano uses an elastic size-tunable pore which is fabricated in a thermoplastic polyurethane membrane. The membranes are penetrated with a needle to create a single pore which is conically shaped.\(^7\) The size and geometry of the fabricated aperture can be modified by modifying the puncturing needle thus allowing the detection of particles ranging from 70 nm to 10 \( \mu \)m over the full range of manufactured pores.\(^6\) The cruciform TP is mounted by eyelets to teeth on the instrument, above the lower fluid cell, the system can be seen in Fig. 1C. The arms can be altered to increase the stretch on the pore, where it has been shown that applying a stretch of 10 mm to the membrane increases the pore opening by 54%.\(^2\)

### 2.1 Analyte size

The conical pore gives rise to an asymmetric current pulse, Fig. 1, with resistance highest at the narrowest pore constriction resulting in a sharp drop in current which tails back toward the original baseline value as the resistance diminishes toward the base of the pore.\(^28\) For a conical pore, the change in the resistance, \( \Delta R \), across the length of the pore, \( L \), is given by eqn (1),\(^28\)

\[
\Delta R = \rho \int_0^L \frac{dz}{A(z)} - R
\]

where \( \rho \) is the resistivity of the electrolyte that is filling the pore, \( A(z) \) is the cross sectional area perpendicular to the pore axis \( z \) and \( R \) is the pore resistance. When no blockade is present, \( R \) is given by eqn (2),\(^28\)

\[
R = \frac{4L\rho}{\pi D_L D_S}
\]

where \( D_L \) and \( D_S \) are the largest and small pore diameters. When a particle traverses through the pore, a blockade event is observed. This blockade is created by the particle displacing a volume of electrolyte which in turn increases the resistance in the circuit, temporarily lowering the current. The blockade magnitude can then be used to size the particles or analyte as the magnitude of the increased resistance is directly related to the size of the analyte. Unlike solid state pores where the size of the pore is always known, the tunable pore must first be characterised before users can accurately determine the size of the analyte using TRPS. This is done using calibration beads of a known size and narrow size distribution and must be done prior to sample analysis and under the same conditions.

### 2.2 Analyte concentration

The frequency of the pulses, \( f \), can be related to the concentration of the analyte, \( C_a \), as well as the velocity of the traversing

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particle, \( v_p \). The velocity term is the sum of the fluidic, \( v_f \), electrophoretic, \( v_e \), and electroosmotic, \( v_o \), velocities, i.e. \( v_p = v_f + v_e + v_o \). Here we typically ignore the contribution from diffusion due to the magnitude of other forces and end effects are not taken into account in the analysis. \( v_p \) can be written as:

\[
v_p = \frac{Q}{\pi \left( \frac{D_h}{2} \right)^2} + \frac{\varepsilon \zeta_{\text{particle}}}{\eta} E - \frac{\varepsilon \zeta_{\text{pore}}}{\eta} E
\]  

(3)

where

\[
Q = \frac{3\pi D_h^3 \Delta P}{128\eta \left( D_h - D_S \right)}
\]  

(3a)

\( \varepsilon \) and \( \eta \) are the permittivity of the solution and kinematic viscosity respectively, \( \Delta P \) is the pressure across the pore, \( \zeta_{\text{pore}} \) and \( \zeta_{\text{particle}} \) are the zeta potential of the channel surface and particle respectively, and \( E \) is the electric field. The pulse frequency, \( f \), is then related to both the velocity and the particle concentration, \( C_0 \) via the equation; \( f = C_0 \times v_p \). For the TRPS system it has been demonstrated that the forces of electrophoresis, electro-osmosis and pressure are usually dominant.\(^{1,15,22,29}\)

### 2.3 Varying the pressure across the pore

The reader will note that the pulse frequency is related to the pressure difference across the pore. With the Izon system there is always an inherent pressure head guaranteed within the setup due to gravity, indicated with \( P_1 \) and \( P_2 \) in Fig. 1A. Using the variable pressure system supplied with the instrument users can vary the ratio between \( P_1 \) and \( P_2 \) across the pore. Willmott \etal., showed that pressure-driven transport can be made dominant,\(^{23}\) which has advantages for studying zeta potential values. It also helps data analysis with samples that contain a low concentration of analyte. Such samples would typically have a low particle count rate, and thus would result in long run time. By applying a pressure to the cell, \( P_1 \gg P_2 \), analytes can be driven through the pore more frequently reducing the total run time. Alternatively if a sample is too concentrated, producing blockade events that are not clearly resolved from one another, or the particles are moving at too great a velocity, reducing the information and signal within the peak width, it is possible to slow the blockade events by the application of vacuum, \( P_2 \gg P_1 \). As a guide the particle rate should ideally not exceed 1000 particles per min, with an optimum range between 500–700 particles per min.

Pressure ratios are controlled by the variable pressure module, VPM. The VPM has a mobile ‘arm’ with a scale of 0–20 cm. The amount of pressure applied is relative to the length of the arm \( e.g. \) if the arm is inserted 5 cm into the system, a pressure of \( 5 \text{ cm}^2 \) is applied. For a negative pressure, \( P_2 \gg P_1 \), the same arm is pulled out of the instrument to the required length in cm. Each cm of pressure is equivalent to approximately 1000 Pa.\(^{21}\) The unit of cm will be used for the remainder of the review when denoting a pressure applied to the TRPS system.

### 2.4 Zeta potential theory

Evident from eqn (3) is the relationship between particle velocity and its zeta potential. Zeta potential is defined as the electrostatic potential at the border between the diffuse layer and compact layer\(^{10}\) (also known as the Stern layer)\(^{30}\) of a colloidal system. Zeta potential is related to the surface charge of the particle and is often used as an indicator of colloidal stability. It also offers a unique and additional parameter to identify biological analytes when their sizes are comparable, as Martin \etal., demonstrated by measuring the velocity of several proteins using a solid state pore system.\(^{25,33}\) The Smoluchowski approach\(^{34}\) defining zeta potential is displayed in eqn (4), where \( \zeta \) is zeta potential, \( \eta \) is dynamic viscosity of the fluid, \( \mu \) is particle mobility and \( \varepsilon \) is the dielectric constant.

\[
\zeta = \frac{\eta \mu}{\varepsilon}
\]  

(4)

The Smoluchowski approach supports that a particle’s zeta potential can be determined from its velocity taking into account convective and electro osmotic forces, as well as the electrophoretic mobility of the particle. The electrophoretic mobility is a measure of the translocation time of the analyte through the pore under an applied electric field, the convective forces are attributed to the flow of the solution through the pore due to gravity and any applied pressures; whereas the electro osmotic forces relate to the flow of liquid through the pore which arises from the charge on the pores surface and the movement of liquid in the electric field. In their seminal paper Vogel and Willmott \etal., developed a method of balancing the electro osmotic and electrophoretic effects by balancing the pressure across the pore allowing zeta potential values to be extracted from the resistive pulse, their methodology is shown in Fig. 2.\(^{25}\)

- **Fig. 2** Schematic of the variable pressure set up used with the qNano pore sensor (a). Pressure in the top fluid cell (b) is precisely controlled via a flexible tubing connection (c) by varying the height difference between the water level in a partially submerged buret (d) and the water level of a large water reservoir (e). The bureet was equilibrated with atmospheric pressure by opening a valve (f). Progression of an experiment where pressure is varied from positive to negative (g). Reprinted with permission from Anal. Chem., 2012, 84(7), 3125–3131. Copyright (2012) American Chemical Society.
### 2.5 Setting up TRPS

TRPS is a relatively simple set up procedure, and users can find the protocol within the user manuals, here we include some useful points that we have adopted as part of our best practice.

The pore membrane is connected to the system via the four arms, Fig. 1, and the desired pore stretch can be obtained (>43 mm, 43 being the smallest distance between the teeth applying no stretch to the membrane), the electrolyte is then placed in the lower fluid cell (75 μL). Care needs to be taken to ensure no bubbles are introduced into the electrolyte, if bubbles do occur the electrolyte needs to be replaced. If no bubbles are present, the upper fluid cell can be placed on top and twisted into place. Once the upper fluid cell is connected and before you place liquid into the upper cell switch on the instrument. The current should be ~0.5 nA and stable. If the current is drifting or unstable, there may be a fluid leak in the system and the instrument needs to be turned off, taken apart, cleaned, and dried thoroughly. The electrolyte solution can then be placed into the upper fluid cell (40 μL). The Faraday cage is placed over the fluid cell to reduce background noise and the system is switched on using the computer software. Upon the application of a voltage a stable baseline should be observed. Sufficient voltage needs to be applied to ensure a baseline current > 50 nA. If the baseline is fluctuating rapidly or not settling at a current, it is not stable and a number of troubleshooting methods can be applied (Table 1).

### 3. Applications of TRPS

#### 3.1 Cells/bacteria

Optical density, OD, measurements are commonly used in monitoring bacterial growth due to its ease of implementation. The OD measurements allow the bacterial growth to be followed in real time, however the accuracy of these measurements can be hindered by low sensitivity. OD measurements use an approximation of the cell number and cell size by changes in the light scattering from the sample. Plate counts and microscopes are employed when an account of the bacteria present is needed; these are usually time-consuming and laborious as a stain on the bacteria is usually needed and requires manual counting. Other modern bacterial growth monitoring methods such as flow cytometry and microscopy are also common in the literature. Allen et al., developed a method using TRPS to monitor the growth of two commonly used bacteria; Gram-positive *Bacillus subtilis* str.168 (BSU168) and Gram-negative *Escherichia coli* str. DH5α (DH5α), TRPS was used to monitor the bacterial cell concentration and cell volume, both of which can be analysed simultaneously, see Fig. 3. The cell volume dynamics and the level of growth can be tracked in real time.

#### Table 1 Reference guide to problems and solutions

<table>
<thead>
<tr>
<th>Problem</th>
<th>Solution</th>
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| Introduced particles but can’t see peaks | - Check pore classification is appropriate for size of interest  
- Reduce stretch (minimum 43 mm)  
- Sample may be too concentrated, clean pore and dilute sample 1 : 100; if resulting particle rate is too slow apply pressure |
| Frequent blockages | - Clean pore thoroughly  
- Check for bubbles in both fluid cells  
- Ensure sample is well dispersed – try vortexing, sonicating or the addition of a surfactant – or all three!  
- Increase pore stretch or change to a larger pore if necessary  
- Try additional pressure |
| Unclean pore | - Increase stretch and voltage where possible  
- Replace the sample/electrolyte with fresh electrolyte buffer several times  
- Apply pressure/vacuum to the pore to dislodge any particles ‘stuck’ in the pore |
| Rate trace not linear | - Add pressure for calibration files  
- Check for bubbles  
- Sonicate and vortex sample  
- Dilute sample to reduce risk of blockages |
| Bubbles frequent | - Possibility of too much surfactant, reduce concentration  
- Warm working conditions (>30 °C) can result in bubbles due to gradual evaporation of buffer |
| Current rises rapidly/saturates | - Fluid leak, remove fluid cell from the TRPS instrument and dry thoroughly  
- Decrease applied voltage |
| Current drops rapidly/Current drops to zero | - Pore may be partially blocked, troubleshoot to remove the blockage, increasing the stretch and applying a pressure/vacuum can aid this process  
- Air bubbles in the upper or lower fluid cell. Remove the liquid from the appropriate fluid cell and replace ensuring no bubbles are present |

bacterial cell chain formation can be observed by tracking the spread of the cell volume histogram. Coefficient of variation, CV, was used to measure the level of dispersion and precision of the concentration measured by TRPS where a CV lower than 5% is considered acceptable; CVs observed for the samples were lower than 2%. The low CV was a good indicator that TRPS is reliable for measuring the concentration of the bacteria present. When using TRPS, a measurement can be made within a few minutes which is in contrast to traditional colony-plating methods which involve long preparation steps of agar plates, incubation and counting. The concentration measured by TRPS was different than the concentration measured by OD and colony plating, which is highlighted in Fig. 3. The difference is due to TRPS counting all the cells present in the liquid and cannot differentiate between dead and live cells as colony plating does; in addition cell counting methods are often approximated due to the chosen field of view.

3.2 Viruses

Sub 100 nm sized particles are generally a challenge for many nanoparticle characterisation techniques. Viruses are infectious agents made up of nucleic acids and are particularly valuable in scientific and medical research. The virus’ genetic material is contained within a protein shell (capsid) and this as a whole is known as a virion or virus particle, VP. Virus like particles, VLPs, are also available with similar capsid properties to VPs. VLPs are self-assembled protein structures with similar, and sometimes identical, structure to their resident virus and can be applied to fields including gene therapy and vaccine development. VLPs have gained further interest in the development of nanomaterials because of their small size (10–200 nm), construction flexibility and structural uniformity.

Analytical tools for the detection and characterisation of viruses and VLPs are constantly being developed and improved. These included ELISA, real-time PCR, loop-mediated isothermal amplification (LAMP), multiplex tools incorporating bead arrays, and next generation sequencing (NGS). One of the main challenges for virus detection comes from the small sizes of virions and VLPs. Small particle analysis by TRPS systems has been developed recently by Vogel et al., successfully detecting and sizing virions 70–95 nm in diameter, producing highly reproducible data in agreement with both optical methods and TEM (within 6%). Optical methods are common but are generally averaging techniques that don’t allow for the versatility of particle-by-particle analysis available from TRPS.

The ability to detect individual 70 nm particles is an exciting prospect for nanotechnology and current TRPS developments are allowing for even smaller particles to be analysed on a particle-by-particle basis.

3.3 Cellular vesicles

Cells release numerous types of membrane particles under physiological and pathological conditions. Originally thought to be an artefact of the body, research is now demonstrating that these microvesicles, MVs, may be part of the cell signalling process, facilitating cellular messaging and the exchange of RNAs which may even precede the release of protein inflammation markers. MVs have recently seen a surge in interest as they may act as clinically relevant biomarkers and their properties such as size, concentration and composition could provide important physiological information and be of potential use in early diagnostics. Although their prevalence or characteristics may be well tied to a range of disorders, a gold standard technique for their characterisation has yet to emerge. MVs range in size from around 20 nm to 1 μm and at current there is a lack of availability of techniques which can accurately characterise MVs in terms of their size and concentration with concentration analyses largely varying from instrument to instrument due to the working range of the method in question and differences in the minimum detected size.

For many years the gold standard of particle characterisation in terms of size accuracy has been electron microscopy. However, these methods have severely limited abilities to quantify their concentration. As the concentration of circulating MVs has been reported to vary in several different physiological states such as hypercholesterolaemia, ath erosclerosis and even exposure to pollutants, the lack of accurate concentration analysis is a significant limitation, requiring a second method to attain concentration data. Flow cytometry is a high-throughput method for identifying and quantifying analytes
based upon the scattering of light or the fluorescence signal of the particles. Whilst flow cytometry has previously been used to quantify MVs, a lack of resolution below 200 nm lends bias to any size or concentration data as many subtypes of MV are believed to lie below this threshold.  

The application of TRPS within the field of MV characterisation is highly advantageous due to the ability to easily fit a range of pores which can detect particles as small as 70 nm providing a particle-by-particle analysis to elucidate concentration. By using several pores sequentially it is possible to analyse a wide range of sizes of MVs and due to particle-by-particle analysis, generate an accurate size distribution despite the polydispersity of the sample. Using TRPS makes it possible to attain accurate size and concentration data for a wide range of vesicles, and in conjunction with complimentary techniques a rich level of information can be obtained. Szabó et al., investigated the cell-signalling potential of MVs and conducted an extensive study using a wide range of techniques to gain a full picture of the effects of MVs on the gene expression of recipient cells, see Fig. 4. TRPS was used to determine the concentration and size distribution of the MVs using two membranes. It was found that recorded size distributions by TRPS were comparable to values obtained by SEM and TEM with the mean diameter lying at around 350 nm.

In further support of TRPS as a new tool in MV characterisation, Connolly et al., measured low-density-lipoprotein (LDL) cholesterol linked to familial hypercholesterolaemia using TRPS for the MV size and concentration, flow cytometry to determine MV origin, and gas chromatography to monitor the fatty acid composition of the MVs; this multifaceted approach is likely the best way to gain a full view of the vesicles being investigated. This study was able to monitor the clinically relevant progression of apheresis treatment for hypercholesterolaemia by monitoring MV concentration by TRPS.

3.4 Particles based bioassays – DNA extraction/detection

Nanoparticle based bioassays are being increasingly developed for point-of-care assays. The immobilisation of biological components onto their surfaces allows them to be used as drug delivery agents, bioimaging substrates or to be incorporated into a range of sensing technologies. The development of superparamagnetic beads, SPBs, and magnetophoresis devices have made it possible to rapidly and efficiently separate cells, proteins and DNA from complex mixtures, such as, plasma, urine, and culture media, in a manner that does not require complicated equipment. By functionalising the SPBs surface with DNA they can be used to extract target DNA from solution. TRPS is particularly well suited to the detection of DNA on the surface of particles. Although there exists a wealth of available technologies to sequence and characterise DNA samples with high accuracy, at current many of these techniques require PCR, gel electrophoresis and fluorescence which are contributors to lengthy processing and expense involved in DNA analysis.

Several groups have engaged in work with RPS and TRPS technology to quantify DNA hybridisation and to monitor particle surface modifications. The benefits of using TRPS technology over conventional techniques is its ability to generate a label-free signal, fast run time (in most cases sub 5 minutes). Booth et al., devised a method which incorporated the use of an applied vacuum to be able to elucidate zeta potential of beads under several different conditions. By applying a positive potential bias across the pore, polyanion DNA lends negative charge to the beads which in turn increases electrophoretic mobility. As a vacuum is applied and is gradually
The frequency of particles moving through the pore slows, stops and then eventually reverses as beads are pulled back up from the underside of the pore. This inflection point is relative to the surface charge of the beads as when negative charge is increased the amount of vacuum applied must also be increased to overcome the electrophoretic mobility of each particle.\textsuperscript{21,67} Using this variable pressure method it was possible to measure the increased charge loading resultant from DNA hybridisation.

An additional method utilising the hybridisation of DNA concerns the specific creation of aggregates using complementary sequences to join groups of beads together.\textsuperscript{50} Agglutination assays are easily adaptable to TRPS, aggregate size is able to be easily determined by monitoring the increase in volume as each aggregate passes through the pore\textsuperscript{48,65} via alterations to the magnitude of the peak and the frequency of particles through the pore and has been employed for the study of Au particle agglutination.\textsuperscript{79} Previous work employs fluorescence and light scattering that both require an additional label for analysis,\textsuperscript{77} whereas in TRPS the aggregate is in effect the label and there is no need for additional markers; in this sense it is “label-free”. An additional consideration in the use of aggregation assays is the ability to bring each of the components into close proximity, one such way to do this is the employ SPBs as outlined in a proof-of-concept assay in which 1 and 3 \(\mu\)m SPBs were coated in avidin and then incubated in the presence of a range of concentrations of biotin.\textsuperscript{69} It was found that the application of a permanent magnet and rolling of sample vials caused increased aggregation due to the beads being brought into closer proximity to each other, this action was termed magnet assisted aggregation (MAS).

A similar technique has been used \textit{via} TRPS to monitor single nucleotide polymorphisms (SNPs) using the highly selective aggregation of AuNPs. This was completed by using probes with a controlled number per AuNP and with a specific sequence and length such that aggregation occurs only in the presence of a complete complement target,\textsuperscript{63} see Fig. 5. The use of TRPS was advantageous over the use of a solid state pore for several reasons worth discussing – firstly, the study of potentially large aggregates poses the threat of blockage; however, by being able to stretch the pore in real time it is possible to temporarily pause recording, open the pore, allow the blockage to pass, restore the desired stretch and resume recording. In addition, in this study it was possible to tune the applied stretch so that single particles are not visible above the level of baseline noise and only aggregates are visible. In theory this proof-of-concept method could be utilised to target any desired SNP by tailoring the capture probes of choice.

![Fig. 5 Schematic illustration of nanopore-based single-nucleotide detection using a nAu–DNA probe. The ssDNA sequences on nAu-100b and nAu-18b probes were designed to be complementary to the mutant (mut) sequence and single mismatched to the wild-type (wt) sequence. In the presence of a perfectly matched (PM) target, a well-mutant (mut) sequence and single mismatched to the wild-type (wt) sequence.](image)

### 3.5 Confirming the DNA is on the particles

Due to the negative sugar–phosphate backbone, functionalising DNA onto nanoparticles can alter their behaviour within TRPS due to a change in their surface charge. As the surface charge of the bead directly impacts upon the pulse frequency, eqn (3), and the pulse width, FWHM, it is relatively simple to confirm the presence of DNA on the beads. To do this user must first ensure that the increase in pulse frequency is due to the presence of the DNA and not caused by a change in particle concentration. First their concentration is to be verified using calibration beads with the application of large pressure \(i.e. P_1 \gg P_2\) Fig. 1, typically done using a pressure greater than 5 cm on the pressure module. This pressure ensures that the dominant force acting upon the beads is the fluidic component of eqn (3). Once the concentration has been found, a sample of beads before and after the modification of DNA is prepared to the same concentration. Both samples are then run under no additional pressure (0 cm) at a range of voltages to produce a plot of pulse frequency vs. voltage, see Fig. 6. The presence of DNA on the particle is confirmed when there is a larger change in rate as a function of voltage.

3.6 Particle–aptamer–protein studies

Many traditional bead-based protein assays have revolved around the use of antibodies. Where previously antibodies have been the capture probe of choice, aptamer technologies are gaining interest.\textsuperscript{72–75} Aptamers are conventionally generated through the process known as SELEX\textsuperscript{76–79} (systematic evolution of ligands by exponential enrichment), where strong binding sequences are evolved/enriched from extensive libraries, or by CLADE (closed loop aptameric directed evolution) which produces the aptamers ‘on-chip’.\textsuperscript{80–82} Due to their comparable selectivity, stability and cost; over the last two decades, aptamers have started to challenge antibodies in their use on many technology platforms. As discussed above, the modification of particle surfaces with aptamers should be a process well suited to TRPS technologies.

Billinge \textit{et al.}, demonstrated TRPS as a label-free detection platform for the detection of the thrombin protein using SPBs coated with thrombin aptamer.\textsuperscript{83} When the thrombin protein was introduced to the aptamer-coated beads, a decrease in pulse frequency was observed. The isoelectric point of thrombin lies at pH 7.1,\textsuperscript{84} suggesting that in pH 7.4 PBST buffer, as used in the study, the overall surface charge of the thrombin molecule should be largely neutral. The shielding of the negative DNA aptamer with the protein reduced the electrophoretic mobility of the bead moving through the pore, reflected in a reduced frequency, $J$, and increased FWHM.

Alsager \textit{et al.}, developed an assay to 17$\beta$-estradiol (E2) using aptamer-coated carboxyl beads monitored with Dynamic Light Scattering (DLS) and TRPS to observe changes in zeta potential. In addition to the changes in electrophoretic mobility observed of ligands by exponential enrichment), where strong binding sequences are evolved/enriched from extensive libraries, or by CLADE (closed loop aptameric directed evolution) which produces the aptamers ‘on-chip’. Due to their comparable selectivity, stability and cost; over the last two decades, aptamers have started to challenge antibodies in their use on many technology platforms. As discussed above, the modification of particle surfaces with aptamers should be a process well suited to TRPS technologies.

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\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure6.png}
\caption{Example of the relationship between applied voltage and particle rate, for beads of identical concentrations with and without the DNA on their surface.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure7.png}
\caption{Agglutination assay data collected at a stretch of 45.5 mm and potential of 0.14 V, where red (i) indicates $i_p$ and blue (ii) indicates FWHM. (A) Variation in $\Delta_i$ and FWHM for 400 fM AuNi rods (1.23 $\mu$m long with CV 20%, Ni content 15% by length) as assay time is increased in the absence of an analyte. (B) The same rods as (A) at assay time 10 min. Ni segments are functionalized with avidin and the concentration of the biotinylated-BSA analyte is varied. Dashed lines represent a 10 min assay with a non-biotinylated target. (C) A biotin–avidin assay at 10 min as in (B), using 500 fM AuNiAu rods (0.82 $\mu$m long with CV 14%, Ni content 18% by length) in the side-on configuration. (D) 150 fM AuNi rods (1.1 $\mu$m long with CV 20%, Ni content 14% by length) at assay time 10 min. Ni segments are functionalized with PDGF aptamer, and the analyte is PDGF. The circled data points at 100 fM indicate the change in FWHM and $\Delta_i$ for the same rods using a control protein. Error bars show the d25 and d75, values for each data point, lines joining data points are drawn to guide the eye. Reprinted with permission from Small, 2012, 8, 2436–2444 ©2012 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim.}
\end{figure}
by Billinge et al., a change in the size as the aptamer bound to its target was also observed. TRPS was able to resolve a 20 nm increase in the mode diameter of beads once the aptamer had been bound to the beads, followed by a reduction in size as the target analyte E2 was introduced.65

Additional aptamer-bead based assays are able to be designed such that if an analyte contains multiple binding epitopes, aggregate formation can be monitored. Platt et al., used a specific class of nanomaterial termed nanorods to detect femtomolar levels of homodimeric PDGF-BB by monitoring specific aggregation of nanorods.66 In the same study the authors also demonstrated the ability to generate aggregates of specific orientations and differentiate between these in the presence of different analytes; see Fig. 7.

TRPS has also been used to obtain kinetic information for aptamer–target interactions, and recorded results similar to traditional SPR techniques.67 A recent development of this TRPS-based assay takes advantage of the excellent size discrimination inherent in a particle-by-particle system. Billinge and Platt have demonstrated the ability to successfully analyse the binding of two aptamers to their respective proteins simultaneously; by using the bead size as a label, two different populations of beads were selectively coated with aptamer and incubated with a combination of different protein concentrations.68 These key proof-of-concept works demonstrate the ability of one label-free approach to measure virtually any aptamer target by tailoring the aptamer of choice.

4. Conclusions

TRPS is a technology platform that is becoming more widely accepted amongst analytical laboratories as a common platform for analysis, one of the reasons being the many variants of samples and analytes that can be analysed. Current TRPS users have contributed greatly towards the validation of the technique for future studies with the technology. In our opinion we are at a tipping point where the data from TRPS no longer needs to be verified alongside DLS, TEM or other technologies. We predict that as this technique continues to develop within the individual fields of MV, protein and DNA analysis, TRPS will become more capable of multiplexing across different ohmic fields, and it is this exciting possibility that makes TRPS an exciting platform to work with.

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Particle-by-Particle Charge Analysis of DNA-Modified Nanoparticles Using Tunable Resistive Pulse Sensing

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ABSTRACT: Resistive pulse sensors, RPS, are allowing the transport mechanism of molecules, proteins and even nanoparticles to be characterized as they traverse pores. Previous work using RPS has shown that the size, concentration and zeta potential of the analyte can be measured. Here we use tunable resistive pulse sensing (TRPS) which utilizes a tunable pore to monitor the translocation times of nanoparticles with DNA modified surfaces. We start by demonstrating that the translocation times of particles can be used to infer the zeta potential of known standards and then apply the method to measure the change in zeta potential of DNA modified nanoparticles. By measuring the translocation times of DNA modified nanoparticles as a function of packing density, length, structure, and hybridization time, we observe a clear difference in zeta potential using both mean values and population distributions as a function of the DNA structure. We demonstrate the ability to resolve the signals for ssDNA, dsDNA, small changes in base length for nucleotides between 15 and 40 bases long, and even the discrimination between partial and fully complementary target sequences. Such a method has potential and applications in sensors for the monitoring of nanoparticles in both medical and environmental samples.

1. INTRODUCTION

The immobilization of oligonucleotides onto surfaces is a key design to many technologies within DNA sequencing,1,2 DNA–protein interactions,3−5 biosensing,6−9 and targeted drug delivery.10−12 The functionalization of DNA onto nanoparticle surfaces is now a common practice, and within the field of biosensors alone the number of strategies for immobilization, type of nanomaterial, and detection platform are varied enough to fill several reviews.13−19 One family of nanomaterials favored with purification strategies is superparamagnetic particles, SPPs. These particles allow for the removal of specific analytes from complex sample matrices using nothing more complicated than a hand-held magnet,14,20−25 and the use of SPPs has become increasingly common. When they are incorporated into fluidic devices they can be used to continuously sort cells and DNA from liquids24 and are integrated into a variety of detection platforms.25

When using nanomaterials in bioassays, the material must remain suspended in the solution for it to capture the analyte. A particle’s surface chemistry design is important to avoid sedimentation of irreversible aggregation; there are two mechanisms available to prevent this. First is the use of steric stabilization by placing a neutral polymer onto the particle surface, and the second depends upon charge stabilization whereby the repulsive Coulombic forces overcome the attractive van der Waals forces.26−27

For charge stabilized particles, a typical measurement used to represent the surface charge, and infer stability, is zeta potential. The zeta potential represents the value of the electrostatic potential at the plane of shear and typically for nanoparticle systems, zeta potential values of ±30 mV are representative of stabilized particles.28 When a polyelectrolyte, such as DNA, is immobilized onto the surface of the nanomaterials the DNA can take on two roles. The first is the more natural of the two as a capture probe, designed to hybridize to target DNA. The second is a passive role where the inherent charge on the phosphate backbone can act as a stabilizer by creating a high charge density on the particle surfaces, helping suspend them in solution.29 In doing this it is important to consider the structure of the DNA immobilized onto a nanomaterial’s surface. Single-stranded and double-stranded DNA varies in persistence length, which affects the stability and flexibility of the polymer when immobilized to a surface. For example, dsDNA has a 50-fold higher persistence length than ssDNA,30,31 making it a far more rigid polymer. As well as the persistence length, the

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contour length also varies between ssDNA and dsDNA, and both these parameters will affect the plane of shear and thus zeta potential.32

When using nanoparticle systems a mean population zeta potential will not allow the true measure of the ligand distribution across all of the particles to be interpreted, and in a typical reaction the ligand density would follow a Gaussian distribution.53–55 The spread of the population can have an effect on the reaction kinetics, stability and sensitivity of nanoparticle based assays.36–38 To build up a true measure of the spread of zeta potential values for a given particle population, the zeta potential of each individual particle has to be measured, and this aspect is challenging, although electrophoretic and electrochemical techniques allow insight into these measurements.39,39 Electrophoresis studies have demonstrated the ability to separate ssDNA and dsDNA modified particles, and probe the structure of the ssDNA surfaces.40–42 Alternative technologies for monitoring particle-by-particle zeta potentials rely upon particle tracking technologies that monitor the speed of the particles in an applied electric field.43

A relatively recent technology to be developed for the characterization of nanoparticles is based upon tunable resistive pulse sensing (TRPS).44–51 TRPS is based on polyurethane elastomeric membranes in which the pore geometry can be altered in real time. The brief set up and theory for TRPS technologies is as follows: a stable ionic current is established by two electrodes, separated by a pore; as particles/analytes translocate the pore they temporarily occlude ions, leading to a transient decrease in current known as a “blockade event”, examples of which can be seen in Figure 1a. In the TRPS arrangement used here, the pore is mounted laterally so that particles typically move from the upper fluid cell into the lower fluid cell, aided by an inherent pressure head due to 40 μL of liquid in the upper fluid cell of approximately 50 Pa,52 and a positive or negative bias is applied via an electrode under the pore. By monitoring changes in blockade width or full width half-maximum (fwhm), blockade magnitude (ΔΔt) and blockade frequency (events/min) it is possible to elucidate the zeta potential,53,54 size,49 and concentration10 of colloidal dispersions in situ.19

The methodology for measuring zeta potential using RPS technologies has seen an evolution of techniques,44,54,55 and here we use a similar concept as was published by Arjmandi et al. using pyramidal pores.56 In brief, a calibration based zeta potential method is applied, based on the measurement of signal durations of translocation events as a function of voltage. The electrophoretic mobility is calculated from the derivative of medium particle velocity and applied electric field. The zeta potential of each particle can then be obtained from the measured electrophoretic mobility using the Smoluchowski approximation.44,57 The calculated zeta potential only depends on the measured pulse duration and is independent of the magnitude of the pulse, meaning that simultaneous size and charge measurements can easily and reliably be carried out. TRPS’s particle-by-particle nature means that subpopulations with different zeta potential are able to be resolved, while ensemble methods such as phase analysis light scattering or PALS will only report an average zeta potential.

We adapt and apply the theory by first demonstrating its use with calibration particles and then move to measure changes in zeta potential for DNA modified nanoparticles. We go on to measure the change in zeta potential as a function of DNA concentration on the particles surface. We observe that the measured zeta potential is correlated to the concentration of DNA, and as the technique also provides a particle-by-particle analysis, the distribution of the zeta potential across the sample population is also produced. As we increase the concentration of DNA, a more symmetrical Gaussian distribution of charge is produced, indicating a more uniform ligand distribution around the nanoparticles. By measuring the zeta potential and shape of the distribution, we go on to measure the effects of oligonucleotide length and apply our method to the detection of dsDNA. By controlling the packing density of the capture probe (CP) on the particle surface and the mechanism by which the CP hybridizes to the target, the sensitivity of the instrument can allow for the detection of target DNA in assay times under 30 min. Finally we demonstrate that by designing the length and position of the complementary section to the target we can improve the signal and detection.

The method will have an impact on designing particle based assays and the technology shows potential to study zeta potentials on biological analytes, with clear applications in fields of bioassays; as well as the monitoring of nanomaterials in nanotoxicology and nanomedicine where a clear understanding of the particle surface charge and size can have an influence on the efficiency and toxicology of particle based drugs.

2. MATERIALS AND METHODS

2.1. Chemicals and Reagents. The buffer used was phosphate buffered saline with Tween-20 as a surfactant (1 × PBST (0.01 M phosphate buffer, 0.0027 M Potassium Chloride, 0.137 M Sodium Chloride, pH 7.4 with 0.05 (v/v)% Tween-20 in 200 mL deionized water (18.2 Ω cm))). PBS tablets (P4417) and Tween-20 (P1379)
were purchased from Sigma-Aldrich, U.K. Streptavidin coated superparamagnetic particles (120 nm, 4352 pmol/mg binding capacity, product 03121) were purchased from Ademtech, France.

2.2. Carboxyl Polystyrene Particle Standards. Carboxylated polystyrene particles with a mean nominal diameter of 220 nm were purchased from Bangs Laboratories, U.S. and are denoted as CPC200. The specific surface charge as determined by the manufacturer was ∼86 μeq/g, equivalent to a surface density of 3.2 × 10⁻¹⁹ C/nm². The CPC200s were measured at a concentration of 1 × 10¹⁰ particles/mL.

2.3. Custom DNA Oligonucleotides. All the oligonucleotides used in this study were purchased by lyophilized powders (100 pmol/μL) from Sigma-Aldrich, U.K. with customized DNA sequences fit for purpose detailed below, please note the abbreviation [Bn] is relative to a biotin modification; S′NNNNNNNNNN[Bn]³⁻ (VL0, 10 bases), S′TGAGGATGGTGTTGGGCTGCCCTTTT[Bn]³⁻ (VL36, 36 bases), S′ATACATGCTAATCTAGTG-GCCCGTTCCGTATGTGTTGGCTGGCCAG[Bn]³⁻ (VL50, 50 bases), S′ATGGTTAACCTCACTACGGTGCG[Bn]³⁻ (VL25, 25 bases), S′GCCACCGTTAGTGAGGTATTTAACCAT³⁻ (cDNA, 25 bases), S′GTAGTGGATT³⁻ (MidT, 10 bases), S′GGTTTAAACCAT³⁻ (EndT, 10 bases), and S′GTAGGTTTTAACATT-TTTTTTTTTTTTTTTT³⁻ (OverT, 30 bases).

2.4. Phase Analysis Light Scattering (PALS). CPC200 zeta potentials were measured on a Malvern Zetasizer Nano ZS. PALS analysis was used to determine the average zeta potential of the carboxylated polystyrene standards dispersed in PBS electrolyte.

2.5. Hybridizing DNA to Streptavidin Coated Particles. 120 nm diameter streptavidin coated particles (03121, Ademtech, France) were diluted to a concentration of approximately 1 × 10¹⁰ particles/mL. The diluted particle solutions were then vortexed for 30 s and sonicated for 2 min, to ensure monodispersity.

The biotinylated DNA capture probe was added to the streptavidin coated particles (4352 pmol/mg binding capacity—determined by the supplier) at the required concentration. The samples were then placed on a rotary wheel for 30 min. Any unbound DNA remaining in solution was then removed via magnetic separation by placing the samples onto a Magrack (GE Healthcare, U.K.) for 30 min. The supernatant was then removed and replaced with new buffer (PBST).

2.6. Addition of Complementary Target DNA. Target DNA was added in excess (500 nM) to ensure the maximum possible target binding was reached. The samples were then placed on a rotary wheel at room temperature to investigate the effect of DNA hybridization time.

2.7. TRPS Setup. All measurements were conducted using the qNano (Izon Science Ltd., NZ) combining tunable nanopores with qNano average velocities and electric field measurements. The qNano was suspended in the buffer cell always contained the electrolyte buffer (150 mm PBS) and the electrolyte buffer (150 mm PBS) and an adjustable gap, and the streaming potential was measured for a range of applied pressures within a cyclic pressure sweep. The zeta potential was evaluated with the Surpass Visiolab software, applying an adjustable gap, and the streaming potential was measured for a range of applied pressures within a cyclic pressure sweep. The zeta potential was evaluated with the Surpass Visiolab software, applying a calibration particle with known zeta potential. Samples of particles with a wide spectrum of zeta potentials, potentially reaching from positive to negative values and/or very dilute suspensions, may result in the translocation of particles through a nanopore as a function of applied voltage, with particle velocity and electric field being averaged over the entire sensing zone of a regular conical pore. The electric field, \( E_L \), can be determined using the calculation of pore resistance, so that \( E_L \) is entirely parallel to the z-axis, \( I_0 \) and \( R \) being the electric field component along the pore axis, electric current, and resistance, respectively. \(^{60}\)

For a voltage \( V_0 \) of 0.5 V, a small pore opening diameter of 0.8 μm, a large pore opening diameter of 40 μm, and a membrane thickness of 250 μm, the maximum electric field is approximately 10⁵ V/m. Please note that the above pore dimensions are estimates, which are in accordance with SEM images of pores with similar dimensions to the ones used for this study. The electrophoretic mobility is the derivative of 1/T (with T being the signal duration) and can be determined using the Henry equation. Therefore, Henry’s equation is used to relate the particle zeta potentials with the measured electrophoretic mobility of single particles. \(^{60}\)

We are using a related approach, in which we are considering the effects of electroosmosis and convection (through an applied pressure) in addition to electrophoresis when calculating the zeta potential of single particles. Samples of particles with a wide spectrum of zeta potentials, potentially reaching from positive to negative values and/or very dilute suspensions, may require the application of an external pressure in order to capture the whole spectrum of particle zeta potentials. Also, without any net pressure, most neutral particles might not translocate the pore and hence are not measured, skewing the results.

Average velocities and electric fields at multiple points through the sensing zone (as opposed to only at the end of the sensing zone, see Figure 1b) will help to reduce errors that

3. RESULTS AND DISCUSSION

3.1. Zeta Calculation. In this study we are using a method related to Arjmandi et al., \(^{56}\) who described a calibration based method of measuring particle zeta potentials using resistive pulse sensing. This method is based on measuring the duration of the translocation of particles through a nanopore as a function of applied voltage, with particle velocity and electric field being averaged over the entire sensing zone of a regular conical pore. The electric field, \( E_L \), can be determined using the calculation of pore resistance, so that \( E_L \) is entirely parallel to the z-axis, \( I_0 \) and \( R \) being the electric field component along the pore axis, electric current, and resistance, respectively. \(^{56}\)

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Average velocities and electric fields at multiple points through the sensing zone (as opposed to only at the end of the sensing zone, see Figure 1b) will help to reduce errors that
result from rogue events such as instantaneous background noise. In other words, multi point analysis serves as a sort of quality control of the acquired zeta potentials. The calibration of the pore is based on measuring the linear dependence of $1/T_x$ vs voltage, $V_x$ using standard carboxylated polystyrene particles with a known average zeta potential (Figure 1c). The calibration process is summarized in the Supporting Information (eqs A.1–A.6). From this the electrokinetic particle velocities of sample, $(v_{x,d}^j)_{Sample}$ and calibration, $(v_{x,d}^j)_{Cal}$, are related with their zeta potentials, $\xi_{x,net,Sample}$ and $\xi_{x,net,Cal}$ (eq 1), assuming a linear relationship between velocity (mobility) and zeta potential as given in the Smoluchowski approximation.53,57

$$\frac{(v_{x,d}^j)_{Sample}}{(v_{x,d}^j)_{Cal}} = \frac{\xi_{x,net,Sample}}{\xi_{x,net,Cal}}$$

The net zeta potentials for both sample and calibration particles are the differences in the respective particle zeta potentials and the membrane zeta potential, $\xi_m$ (eq 2)

$$\xi_{x,net,Sample} = \xi_{x,net,Cal} + \xi_m \tag{2}$$

The zeta potential of each sample particle $i$, $\xi_{x,Sample}^i$, is given by averaging respective zeta potential values, calculated at various locations within the pore (eq 3), with $l_x$ being the position within the pore reached after time, $t = T_x$. Please note that $l_x$ is set to equal 0 right at the narrow pore entrance of the conical pore, where the signal magnitude reaches its maximum, as shown in Figure 1b. Zeta potentials are evaluated by taking the average at several discrete points, $l_x$.

$$\xi_{x,Sample} = \frac{\sum x \xi_{x,Sample}^i}{\sum x} = \frac{\sum x (v_{x,Sample}^V - v_{x,Cal}^V)/(v_{x,Cal}^V)}{\sum x} \xi_{x,net,Cal} + \xi_m \tag{3}$$

$v_{x,Sample}^V$ is the sum of the time averaged electrokinetic (electroosmotic and electrophoretic) and convection velocity components of sample particulates at position $l_x$ within the pore (eq 4).

$$v_{x,Sample}^V = \frac{l_x}{T_x} = \frac{\int_0^{T_x} v(t) \, dt}{T_x} \tag{4}$$

$v_{x,Cal}^V$, $v_{x,Cal}^P$, and $V$ are electrokinetic velocity per unit voltage, convective velocity per unit pressure, applied pressure, and voltage for the sample runs, respectively. The electrokinetic velocity per unit voltage is equivalent to the electrokinetic mobility, which is the sum of electroosmotic and electrophoretic mobility. $v_{x,Cal}^V$ and $v_{x,Cal}^P$ are calculated by averaging typically over more than 500 calibration particles. $\xi_{x,net,Cal}$ and $\xi_m$ are the zeta potentials of polystyrene standard particles and the membrane, respectively. The zeta potentials of polystyrene standards and the thermoplastic polyurethane membrane were measured using PALS and streaming potential techniques, to be $-20$ and $-11$ mV respectively (see the Supporting Information).

3.2. Zeta Potential of DNA Modified Particles. To test our method on DNA modified particles we first performed a series of measurements increasing the concentration of ssDNA (25 bases in length) on the streptavidin coated particle surfaces.

The concentration of DNA, termed here CP, was increased from 10–210 nM, while the particle concentration remained constant. At DNA concentrations over 188 nM the theoretical binding capacity of the streptavidin particles (as given by the supplier) has been reached (see the Supporting Information, Figures A.2 and A.3a for the size and charge distributions of the streptavidin coated particles without DNA). At the highest concentration of DNA added to the particles there is ~12648 pieces of DNA/particle, if all of these are attached to the surface of the particles it would equate to 1 DNA molecule every 2 nm across the particle surface. At lower concentrations of the DNA this ratio changes to 602/particle at 10 nM, and 4517/particle for 75 nM. Figure 2 is an example of size and zeta potential data that can be captured simultaneously in a single TRPS measurement. The blue bars/data points show the data at the lowest and middle concentration of CP (10 nM and 47 nM respectively) and the green bars/data points show results from the highest concentration of CP measured (210 nM). Please note that each data point in Figure 2 represents a single particle.

From this, it can be found that although there are no significant size changes observed between the samples, there are significant changes observed in the zeta potential. The inferred zeta potentials from the measured velocities of the samples at varying CP concentration are shown in Figure 3a,b, respectively. The particle velocities are determined from $1/T_{a50}$ (see the Supporting Information), which is an estimate of the average particle speeds. The measured zeta potential in Figure 3a shows that as the DNA concentration is increased, the larger the absolute zeta potential, and follows the expected trend based on the measured particle velocities; similar data for repeat experiments are given in Figure A.4. This is attributed to each phosphate group contributing to a negative point charge, thereby increasing the charge density of the particle surface, as described by Graham’s equation. Surface charge densities were calculated using Graham’s equation.$^{50,61}$ With mean zeta potentials not exceeding an absolute value of 40 mV the respective absolute surface charge densities lie below 0.035 C/
m², and acknowledge that while counterion condensation may play a role under these situations it is beyond the scope of the study to describe in detail. Figure 3c shows a series of histograms of frequency versus measured zeta potential for each concentration of DNA. The distribution at low concentrations of DNA can be characterized as narrow with a long skewed tail, which may in part be due to the particles themselves not having a uniform coating of streptavidin. As the concentration of DNA is increased the distribution changes, with the median skew value going from $-0.66$, $-0.51$, and $-0.36$ for 10, 95, and 210 nM, respectively. It should be noted here that the skewed histogram data in the figure may be an inherent property of the particles themselves not having a uniform coating of the streptavidin protein, as shown in Figure A3a. The charge histograms for all of CP concentrations studied are shown in Figures A.4 and A.5. Particle-by-particle measurements provide more detailed analysis of a sample solution. Charge distribution histograms are used to represent the spread of data among a given sample population. The zeta potential of the sample can then be analyzed in more depth.

A similar relationship between the length of the ssDNA and measured zeta potential should also exist, that is as the length of the DNA increases, the zeta potential is also predicted to increase. Steinbock et al. have previously investigated the effect of long double-stranded DNA strands (4 and 6 kilo base pairs) hybridized to colloids using microparticles and a microcapillary base Coulter counter system. They found that DNA coated microparticles displayed a much smaller change in conductance values due to the additional charge in the system. To investigate the sensitivity of TRPS in its ability to measure changes in zeta potential, we are focusing on much smaller strands of single-stranded DNA. The ssDNA oligonucleotides in this study were 10, 25, 36, and 50 bases in length equivalent to 7.0, 17.5, 25.2, and 35 nm in length respectively if the ssDNA is fully extended. Figure 4a shows how the zeta potential increases when the length of the DNA is increased alongside the relative particle velocity displayed in Figure 4b. In this example (red bars), the concentration of DNA added to the particles is in excess of the binding capacity. Figure 4a, blue bars, illustrate the same effect, i.e. as the length of the DNA increases so does the measured zeta potential. However, in this experiment, the DNA is at 75 nM, which is lower than the theoretical binding capacity of the particles. At this lower concentration the DNA is much more flexible and can exist in its condensed mushroom form. Figure 4c shows the charge distribution histograms for the densely packed DNA particles (distributions for the 75 nM DNA are

![Figure 3.](image)

*Figure 3. (a) Mean zeta potential vs capture probe concentration. (b) $1/T_{0.50}$ see Figure 1b, estimating average particle speed vs capture probe concentration. (c) Charge distributions among the sample population shown in panel a increasing in DNA concentration from left to right. 537, 605, 585, and 588 particles were measured for the samples containing 10, 47, 95, and 210 nM DNA, respectively.*

![Figure 4.](image)

*Figure 4. (a) Mean zeta potential vs capture probe base length. Blue bars are representative of a 75 nM DNA concentration and red bars represent a 210 nM DNA concentration. (b) $1/T_{0.50}$ see Figure 1b, estimating average particle speed vs capture probe base length. (c) The charge distribution of varied DNA base length using 210 nM DNA. 676, 1001, 996, and 693 particles were measured for the 10, 25, 36, and 50mer, respectively. Error bars represent s.d. where $n = 2$.***
given in Figure A.6, and distributions from multiple runs are presented in Figure A.7.

The width of the distribution increases as the length of the DNA increases (D90/10 goes from 2.87 to 5.3 for 10 and 50 base lengths respectively), we attribute this observation to the steric hindrance of the longer strands, preventing a higher packing density of the DNA around the particles due to their radius of gyration, effectively blocking the binding of the DNA onto the surface. It is also interesting to note that the median skewness values go from $-0.42$ to $-0.41$, $-0.12$, and $0.45$ for 10, 25, 35, and 50 bases, respectively. The particle charge distributions for the lower DNA concentration (75 nM) and comparative data sets for the higher concentration (210 nM) illustrating the reproducibility of the data are given in the Supporting Information, Figures A.6 and A.7.

3.3. Detecting Target DNA Hybridization. It was then investigated if the technique could discriminate between ssDNA and dsDNA. Initially the capture probe length was kept constant at 25 bases in length, and was always added in excess of the binding capacity of the particles. Any unbound capture probe in solution was removed before the target probe was incubated with the particles. As can be seen in Figure Sai and ii, the formation of dsDNA can be measured by an increase in zeta potential for assay hybridization times of 16 h (green triangles) and for hybridization times as short as 30 min (red squares). The change in structure from ssDNA to dsDNA is a 50-fold increase in persistence length, and this will result in the hydrodynamic radius of the particle upon forming dsDNA to increase. Two competing factors then affect the surface charge density. The first is the elongation of the DNA upon forming the dsDNA structure which has the effect of spacing out the charged phosphate groups away from the particle’s surface, resulting in a decrease in charge density. However, this is countered by the addition of a second strand of DNA doubling the number of point charges resulting in a net increase in electrophoretic mobility in solution, and thus resulting in increased velocities and larger zeta potential values.

This is similar to the work done by Booth et al. demonstrating the detection of target-probe DNA hybridization and successfully discriminating between “probe” and “target-probe” hybridized particles using TRPS. However, in these previous examples the experiments utilized a 23mer capture probe and 50mer target, as such the captured DNA extended out into solution and was predominantly ssDNA. Here we were curious as to the ability of the technique to discriminate between ssDNA and dsDNA, as well as overhanging DNA. We investigated a range of DNA targets binding to various positions of the capture probe, to determine the sensitivity and reliability of a zeta potential measurement for the detection of varied DNA hybridization. We termed these target probes as cDNA (fully complementary), MidT (binds to the middle of the CP), EndT (binds to the end of the CP furthest from the particle surface) and OverT (binds to the end 10 bases of the CP and overhangs into solution by 15 bases). The results for these measured zeta potential values are plotted in Figure Saii–v. As we add target DNA in each of the hybridization experiments to form dsDNA, be it at the middle or end of the CP, there is a larger zeta potential recorded. The magnitude of change in zeta potential is always greatest with the longer hybridization times. Of interest is the fact that the overhanging DNA sequence (OverT) gives the largest negative zeta potential of all the samples despite being the longest length. Increasing the length of the DNA could have slowed the speed at which the particles traverse the pore due to additional drag effects and lowered the recorded zeta potential. However, the result indicates that the increase in charge due to the additional 30 bases has a more dominant effect on particle translocation.

Figure 5. (a) Relative change in mean zeta potential (mV) from DNA capture probe (CP, 250 nM) to when a variety of targets are hybridized for 30 min (red squares) and hybridized for 16 h (green triangles). The relative change in zeta potential was also investigated for a lower concentration (75 nM) and 30 min hybridization time (blue diamonds). (b) Charge distributions for each target at 250 nM CP concentrations and a hybridization time of 16 h. 500, 990, 592, 707, and 964 particles were measured for samples (i–v), respectively, with median skewness values of $-0.073$, $-0.59$, $-0.41$, $-0.49$, and $-0.31$. Error bars represent st.dev where $n = 3$. DOI: 10.1021/acs.langmuir.5b03024 Langmuir 2016, 32, 1082–1090
times. One suggestion for this observation could be due to the ssDNA having a lower persistence length. The overhanging ssDNA may coil/fold back toward the particle. This folding in effect increased the surface charge density around the particle, thus increasing the electrophoretic velocity through the creation of a “hairy layer mechanism.”66 The ssDNA within the overhanging DNA is also further from the particle’s surface than the DNA in any other experiment. Given the curvature of the particle the distance between each DNA molecule will increase, and this room allowing for the DNA to fold back may explain the enhanced effect over MidT and EndT experiments.

A surprising aspect of the data was the ability to distinguish between dsDNA formed at the end or middle of the capture probe. The target DNA that bound to the end of the CP (EndT), recorded a smaller zeta potential than a same sized target that was hybridized to the middle of the CP (MidT). We attribute this to the effects of persistence lengths and the location of the dsDNA in the capture probe. The MidT dsDNA leaves a section of ssDNA exposed to the solution. This is more flexible and we hypothesize that when the dsDNA is in the middle of the DNA, the single stranded end section coils/folds back to increase the charge density around the particle,66 thus creating a larger zeta potential. In the case where the dsDNA is at the end of the sequence, the ability of the DNA to fold back on itself is restricted and forms a more rigid elongated oligomer across the entire length of the DNA, moving the charge away from the surface and lowering the surface charge density.

The observation that MidT produces a larger shift in zeta potential could influence the design of future assays on TRPS systems. The change in distribution shape indicates a difference in the DNA hybridization or DNA target itself. Figure 5b shows the change in charge histogram shape, dependent on the target DNA hybridized to the CP. The ability to monitor any of these discrete differences is an insightful prospect for future colloid and nucleotide research.

As well as designing the location to capture target DNA, there is also an interesting observation on the effect of DNA density on hybridization kinetics. Previous studies have illustrated that the kinetics of target DNA capture is influenced by DNA probe density at a surface.57−70 At high DNA probe densities, the ssDNA forms a dense packed polymer brush,56 the DNA forms a rigid polymer coating whose thickness is equal to the length of the extended DNA sequence, \( H \).72 The effects on packing density then determine the electrostatic potential, the position of the shear plane and the kinetics of target DNA hybridization. The significance of the zeta potential at polyelectrolyte layers becomes more complicated, and when the debye length, \( \kappa^{-1} \), is sufficiently lower than the polyelectrolyte layer thickness, \( H \), i.e. \( \kappa^{-1}/H \ll 1 \), the measured zeta potential may no longer reflect the stern potential, as the plane of shear is shifted to distances further away from the particle’s surface.73 Therefore, at polyelectrolyte surfaces, the term zeta potential in effect loses its original meaning. As the density of the DNA packing decreases the plane of shear may enter the DNA layer.3

Figure 6 shows the effect of hybridizing an excess of target cDNA to different packing densities of CP. At a low capture probe concentration and a target hybridization time of 30 min (shown by Figure 6a), the charge distributions were much narrower in shape with less of a tailing effect observed. In agreement with previous studies,74 when the DNA capture probe concentration is lower, there is a faster rate of reaction, resulting in a much narrower charge distribution histogram. At high capture probe concentrations, it is difficult to observe an increase in zeta potential for small hybridization reaction times, thus for quick assay times, low packing densities of CP produce better results.

Panels b and c in Figure 6 both display results observed at high CP concentrations and show the charge distribution widening as the target hybridization time increases from 30 min to 16 h. This is due to the increase in the amount of time the target DNA has to reach the required orientation to achieve successful complementary DNA binding. The more time there is for this to happen, the higher the proportion of target DNA that can successfully bind to the capture probe resulting in a larger amount of dsDNA present on the particles. This increases the particle velocity through the nanopore, thus resulting in a larger absolute zeta potential.

A more Gaussian charge distribution was seen for particles analyzed in panels a and c in Figure 6 than in panel b, with skewness values of +0.1, −0.41, and −0.37, respectively. We attribute this to an increase in ability to form dsDNA, and then detect its presence on a particle. For example with a hybridization time of 30 min and a lower capture probe concentration (Figure 6a), the presence of dsDNA is easily detected. This may be due to two factors, first there being less steric hindrance for the target DNA to approach the particle allowing the rate of dsDNA formation to be increased, and second the resolution of the technique to measure the incremental addition of dsDNA against a particle of lower charge, compared to a high density ssDNA covered particle in 6b. Increasing the target hybridization time to 16 h (Figure 6c) using the high concentrations of capture probe allows the target to have more time to hybridize and thus more dsDNA is present on the surface.

4. CONCLUSIONS

We have demonstrated that TRPS can successfully detect and characterize both unmodified and DNA-modified particles in a single, real-time measurement. Charge distributions, rather than a single mean zeta potential value allow for more information to be extracted from a sample data set using a particle-by-particle perspective. DNA-based surface modifications to a nanoparticle
affect the behavior of the nanoparticles in an electrolyte solution and their mobility through a nanopore; and by optimizing the hybridization time and DNA packing density on a surface, we measure the successful capture of target DNA after just 30 min incubation time. Successful analyte capture after such short incubation times is advantageous and shows great potential for medical applications, such as point of care assays, for example.

■ ASSOCIATED CONTENT

1 Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.langmuir.5b03024.

Supplementary figures and data associated with this article (PDF)

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Characterisation of the protein corona using tunable resistive pulse sensing: determining the change and distribution of a particle’s surface charge

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Abstract The zeta potential of the protein corona around carboxyl particles has been measured using tunable resistive pulse sensing (TRPS). A simple and rapid assay for characterising zeta potentials within buffer, serum and plasma is presented monitoring the change, magnitude and distribution of proteins on the particle surface. First, we measure the change in zeta potential of carboxyl-functionalised nanoparticles in solutions that contain biologically relevant concentrations of individual proteins, typically constituted in plasma and serum, and observe a significant difference in distributions and zeta values between room temperature and 37 °C assays. The effect is protein dependent, and the largest difference between the two temperatures is recorded for the γ-globulin protein where the mean zeta potential changes from −16.7 to −9.0 mV for 25 and 37 °C, respectively. This method is further applied to monitor particles placed into serum and/or plasma. A temperature-dependent change is again observed with serum showing a 4.9 mV difference in zeta potential between samples incubated at 25 and 37 °C; this shift was larger than that observed for samples in plasma (0.4 mV). Finally, we monitor the kinetics of the corona reorientation for particles initially placed into serum and then adding 5 % (V/V) plasma. The technology presented offers an interesting insight into protein corona structure and kinetics of formation measured in biologically relevant solutions, i.e. high protein, high salt levels, and its particle-by-particle analysis gives a measure of the distribution of particle zeta potential that may offer a better understanding of the behaviour of nanoparticles in solution.

Keywords Biosensor · TRPS · Zeta potential · Protein corona · Tunable pores

Introduction

In recent years, synthesis methods for nanoparticles have evolved to the extent that particle size, shape and composition can be easily modified [1–4] and this had led in turn to great advances in the field of diagnostics [5, 6], drug delivery [7–9] and technology platforms [10, 11]. With the desire to understand and improve nanomaterials comes a need for characterisation platforms to offer rapid analysis of size, charge and shape. Ensemble techniques that take measurements on several particles simultaneously and provide an average measurement can underestimate subpopulations within multimodal samples [12, 13], and a raft of technologies have appeared to help tackle this [14, 15]. Such technologies now offer an ability to quantify the population of particles with single particle resolution building an understanding that not all particles are created equal and there exists distributions such as particle size or ligand density.

One such technology is based on the Coulter Counter principle, referred to as resistive pulse sensing (RPS) [16–18]. The technique allows the characterisation of proteins, inorganic ions, colloids and nanoparticles within their natural environment. Two categories of resistive pulse sensors exist.
that utilise either biological [19, 20] or inorganic nanopores [21–23]. Here, we describe a recent adaptation to inorganic pores that uses a tunable elastomeric pore termed tunable resistive pulse sensing (TRPS) [14, 24–36]; the pore can be stretched in real time to suit the sample. The brief setup and theory for TRPS technologies is as follows: A stable ionic current is established by two electrodes, separated by a pore; as particles/analytes translocate the pore, they temporarily occlude ions, leading to a transient decrease in current known as a ‘blockade event’, examples of which can be seen in Fig. 1. In the TRPS arrangement used here, the pore is mounted laterally so that particles typically move from the upper fluid cell into the lower fluid cell, aided by an inherent pressure head due to 40 μl of liquid in the upper fluid cell of approximately 50 Pa [35]. By monitoring changes in blockade width, blockade magnitude (ΔIp) and blockade frequency (events/min), it is possible to elucidate the zeta potential, size and concentration of colloidal dispersions in situ [14, 37, 38]. By controlling the aspect ratio of the pore, resistive pulse sensors have been used to measure analytes that range from single molecules, DNA, proteins, cellular vesicles to cell bacteria and viruses; detailed reviews on the types of analytes and applications can be found elsewhere [24, 36, 39, 40]. TRPS is becoming an increasingly common variation of RPS for the characterisation of biological and inorganic nanomaterials [24, 36] and since its conception has been tested against alternative technologies such as DLS/ PALS [14, 15, 41–44], TEM [33], and ultracentrifugation [44] for the characterisation of nanomaterials [15, 45].

The how and where of measuring the properties of particles are important to consider as changing pH, ionic strength or temperature, or purifying particles can give a misrepresentation of their behaviour in their natural environment. In the case of nanomaterials that are intended to be used in vivo, it is not properties within synthesis processes that determine their biological activity, but how they interact with proteins upon entering the body. Upon the addition of nanoparticles to biological fluids, there is an almost immediate fouling of their surfaces with proteins, peptides and other cellular apparatus forming a layer known as the protein corona [46–48]. The composition of the corona has been shown to determine the eventual properties of the particles [49–51] and has been reported as critically affecting pathophysiological effects of nanoparticles [52]. The structure of the protein corona can be dynamic and complex and is different for particles of the same composition but with different surface chemistries and size in the same solution [46, 53]. Detailed studies of the corona have been performed using an array of technologies including mass spectroscopy [54, 55]. Various techniques have been used to look at a range of specificities of protein coronas, for example, protein corona thickness has been investigated using ensemble techniques such as dynamic light scattering (DLS) and differential centrifugal sedimentation.

Fig. 1 Particles in the presence of human plasma and serum showing the formation of both a ‘hard’ and ‘soft’ protein corona. I1.0, I0.8, I0.6, I0.4, I0.2 represent the position of the particle as it translocates the pore (where I1.0 is the narrow pore entrance) and are relative to T1.0, T0.8, T0.6, T0.4, T0.2, which represent the time taken (ms) for the particle to reach that position. T1.0 is equivalent to dRmax when the blockade event is at 100 % magnitude; T0.8, T0.6, T0.4, T0.2 correspond to when the blockade is 80, 60, 40, and 20 % of its dRmax and indicates the particle traversing the pore.
Protein corona conformation has been studied using circular dichroism (CD) and fluorescence quenching [57, 58]; the affinity has also been a popular characterisation property of protein coronas and has previously been measured using size exclusion chromatography (SEC), surface plasmon resonance (SPR) and isothermal calorimetry (ITC) [49, 56, 59]. A frequent and easy value used to characterise the corona is zeta potential [46, 48, 50, 54, 60]. The zeta potential represents the value of the electrostatic potential at the plane of shear, and typically for nanoparticle systems, zeta potential values of ±30 mV are representative of stabilised particles [61].

When nanoparticles are introduced to biological fluids, the protein corona is formed in a series of layers, otherwise known as the ‘hard’ and ‘soft’ corona. Proteins forming the hard corona are those with a higher affinity that interact directly with the nanoparticle surfaces, whereas proteins forming the soft corona are those engaging in weaker protein–protein interactions with the hard corona [56, 62]. It has previously been found that a vast range of particles bind successfully to apolipoproteins in physiological fluids [55]. Formation of a protein corona alters the size, aggregation properties and surface properties of nanoparticles [63], thus creating a new biological distinctiveness for further application. There are 5 main components that define the composition of a protein corona: thickness and density, identity and quantity, orientation, conformation and affinities [63].

Protein adsorption kinetics play a prominent role in this study and are key to understanding the binding mechanisms that will occur in a natural environment. Although this process is time-dependent, the kinetics rely on $k_{\text{on}}$ and $k_{\text{off}}$ parameters, indicating the rate constants for adsorption and desorption of proteins. $k_{\text{on}}$ is largely dependent on how often the protein contacts the nanoparticle surface, as well as the probability of successful binding between the two materials [64]. The strength of the protein–nanoparticle interaction defines $k_{\text{off}}$ [64], and a strong, high-energy interaction will exert a low $k_{\text{off}}$ value. Understanding the kinetics of formation and protein corona composition is important to understand processes nanoparticles may undertake when introduced into the body and into physiological conditions.

Here, we present a protocol for the rapid analysis of the corona zeta potential and demonstrate its versatility by making the measurement in solutions that mimic the natural environment, i.e. high ionic strength and high protein composition. By making comparable measurements of carboxyl polystyrene nanoparticles in a range of incubation temperatures and with different proteins, a clear difference in magnitude and variation of zeta potential within the particle population was observed; the three proteins chosen to demonstrate this are the most predominant (in terms of quantity) proteins in normal human plasma and we perform the experiment concentrations that would reflect normal plasma. The ability to have individual particle resolution provides an opportunity to see the full variation of zeta potential in a single sample. The findings highlight the need to monitor the protein corona and its formation at biologically relevant temperatures and suggest that the kinetics of protein adsorption and spread in zeta potential values varies for each of the proteins and biological mediums studied. Finally, we show the scope of the technology by monitoring the change in the hard and soft corona elements interacting with the particles through incubation in serum, followed by the addition of a small amount (5 % (V/V)) of plasma. It is known that protein components of a higher concentration or affinity to the particle can remove and restructure the soft corona that is formed in biological fluid [55], and we monitor the rate of this change and the kinetic effects that eventually settle on a new zeta potential value.

### Materials and methods

#### Chemicals and reagents

The initial buffer used for particle analysis was phosphate buffered saline (1× PBS tablet (0.01 M phosphate buffer, 0.0027 M potassium chloride, 0.137 M sodium chloride) in 200 mL deionised water (18.2 MΩ cm)). PBS tablets (P4417) were purchased from Sigma-Aldrich, UK.

**Carboxyl polystyrene standards**

Carboxylated polystyrene particles, denoted as CPC200, with a mean nominal diameter of 210 nm and stock concentration of $1 \times 10^{12}$ particles/mL, were purchased from Bangs Laboratories, USA and used as a calibrant for zeta potential analysis, as well as the sample particles. CPC200s were vortexed for 30 s followed by a 2 min sonication to ensure monodispersity prior to any TRPS analysis or sample incubation.

**Isolated proteins**

All isolated proteins studied were purchased from Sigma-Aldrich, UK, without modification or purification unless stated otherwise: fibrinogen from human plasma (F3879), albumin from human serum (A9511) and γ-globulin from human blood (G4386).

**Human plasma and serum samples**

Blood samples were collected and prepared at Peterborough City Hospital Pathology Laboratory, UK. Plasma collection was completed using blood from a healthy volunteer donor that was collected in citrate medium (Sarstedt, UK) and centrifuged at 3000 rpm for 8 min. Serum was gathered using blood from a healthy volunteer donor that was collected into a Sarstedt monovette/collection tube, and was centrifuged at

...
3000 rpm for 6 min. The supernatants from each sample were transferred into separate sample vials and stored at room temperature prior to use.

**Isolated protein studies**

Using PBS buffer, isolated albumin, fibrinogen and γ-globulin samples were prepared to give the following concentrations: 43, 3.2 and 20 g/L, respectively, as to mimic protein concentrations found in human blood. The concentrations of proteins were measured from human plasma and serum samples. The samples used in this study were analysed by an Instrument Laboratory ACL TOP CTS500 coagulation analyser (Werfen, Spain) to obtain the fibrinogen concentration. Albumin and immunoglobulin levels were taken from test serum samples that were analysed by a Roche Cobas 6000 Analyser (Roche Diagnostics, Switzerland). CPC200s were added resulting in a final concentration of $1 \times 10^{10}$ particles/mL. Each sample was vortexed for 30 s and sonicated for 1 min before incubation. Samples were then incubated at 25 and 37 °C in a mini dry bath (Benchmark Scientific, USA) for 10 min prior to TRPS analysis.

**Serum and plasma studies**

Human plasma and serum were prepared immediately before the experiments to minimise ex vivo artefactual changes. The prepared plasma and serum were separately diluted 10-fold with PBS before CPC200s were added to both samples resulting in a final particle concentration of $1 \times 10^{10}$ particles/mL, herein these solutions are referred to as serum and plasma. Samples were vortexed for 30 s and sonicated for 1 min, followed by incubation in a mini dry bath (Benchmark Scientific, USA) at 25 and 37 °C for 10 min before being removed for TRPS analysis. It should be noted that it is possible for some proteins in human plasma and serum to interact and adsorb onto the pore walls; therefore, a control measurement of CPC200s in PBS (of known zeta potential, -20 mV) was completed before and after each protein/plasma/serum sample to establish if any changes had occurred to the pore itself.

**Plasma spiking assay**

Human serum was 10× diluted in PBS before CPC200s were added to a final concentration of $1 \times 10^{10}$ particles/mL. Samples were vortexed for 30 s and sonicated for 1 min before being incubated for 10 min at 25 and 37 °C in a mini dry bath (Benchmark Scientific, USA). At 10 min, 5% (V/V) human plasma was added to the serum samples and the samples were vortexed for 30 s. TRPS measurements were completed once the plasma had incubated with the serum sample for 5, 10, 15, 20, 30 and 60 min.

**Tunable resistive pulse sensing**

All measurements were completed using the qNano (Izon Science Ltd, NZ). The system utilises tunable nanopores with propriety data capturing software (Izon Control Suite v3.1.2.53). In all experiments, the lower fluid cell contained 80 μL of PBS buffer, ensuring no bubbles were present. When a sample measurement was being taken, the upper fluid cell contained 40 μL of the sample (suspended in PBS buffer). After each measurement was taken, the nanopore was washed several times by removing and replacing 40 μL of buffer, each time applying varied pressures until no particles were observed. This was performed several times to remove any residual particles in the system and thus ensure no cross-contamination between samples. The nanopores used throughout all experiments were capable of detecting particles within the size range of 100–300 nm (as stated by the manufacturer, Izon Science Ltd) and denoted as an NP200. To account for the variation in the manufacturing of the nanopores, appropriate stretch (44–46 mm), voltage and pressure were applied in all experiments; the conditions were matched as to the blockade magnitudes of CPC200s in PBS being of a similar size throughout all experiments. All samples were vortexed for 30 s and sonicated for 2 min prior to analysis.

**Zeta potential measurements using TRPS**

When carrying out zeta potential measurements, the nanopore stretch was kept the same for a particular dataset and nanopore between calibration and sample measurements. To calibrate a nanopore for zeta potential analysis, the calibration particles, of known size and zeta potential, were measured in PBS at 3 applied voltages; the particles measured at the highest voltage were measured at 2 external pressures (in addition to the inherent 47 Pa pressure head on the system). When running the samples, the blockade magnitudes were ensured to be at least 100× larger than the respective background noise of ca. 10 pA. In accordance with the calibration runs, the samples were run at the highest calibration voltage. Calibration measurements were completed when a new nanopore (NP200) was introduced to ensure conditions were matched so the blockade magnitudes of CPC200s in PBS were of a similar size to other NP200s used for this study. A CPC200 sample in PBS was run after each protein/plasma/serum sample to ensure the zeta potential of the pore remained unchanged and as such did not affect the measured zeta potential of further samples.

**Results and discussion**

Zeta potential values were determined from the particle velocities as they traversed the nanopore; a full description of the protocol and theory can be found elsewhere [21, 38]. Briefly,
the duration of particle translocation is measured as a function of applied voltage, taking an average electric field and average particle velocities over the entire sensing zone that is a regular conical pore. Each particle’s electrophoretic mobility is derived from $I/T$, where $T$ is the blockade duration and voltage, multiplied by the square of the sensing zone length, $L$, as part of a calibration constant. Figure 1 shows the conical sensing zone and an example of the blockade detection times, $T$, as a result of a blockade event at various positions, $I$, in the nanopore. $T_{0.6}$ for example is equivalent to when the blockade is 100% in magnitude and is indicative of $I_{0.6}$, the position to which the particle is approaching the pore entrance. $T_{0.6}$ relates to position $I_{0.6}$ where the blockade is 60% in magnitude and the particle has traversed 40% of the pore. It is important to note each blockade depicted in the signal trace is indicative of a single particle as it passes through the pore, highlighting the advantages of using particle-by-particle technologies such as TRPS.

Average velocities determined across multiple reference points within the nanopore vastly reduce any errors in this zeta potential calculation process [38]. The calibration of the pore itself is based on a linear relationship between $I/T$ and voltage, $V$, at each blockade reference point. Equation 1 shows the direct relationship between particle velocities and their zeta potentials, $(v_x)_{el Cal}$ and $(v_x)_{el Sample}$ are the particle velocities of calibration and sample particles, respectively, and $ξ_{net Cal}$ and $ξ_{net Sample}$ represent their zeta potential values [38].

$$\frac{(v_x)_{el Sample}}{(v_x)_{el Cal}} = \frac{ξ_i Sample}{ξ_i Cal}$$  \hspace{1cm} (1)

Equation 2 shows the zeta potentials measured at each of the blockade reference points can then be used to determine the zeta potential of each individual particle, $i$, as it passes through the pore, $ξ_{Sample}$

$$ξ_i Sample = \frac{ξ_i Sample}{ξ_i Cal} = \frac{\sum x \left( (v_x)_{Sample} - (v_x)_{Cal} \times P \right) / (v_x)_{Cal} \times V}{\sum x}$$

$$\times ξ_{net Cal} + ξ_m$$  \hspace{1cm} (2)

Where $v_x Cal$,$v_x Sample$, $P$ and $V$ are electrokinetic velocity per unit voltage, convective velocity per unit pressure, applied pressure and voltage, respectively. $I_x$ is the position of the particle in the nanopore after time $t = T_x v_x Sample$ is the sum of the particle velocities at relative positions, $I_x$ [38].

The proteins used in this study were chosen based on their relative abundances in both blood plasma and serum samples. Albumin and γ-globulin are present in both plasma and serum samples at approximately 4 and 2%, whereas fibrinogen (as well as other clumping factors) is only found in plasma at approximately 0.4%. Zeta potential values measured for particles incubated with each of the isolated proteins are shown in Fig. 2 (for reference the starting zeta potential of a CPC200 in PBS is $-20 mV$).

When the particles were incubated with each of the proteins separately at 25°C, both fibrinogen and γ-globulin showed a relatively small change in mean zeta potential from particles in PBS buffer, differences of 3.2 and 3.6 mV, respectively. The size and zeta potential distributions of CPC200 carboxyl particles in PBS are shown in Fig. 3. Albumin was seen to have a much larger effect on the particle zeta potentials at 25°C as the zeta values were reduced by 9.2 mV from the PBS control. Albumin was at the highest concentration at 40 g/L in comparison to the fibrinogen and γ-globulin samples only having protein concentrations of 4 and 20 g/L respectively. The protein concentrations were chosen to replicate the typical composition usually found in the human body, although it should be noted that the concentration of proteins to that of the particles in each experiment was always in a large excess as to coat each surface of every particle. The proteins were also investigated at a constant concentration (5 g/L) at a 25°C incubation temperature for 10 min to determine whether protein concentration had an effect on the protein corona on the particles, results of which are shown in the Electronic Supplementary Material (ESM), Fig. S1. From this, it was found that the relative change in zeta potential (from a control of the particles just in PBS) was smallest for fibrinogen and γ-globulin with values of 4.3 and 4.9 mV, respectively. The largest change in zeta potential was again observed for the albumin protein with a difference of 8.9 mV. These comparable changes show the results are protein specific and not related to the concentration at these levels. It was therefore expected that the proteins would adsorb onto the particle surface, forming the protein corona. Any such protein corona would change the surface charge density on the particles and be measured by a change in particle velocity, which in turn is plotted as the zeta potential. At 25°C, the small zeta potential changes for fibrinogen and γ-globulin samples are more than likely because of the protein isoelectric points and their behaviour at physiological pH. Albumin has an isoelectric point of 4.7 whereas fibrinogen and γ-globulin have isoelectric points of 5.8 and 6.6, respectively [65]. Previous reports have found that as the adsorption pH moves away from the protein isoelectric points, the adsorbed molecules will occupy a larger area of the surface. This is due to internal electrostatic repulsions and thus a lower structural stability [66]. Our samples were all suspended in PBS buffer at pH 7.4, and therefore, the albumin is expected to occupy the largest area of the nanoparticle surface as the adsorption is occurring at a pH furthest from its isoelectric point. This may be the reason the albumin shows the largest change in zeta potential after a 25°C
incubation in comparison to the smaller changes observed for fibrinogen and γ-globulin (isoelectric points closer to 7.4).

Particles were also incubated with each of the proteins at a higher temperature of 37 °C; it was hypothesised that as the proteins are present in such a large excess that the incubation time of 10 min would be enough to coat the particles with a monolayer of protein, and that the temperature would have little effect on the result. In contrast, at 37 °C, there were significant differences from values at 25 °C and each protein produced varying shifts in zeta potential values. At the elevated temperature, γ-globulin was seen to have the largest reduction in zeta potential from a value of −20.3 mV (particles in PBS) to −9.0 mV. This is of particular interest as these results indicate each protein interacts with the particle surface uniquely, having direct implications on the particle zeta potential. γ-Globulin also showed the largest change in zeta potential as a function of incubation temperature between 25 and 37 °C (5.0 mV), whereas albumin showed the smallest change (1.3 mV). The distribution of zeta potentials for each isolated protein at 25 and at 37 °C are shown in the ESM, Fig. S2.

The particle-by-particle nature of TRPS allows for individual particles to be analysed, as well providing a measure of the spread in values across the sample population. Figure 3 depicts the zeta potential versus particle size plots for the given sample populations summarised in Fig. 2. Note here that each data point in Fig. 3 is representative of a single particle. Whilst the distribution of the values does not change as the incubation temperature increases, the shift in mean zeta potential as the incubation temperature was significant. This shift may be due to the affinities of the proteins for the particle surface being affected by the incubation temperature. Previous studies have found that negative particles have

Fig. 2 Mean zeta potential (mV) versus the protein the particles were incubated with. The blue bars show results for a 10-min particle incubation at 25 °C and the red bars show the mean zeta potential values for particles incubated with the proteins for 10 min at 37 °C. The green lines represent the measured mean zeta potential for calibration particles of known zeta potential (~−20 mV) in PBS that were run after each protein sample to show the protein samples were not having a direct effect on the pore walls themselves that may influence the recorded zeta potentials of future samples run on the same pore. Error bars are representative of the st.dev where n = 3.

Fig. 3 Zeta potential (mV) versus particle size (nm). The red, blue and green datasets are zeta potential distributions for CPC200s incubated for 10 min with fibrinogen, γ-globulin and albumin, respectively at (a) 25 °C and (b) 37 °C. The black data points represent CPC200s in PBS for both figure parts a and b.
maximum protein adsorption at 15, 35 and 37 °C [67] and explain why the CPC200s incubated at 37 °C in each protein medium shifted to a smaller zeta potential value more so than those incubated at 25 °C. When proteins have a higher affinity to the particle surface, there is either the formation of a robust hard corona, or slower release of the proteins from the surface once absorbed. The hard corona layer will alter the particle surface chemistry and will result in a slower particle translocation velocity through the pore due to shielding of the negative particle surface, which consequently results in a smaller zeta potential value. Interestingly, at the 25 °C incubation (Fig. 3a), the γ-globulin and particularly the fibrinogen sample showed a wider spread of data than those samples incubated at 37 °C (Fig. 3b). Figure 3b also shows that at elevated temperatures, a thicker protein corona layer is formed resulting in an increase in particle size. These results suggest the protein binding kinetics may differ as a function of temperature. The population spread may be wider at lower temperatures as the proteins may not have reached maximum levels of adsorption to the particle surface at 25 °C [67], also supporting the small changes in mean zeta potential at 25 °C demonstrated in Fig. 2.

Monitoring individual protein–nanoparticle interactions is interesting but becomes more complex in a medium containing a protein mixture, such as plasma or serum. Both plasma and serum are extracted from blood samples but contain a different composition of proteins. Relevant to this study, serum contains albumin, γ-globulin and apolipoproteins. Plasma has a similar protein composition to serum, but also contains clotting factors such as fibrinogen. Figure 4 shows the measured zeta potentials of CPC200s in PBS and of CPC200s incubated in plasma and serum for 10 min at both 25 and 37 °C.

As seen in the isolated fibrinogen and γ-globulin samples above, only small changes in zeta values were observed for both plasma and serum at 25 °C. Interestingly, at the elevated incubation temperature of 37 °C, the plasma still did not appear to show a significant difference in zeta potential, whereas the sample in serum showed a reduction in zeta potential of 5.9 mV. The most prominent difference between plasma and serum is the presence of clotting factors in plasma; this will have an inherent effect on the protein corona structure and resulting interactions with the particle surface [63]. Protein corona formation is complex in physiological environments.

Fig. 4 (a) Mean zeta potential (mV) versus incubation medium. Comparison of CPC200 particles incubated in PBS (green), plasma and serum for 10 min at 25 °C (blue) and 37 °C (red). Error bars are representative of the st.dev where n = 3. (b) Frequency (%) versus zeta potential (mV). Zeta potential distributions for CPC200s incubated for 10 min at 37 °C in plasma (purple) and serum (pink). Repeat datasets for CPC200s incubated in both plasma and serum at 37 °C for 10 min are illustrated in ESM Fig. S3 and are compared to a zeta potential distribution of CPC200s in PBS only.
as it consists of the simultaneous binding of numerous proteins to the particle surface creating both protein-nanoparticle interactions as well as protein–protein interactions [63].

Proteins within plasma and serum are undergoing a competitive binding assay to the particle’s surface, and proteins of higher concentration and/or affinity will bind to the particle surface more rapidly at the first instance. Protein–protein interactions are also common in plasma and serum samples, and some proteins will have a higher affinity to a subsequent protein over the particle surface. Zeta potential distribution as a function of temperature for the particles incubated with plasma and serum samples are shown in Fig. 5.

When the incubation temperature was increased, the zeta potential for both particles in plasma and serum were smaller. The advantage of distribution studies of a sample population is the discrete differences that can be identified, that cannot be determined immediately from mean values. For example, in Fig. 5a, the distribution shape of the particles incubated with plasma at 25 °C (red) is almost twice as wide as the distribution for 37 °C (purple), yet the mean values only changed by 0.4 mV between temperatures, a negligible difference. The difference in distribution shape can be reflected using median skewness values. The median skewness values for a given sample population of particles incubated in plasma were 0.111 and −0.065 for incubation temperatures of 25 and 37 °C, respectively. Particles in serum showed the same effect and as the incubation temperature was increased, the median skewness values decreased from −0.105 (25 °C) to −0.343 (37 °C).

The protein–nanoparticle interactions in plasma and serum were evidently varied, and to investigate this further, we completed a plasma spiking experiment. This aimed to ascertain if the soft corona formed in the plasma would reorganise in the presence of serum proteins. Figure 6 shows the effect on zeta potential as plasma (5 % \( V/V \)) was used to spike samples containing nanoparticles in serum at various time intervals.

Plasma protein adsorption onto a particle surface is due to the Vroman effect and is defined as the constant change in protein composition based on continuous adsorption and desorption at an interface [68]. There can be both faster and slower stages in this effect dependent on the protein. For example, albumin, \( \gamma \)-globulin and fibrinogen are all proteins that will adsorb rapidly onto a surface based on their high

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**Fig. 5** (a) Zeta potential distributions for CPC200s incubated in plasma for 10 min at 25 °C (red) and 37 °C (purple). (b) Zeta potential distributions for CPC200s incubated in serum for 10 min at 25 °C (green) and 37 °C (blue).
abundances, but are generally replaced by apolipoproteins in a matter of seconds [69] due to their fast dissociation properties. Apolipoproteins, however, although of low abundance, have a much slower dissociation constant and will therefore remain on the potential surface for longer [70]. As with a lot of nanoparticle-based assays, there may be an element of competition between proteins in binding to the nanoparticle surface that will affect the protein corona structure as displacement and exchange reactions may then take place over time. As the hard corona involves the higher affinitive proteins, this should remain adsorbed onto the nanoparticle surface over time and during any biophysical event that may occur [63]. The soft corona involves much weaker protein interactions in the system and will therefore dissociate more rapidly and protein exchange will occur much more readily. This effect is dependent on the relative protein concentrations of all proteins present in the plasma and serum samples. It is well known that protein concentration has a significant effect on the formation of a protein corona when incubated with nanoparticles [55, 71]; when a protein is of high concentration in a given sample, that protein will initially occupy the nanoparticle surface and form a protein corona [55] at a potentially faster rate than those of lower concentrations that may be later exchanged for those at a lower concentration but higher affinity. This effect also depends on the nanomaterial and there have been cases where proteins that have adsorbed first have had the longest residence time [72].

The first measurement was taken after the plasma had been introduced to the serum sample for 5 min. Between 5 and 10 min of the plasma being present (Fig. 6(i-ii)), the zeta potential of the particles was reduced. This is due to the addition of proteins into the sample, a higher concentration of proteins interacting with the particles will result in a slower pore translocation velocity, hence the reduced zeta potential. Figure 6(iii) shows that after 15 min, the zeta potential was reduced to its lowest measured value in this experiment. This is due to some of the plasma proteins displacing those from serum that may have reversibly bound to the particle surface as part of a hard corona layer. The plasma proteins may have been of a higher affinity to those present in the serum sample and therefore form the new hard corona layer [56, 62]. After 20 min and gradually onto 60 min (Fig. 6(iv)), the particle zeta potentials became more negative, indicating an increase in particle translocation velocity through the pore. We attribute this change to the depletion of the soft corona layer as proteins dissociate from loose protein-protein interaction.

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**Fig. 6** The effect of spiking a sample of CPC200s incubated in serum with plasma. (a) Visual representation of the effect of protein displacement and exchange within a protein corona system. (i) Protein corona formed by particle incubation in serum, (ii) introduction of plasma proteins to sample, (iii) displacement of hard corona proteins due to proteins of higher affinities and exchange of soft corona proteins, (iv) depletion of soft corona layer as proteins dissociate from loose protein-protein interaction. (b) Particles were incubated in serum for 10 min and then spiked with 5% (v/v) plasma. Zeta potentials were measured at 5, 10, 15, 20, 30 and 60 min. (i)-(iv) indicate the shift in zeta potential as a result of the effects described in (a).
this result to the weak interactions of the soft corona layers. For example, once the plasma proteins have potentially displaced those in the original hard corona, the displaced proteins will form part of the soft corona and be part of weaker protein–protein interactions. Over time, the soft corona proteins will dissociate more readily away from the particle due to their loose interactions [73], reducing the protein coverage around the particle and thus resulting in a larger zeta potential. The zeta potential becomes larger after this process as there are less bound proteins surrounding the particle to reduce the particle’s translocation velocity. The faster the particle can traverse the pore, the larger the zeta potential value. This is of particular interest as it gathers valuable information on how the different compositions of plasma and serum proteins in a blood sample would affect a nanoparticle and how they behave differently when isolated and in a mixture.

Conclusions

We have demonstrated the effects of more prominent proteins found in protein coronas individually (isolated in PBS) and within their natural environment (within plasma and serum samples) on carboxylated polystyrene nanoparticle surfaces. Protein–nanoparticle interactions involved in the formation of a protein corona have been found to be protein dependent at 25 °C, as well as temperature dependent for each studied protein. Significant changes in particle zeta potentials were observed when all of the proteins interacted with the nanoparticles at 37 °C. TRPS technology has enabled the provision of single particle analysis, as well as information on the zeta potential distributions amongst a given sample population in all experiments carried out, a more detailed insight than some other previously used ensemble techniques. We have found that although a stable hard and soft corona can be formed around particles in serum, we can also track various protein displacement and exchange processes occurring when plasma proteins are introduced to these samples. This has provided more detailed information on the affinities and reaction kinetics of protein coronas dependent on their biological medium and incubation conditions. A further understanding of protein–nanoparticle interactions in complex matrices and in physiological conditions is proving useful for advances in biotechnological assays and therapeutics.

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Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

Human and animal rights and informed consent This work has been performed with the consent of healthy volunteers. The studies were approved by the ethics committee and performed in accordance of the ethical standards or Loughborough University.

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Protein detection using tunable pores: resistive pulses and current rectification†

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We present the first comparison between assays that use resistive pulses or rectification ratios on a tunable pore platform. We compare their ability to quantify the cancer biomarker Vascular Endothelial Growth Factor (VEGF). The first assay measures the electrophoretic mobility of aptamer modified nanoparticles as they traverse the pore. By controlling the aptamer loading on the particle surface, and measuring the speed of each translocation event we are able to observe a change in velocity as low as 18 pM. A second non-particle assay exploits the current rectification properties of conical pores. We report the first use of Layer-by-Layer (LbL) assembly of polyelectrolytes onto the surface of the polyurethane pore. The current rectification ratios demonstrate the presence of the polymers, producing pH and ionic strength-dependent currents. The LbL assembly allows the facile immobilisation of DNA aptamers onto the pore allowing a specific dose response to VEGF. Monitoring changes to the current rectification allows for a rapid detection of 5 pM VEGF. Each assay format offers advantages in their setup and ease of preparation but comparable sensitivities.

Introduction

Interest in nanoscale channels within synthetic materials have grown over the last two decades.1–3 These channels have applications in biosensing,4–6 material characterisation,7,8 quantification of ligand–target interactions,9–11 drug delivery,12 and mimicking biological systems enabling the study of ionic transport within confined geometries.13–16 These nanopores have been created in a range of materials including graphene,17–20 polymers,21 silicon nitride22 and glass.13,14,23,24

The transport of the ions or analyte through the channel can by controlled by tuning the applied potential, pore wall charge, pore size, supporting electrolyte concentration and composition, with a further degree of selectivity by modifying the pore walls with selective ligands.25–27
In addition to the pore properties, the translocation speed and frequency of materials such as small molecules, proteins or nanosized particles are also governed by the analyte size and charge\textsuperscript{5,28–31} Fig. 1a. Nanopore sensors can be categorised into two general areas: Resistive Pulse Sensors, RPS, where the translocation of the analyte creates a characteristic change in resistivity within the pore, Fig. 1a and b, or current flux/rectification studies that monitor current–voltage, $I$–$V$, and can be dominated by the charge on the pore wall to measure, Fig. 1c. The flux of material through the pore is determined by the small and large pore geometries, $d_s$ and $d_l$, pore length, $L$, and analyte charge. By controlling the aspect ratio of the pore, RPS has been used to measure analytes that range from single molecules, DNA, proteins and cellular vesicles to cells, bacteria and viruses; detailed reviews on the types of analytes and applications can be found elsewhere.\textsuperscript{4,5,28,30}

We present a comparison between RPS and rectification ratios on a tunable pore platform. The pore is made of polyurethane, PU, allowing manipulation (stretching) in real time to suit the sample.\textsuperscript{32} The pores are conical in shape and here typically $d_s > 700$ nm. In the first example we utilise an aptamer-modified nanoparticle to detect Vascular Endothelial Growth Factor, VEGF. By measuring the translocation velocities of the aptamer-modified particles the VEGF protein was detected down to 18 pM, equating to circa 10 proteins per particle. In comparison a second strategy was tested by modifying the pores directly with the anti-VEGF aptamer and monitoring the current rectification ratio (measured at $\pm 1.6$ V) in the presence of the VEGF protein. The surfaces of the pores were easily modified using a layer-by-layer (LbL) assembly of polymers, such as polyethylene amine, PEI, and polyacrylic acid–maleic acid and PAAMA. The use of PEI and PAAMA allowed for easy modification, and reversible surface charge of the pores giving a pH- and ionic strength-controlled current flow, with current rectification ratios as high as 3. The LbL assembly was shown to be stable for days, allowing the modification of the pore wall with DNA via standard carbodiimide chemistry. The

\textbf{Fig. 1}  
(a) Schematic of a conical PU pore with small, large pore diameter and pore length, $d_s$, $d_l$, and $L$ respectively. The top surface of the pore is labelled $T_s$. (b) Example of a resistive pulse. (c) Schematic of $I$–$V$ curves for conical pores with different surface charges.
current rectification assay allows for the detection of VEGF down to 5 pM. Despite this comparable sensitivity, RPS offer a larger dynamic range. The scope and ease of each assay format allows for a versatile technology that can be tailored to suite the target analyte.

**Materials and methods**

Poly(ethyleneimine), PEI, low molecular weight, LMW (LMW PEI $M_w \sim 2000$ g mol$^{-1}$, 50% wt, 408 700) and high molecular weight, HMW, (HMW PEI $M_w 750 000$ g mol$^{-1}$, analytical standard, 50% wt, P3143), poly(acrylic acid-co-maleic acid) (PAAMA, $M_w \sim 3000$ g mol$^{-1}$ 50% wt, 416 053), phosphate buffered saline solution (P4417 (0.01 M phosphate buffer, 0.0027 M potassium chloride, 0.137 M...
Fig. 3 (a) Current–voltage curves for HMW PEI and PAAMA layers alongside a blank unmodified pore in 5 mM KCl. (b) Current–voltage curves for layer 2 bilayers alongside a blank unmodified pore. (c) Schematic of the layer–by-layer assembly of PEI and PAAMA.
sodium chloride, pH 7.4), bovine serum albumin (BSA, lyophilized powder, ≥96%, A2153) and 2-(N-morpholino)ethanesulfonic acid hydrate (MES hydrate, ≥99.5%, M2933) fibrinogen from human plasma (F3879), albumin from human serum (A9511) and γ-globulin from human blood (G4386) were purchased from Sigma Aldrich, UK. Tunable conical pores (NP200) were purchased from Izon Science (Christchurch, NZ). Carboxylated polystyrene particles with a mean nominal diameter of 220 nm were purchased from Bangs Laboratories, US and are denoted as CPC200. Potassium chloride (KCl, >99%, P/4240/60) and potassium hydroxide (KOH, 0.1 M, >85%, P/5600/60) were purchased from Fisher Scientific, UK. Hydrochloric acid (HCl, 0.5 M, 37%) was purchased from VWR, UK. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC, 22 980) and Recombinant Human Vascular Endothelial Cell Growth Factor (VEGF, lyophilised, >95%, PHC9394) were purchased from Thermo Scientific, UK. Streptavidin-coated superparamagnetic particles (120 nm, 4352 pmol mg⁻¹ binding capacity, 03 121) were purchased from Ademtech, France. The custom DNA oligonucleotide 5’TGTGGGGGTGGACGGGCCGGGTAGATTTTT (V7t1 amine),³³ was purchased as a lyophilised powder (100 pmol µL⁻¹) from Sigma Aldrich, UK. The sequence was synthesised, with a biotin or amine functional group at the 3’ end.

All reagents were used without further purification and all solutions were prepared in purified water with a resistance of 18.2 MΩ cm (TKA, Smart2Pure). pH of solutions were altered using HCl and KOH and the solutions were measured using a Mettler Toledo easy five pH meter with a Mettler Toledo InLab microelectrode.

### Particle assay

120 nm diameter streptavidin-coated particles were diluted to a concentration of approximately 5 × 10⁹ particles per mL. The diluted particle solutions were then vortexed for 30 s, and sonicated for 2 min, to ensure they were well dispersed. The biotinylated aptamer was added to the streptavidin coated particles (4352 pmol mg⁻¹ binding capacity – determined by the supplier) at 113 and 226 nM for 50 and 100% DNA coverage per particle, respectively. The samples were then placed on a rotary wheel for 30 min. Any unbound DNA remaining in solution was then removed via magnetic separation by placing the samples onto a Magrack (GE Healthcare, UK) for 30 min. The supernatant was then removed and replaced with new buffer (PBS). The VEGF was added at the required concentration and then placed on a rotary wheel for 30 min before being analysed.

<table>
<thead>
<tr>
<th>[KCl] mM</th>
<th>Unmodified pore</th>
<th>PEI – layer 1</th>
<th>PAAMA – layer 1</th>
<th>PEI – layer 2</th>
<th>PAAMA – layer 2</th>
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<tr>
<td>5</td>
<td>1.38</td>
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<tr>
<td>10</td>
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<td>0.67</td>
<td>1.69</td>
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<tr>
<td>50</td>
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<td>0.90</td>
<td>1.10</td>
<td>0.73</td>
<td>2.09</td>
</tr>
<tr>
<td>PBS (pH 7.4)</td>
<td>1.15</td>
<td>0.81</td>
<td>1.10</td>
<td>0.93</td>
<td>1.29</td>
</tr>
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</table>
Fig. 4  (a) $I$–$V$ curves for three different NP200 pores coated with two bilayers. (b) Consecutive scans across the voltage range; starting point is indicated with an arrow. A forward scan refers to moving from $+1.6$ V to $-1.6$ V. Each current was recorded after a 5 second wait to the new voltage. (c) $I$–$V$ curves for an individual pore coated with two bilayers; each day the pore was set up and $I$–$V$ curves recorded before being washed with DI water and air dried. All data obtained at 45 mm stretch, pH 6.8 and 5 mM KCl.
Fig. 5  (a) $I$–$V$ curves for a NP200 pore coated with two bilayers. Measured at pH 6.8 and 5 mM KCl. (b) $I$–$V$ curves for an unmodified pore, PAAMA (two complete bilayers) and PEI (1.5 bilayers). Obtained at 45 mm stretch, pH 3 and 5 mM KCl. (c) $I$–$V$ curves for an unmodified pore, PAAMA (two complete bilayers) and PEI (1.5 bilayers). Obtained at 45 mm stretch, pH 7 and 5 mM KCl.
Table 2  Rectification ratios for pores with different surface chemistry as a function of pH. The concentration of KCl was 5 mM for all experiments. HMW-PEI was used for the bilayers.

<table>
<thead>
<tr>
<th>pH</th>
<th>Unmodified pore</th>
<th>PEI – layer 2</th>
<th>PAAMA – layer 2</th>
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<tbody>
<tr>
<td>3</td>
<td>0.83</td>
<td>0.31</td>
<td>0.73</td>
</tr>
<tr>
<td>7</td>
<td>1.40</td>
<td>0.52</td>
<td>1.98</td>
</tr>
</tbody>
</table>

TRPS set-up

All measurements were conducted using a qNano (Izon Sciences Ltd, NZ) instrument combining tunable nanopores with data capture and analysis software, Izon Control Suite v.3.2. The lower fluid cell contains the electrolyte (75 μL).

![Fig. 6](image_url)

(a – i) Schematic of the DNA-modified pore and (ii) aptamer-VEGF interaction. (b) $I–V$ curves for each layer in the DNA immobilisation. (c) Enlarged section of (b). All data are recorded at 50 mM KCl, pH 6.8, 45 mm stretch.
The upper fluid cell contains 40 μL of sample (which was suspended in the electrolyte) and an inherent pressure on the system (47 Pa) was present when making a measurement. After each sample run, the system was washed by placing 40 μL of the run electrolyte into the upper fluid cell several times with various pressures applied to ensure there were no residual particles remaining and therefore no cross contamination between samples. The membranes are placed into jaws on the qNano instrument and are capable of being stretched. The applied stretch is quantified by the distance between the jaws, with an upstretched distance being 42 mm, any values quoted below are the additional distances in mm applied to the membranes. Most experiments were run at 45 mm. The pore diameters measured in the SEM and in the text were calculated from the current at 45 mm stretch.

**Translocation speed**

The velocity of the particle was calculated by extracting the pulse width. Blockade duration events are recorded from the peak of the blockade back to the baseline.
Fig. 8 (a) Plot of rectification ratio versus VEGF concentration for a third pore. (b) Magnified section of the current between 0 and 1.6 V. KCl 50 mM, pH 6.8. Stretch 45 mm. Each measurement was taken in series on the same pore.

Table 3 Rectification ratios measured for the data shown in Fig. 8

<table>
<thead>
<tr>
<th>Concentration of VEGF</th>
<th>Rectification ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 pM</td>
<td>1.36</td>
</tr>
<tr>
<td>50 pM</td>
<td>1.93</td>
</tr>
<tr>
<td>0.5 nM</td>
<td>3.64</td>
</tr>
<tr>
<td>5 nM</td>
<td>11.60</td>
</tr>
<tr>
<td>50 nM</td>
<td>10.12</td>
</tr>
<tr>
<td>DNA</td>
<td>1.33</td>
</tr>
<tr>
<td>BSA</td>
<td>1.53</td>
</tr>
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</table>
current; the total time gives the blockade duration. Nine time points are recorded along the peak,\textsuperscript{29} relative to different positions within the pore and are denoted $T_{0.90}$, $T_{0.80}$, $T_{0.70}$ etc., here we use one measurement $1/T_{0.5}$ to represent the particle velocity.

**Pore modification**

Conical pores were modified by incubating the pore in polymer solution (5\% wt in H$_2$O) at a stretch of 45 mm for two hours, followed by rinsing the pores with deionised water. The pores were then incubated with a second polymer layer for 2 hours, and again washed with deionised water. This process was repeated until the required number of layers was achieved.

**Modification of PAAMA modified pores with DNA**

The aptamer was dissolved in 100 mM MES buffer (pH 5.9) containing 1 mg mL$^{-1}$ EDC. The final concentration of the DNA was 220 nM. The pores were incubated with the DNA and EDC solution for 2 hours.

**VEGF $I$–$V$ assay**

VEGF was suspended in PBS buffer to give the desired concentration. DNA-modified pores were incubated in VEGF solution, and in each experiment the VEGF solution was only placed on the side of the pore with the small pore opening $D_s$. When multiple solutions of different concentrations of VEGF were used, the lowest concentration was measured first. The VEGF solution was in contact with the pore for 30 minutes with the pore being rinsed with water $\times 3$, and PBS $\times 3$ after each protein concentration. The current rectification property of the pore was then measured, in a range of KCl solutions starting with 5 nM first and working up to 50 mM. When a BSA control was used, 50 nM BSA was incubated first for 30 minutes, with the rectification properties being measured in KCl solutions before adding VEGF to the pore.

**$I$–$V$ measurements**

The pores were mounted between two fluid cells which contain an electrolyte solution. Current–voltage ($I$–$V$) curves were recorded using Izon control suite v3.2, the potential was stepped in 100 mV increments from $+1.6$ to $-1.6$ V and the resulting current measured.

**Results and discussion**

**Assay 1 – measuring translocation times from resistive pulses**

To facilitate sample handling and fast assays some RPS strategies have included nanomaterials by either immobilising the target analytes onto their surface facilitating an analyte-induced aggregation,\textsuperscript{35,36} or measuring the particle translocation speed/frequency upon the binding of the analyte.\textsuperscript{11,30,37–40} With the charge of the particle being a contributing factor in pore translocation speed and frequency, the use of DNA-modified materials and pores has become increasingly popular.\textsuperscript{11,30,41–43} The polyanionic backbone of the DNA can enhance or determine the translocation speed/direction and frequency of the current.\textsuperscript{28}
In the first assay format 120 nm particles were modified with a VEGF aptamer. In two variations on this experiment different surface coverages of VEGF aptamer were used. Under condition one, the streptavidin binding sites on the particles are saturated with biotinylated aptamer (termed FC); in the second, circa half of binding sites (defined by the suppliers specifications) were filled with the aptamer (termed HC). The change in translocation time relative to a particle that was not incubated with the protein, i.e. a blank, is shown in Fig. 2. In previous work we have shown how the translocation velocity can be measured from the pulse width and then converted to zeta potentials using Henry's law. The technique was able to distinguish between DNA coverage, in addition to length and structure i.e. ssDNA or dsDNA, on the particle surface, with the detection of dsDNA down to 10 nM LOD.

In the strategy reported here rather than converting to zeta potentials, we simply measure changes to the relative velocities and infer the presence of the DNA and proteins. The hypothesis was that the binding of the aptamer to the protein target, Fig. 2a, should result in a change in translocation time. Fig. 2b shows the change in translocation times, relative to the blank, versus VEGF concentration for the two coverages of DNA.

There is a decrease in velocity for both sets of aptamer-modified beads as the VEGF concentration increases. The change in aptamer shape from ssDNA to one of a folded tertiary structure upon binding the protein, bringing the DNA closer to the particle surface, could lead to a higher surface change density on the particle. This may result in an increase in particle speed. Conversely, as shown in Fig. 2b, a decrease in particle velocity upon the addition of the protein is observed. The velocity of the particles with HC remains constant at VEGF concentrations above 2 nM. This is attributed to the binding capacity of the beads being reached, and that any additional protein to the solution does not change the numbers on the particle surface, or if it does increase they cannot be measured via this technique. The velocity of the FC beads continues to decrease until a concentration of 18 nM is reached. By tuning the aptamer concentration on the particle surface, the dynamic range of the assay can be extended.

The decrease in velocity is thought to be due several parameters that shield the negative charge on the phosphate backbone, increasing the counter ion condensation onto the DNA. Firstly the conformational change to the DNA structure as it binds the protein requires an increased number of counter ions to stabilise the tertiary structure, and secondly the protein pI is 8.5, and therefore is positively charged at the pH used in the experiment. Both particle assays see a decrease in speed, respective to their blanks, at a concentration of 18 pM.

Previous work using aptamer-modified nanomaterials on the TRPS platform measured a change in particle translocation frequency as the transduction signal. The benefit of the method presented here is in the ability to use data from each individual particle as they translocate the pore. Thus it does not rely upon averaging data across hundreds of particles per min, reducing run times and bias in samples with different particle concentrations. A potential limitation for future assays within complex biological solutions is the tendency of proteins to foul the pore wall or particle surface. The particle translocation speed is a contribution of the electrophoretic speed of the particle and electroosmotic contribution of the pore wall. If the pore wall charge changes throughout the experiment it may impact on the assay’s reproducibility. A solution to this would
be to develop a non-fouling coating on the particle and pore wall, preventing the nonspecific adsorption of proteins. Alternatively, the modification of the pore directly could facilitate the detection of the analyte of choice. Other nanopore systems have either utilised the natural surface functionality of the material or modified the pore walls with gold to allow the facile attachment of materials.\textsuperscript{44} To date there has been no reported modification of tunable polyurethane (PU) pores for RPS studies, although strategies for the modification of PU are available.\textsuperscript{45–47}

**Assay 2 – modification of the pore walls and current rectification**

An alternative to a particle-based assay is to use the change in ionic current through the pore, which can be controlled through electrostatic interactions via the pore wall. This effect is seen to a greater extent within conical pores, and typically recorded for pore openings where the diameter, $D_s$, is equivalent to the electrical double layer (DL) thickness,\textsuperscript{24,48} although the DL does not need to fully extend across the pore opening,\textsuperscript{74} and larger pores exhibit current rectification effects.\textsuperscript{23,49} Rectifying properties of conical pores are described in detail elsewhere,\textsuperscript{48} with the degree of rectification being defined as the ratio of absolute currents recorded at a given negative potential and the identical absolute positive potential. Typically conical pores with charged surfaces do not exhibit ohmic behaviour at lower electrolyte concentrations, and the magnitude of the current through the nanopore at negative potentials is greater or less than the current at positive potentials. The ratios can be tuned by changing the supporting electrolyte, pH, ionic strength and applied voltage, with the current–voltage, $I$–$V$, curves recording a preferred direction of current flow\textsuperscript{24,48} (Fig. 1c).

Tunable pores fabricated from PU have a small negative surface charge\textsuperscript{29,49} at pH > 5, and are conical in shape. The unmodified pores used here had an approx. pore diameter, $d_n$, of 800 nm, at a stretch of 45 mm, calculated from the measured current in 50 mM KCl. This is likely to be an averaged dimension, as in some instances the pore will not be spherical in shape, Fig. S1.\textsuperscript{†}

In Fig. 3a, the black dashed line shows the $I$–$V$ curves of an unmodified pore in 5 mM KCl. A weak current rectification ratio of 1.38 is recorded. The $I$–$V$ curve for an unmodified pore in 50 mM KCl is shown in Fig. 3b, and here the rectification value is 1, illustrating the return of the ohmic response at higher ionic strengths.

In an attempt to introduce a facile method for modifying the surface chemistry of the pore, a LbL assembly using PEI/PAAMA was investigated. This system is well studied having been previously used to modify a range of materials.\textsuperscript{50–55} We favoured this technique over other PU treatments such as plasma or the incorporation of grafting polymers into the matrix via swelling,\textsuperscript{47,56,57} as it allows for a simple and rapid dip-coating strategy. In addition, the LbL assembly would allow the thickness and even the porosity of the PEI/PAAMA bilayer to be controlled in the future.\textsuperscript{51} Here we have opted to use a similar system as Yang \textit{et al.}\textsuperscript{54} and Fu \textit{et al.}\textsuperscript{53} that have shown how the thickness of the bilayers can be controlled ensuring that they do not extend across the pore opening and that the thickness of the bilayers remains in the order of a few nanometres.

Fig. 3c shows a schematic of the bilayer construction with consecutive PEI/PAAMA layers. The resulting $I$–$V$ curves in 5 mM KCl are shown in Fig. 3a alongside the unmodified pore. The addition of PEI onto the pore surface resulted in a change in the preferred direction of current flow, with a reduced and
enhanced current flow through the pore under a negative and positive applied potential, respectively. This is indicative of a positive surface charge. Upon coating with PAAMA, the surface charge switches to being negative, resulting in the preferred direction of current flow being inverted (Fig. 3a). All rectification ratios for the HMW PEI bilayers are listed in Table 1, and are measured at ±1.6 V. The addition of each layer of the LbL assembly caused the preferred current direction, or “on state”, to be switched. The magnitude of the rectification can be used to access the presence and quality of the pore coating and as the number of bilayers increased the rectification ratios improved. It is interesting to note that even with two bilayers added to the pore walls, the pore opening remains unobstructed, as the addition of 210 nm particles to the upper fluid cell results in a standard resistive pulse response as they traverse the pore (data not shown).

Fig. 3b shows the effect of increasing the ionic strength of the solution on the rectification ratio, again values are listed in Table 1. Increasing the ionic strength reduced the rectification value, however it is interesting to note that even at >100 mM KCl with a pore diameter circa 800 nm some rectification was observed. The current rectification may have been enhanced by the nature of the modification of the pores here. The PU pores have a large area (diameter of circa 2.5 mm) on the top surface, $T_s$, as shown in Fig. 1. This surface is also modified along with the inner pore walls via the LbL route. A combination of top surface and pore wall modification has been shown to have a larger effect on the rectification ratio. A similar set of $I$–$V$ curves were obtained using LMW PEI, shown in Fig. S2. However the rectification ratios for each subsequent PEI and PAAMA layer were smaller. This is likely due to the nature of the PEI layer placed onto the surface. The mechanism for LbL assembly goes through island formation before forming a full layer and it is hypothesised that when using the LMW PEI it takes longer to form a fully coated surface layer.

The LbL approach was reproducible on multiple pores (Fig. 4a) and shows little hysteresis through multiple cycles, as shown in Fig. 4b. The LbL coating was also stable over short periods of time even after multiple uses, washes, and being dried and stored overnight, as shown in Fig. 4c. Although multiple uses and storage for over a week resulted in a deterioration of the surface chemistry (Fig. 4c), the same pore could be reused and recoated with comparable results (data shown in Fig. S3).

Given the flexible nature of the PU pore it should be possible to change the rectification ratio by subjecting the pore to a smaller stretch producing a higher rectification ratio. Fig. 5a shows the effect on the $I$–$V$ curves as the stretch is decreased from 5 to 2 mm. As the pore opening, $d_s$, is reduced, the observed current at positive potentials decreases. In addition to the pore size, both the PEI and PAAMA have functional groups that should allow a pH controlled current flow. Fig. 5b and c show the effect of pH on the $I$–$V$ relationship for both the PEI and PAAMA surfaces, for comparison the data for an unmodified pore is given alongside. Lowering the pH of the solution to 3 increases the charge density on the PEI surface as more amines become protonated; this results in an increased current flow at positive potentials and a rectification ratio as low as 0.31. Conversely, increasing the pH to 7 reduced the positive charge on the PEI and the current rectification ratio increases to 0.52. Table 2 lists the rectification ratios for PEI and PAAMA pores at pH 3 and 7. For the PAAMA surface at pH 3, the charge density across the carboxyl groups is reduced and the response which is similar to
the unmodified pore is recorded (Table 2). Increasing the pH to 7 for the PAAMA pores increases the negative charge density of the PAAMA pore wall, with an observed decrease in current flow at positive potentials and a current rectification of 1.98. The unmodified pore shows a current rectification of 0.8 at a pH of 3, indicating the surface groups have become protonated.

For all pores, a particle with 210 nm diameter suspended in PBS was passed through the opening as we built up the layers. The particle’s ability to traverse the opening was interpreted to mean that the pore remained unblocked with a thick bilayer mesh, or that the bilayers have not restricted the pore orifice to a large extent.

The current flow through the pore has two contributing factors; the electro-osmotic flow, EOF, across the pore surface and migration of ions through the centre. Whilst others have shown the EOF to be a small contributing factor to current rectification in smaller pores,24 and that a combined effect of the charge on the pore wall and top surface has the largest contribution to the current rectification.24 In the work reported here, a large area of $T_s$ is also modified with the bilayers and future work may be needed to ascertain if this has a larger contribution in the PU pores. The observation of rectification behaviour does illustrate that the enriched ion zone at the pore mouth used to describe the rectification properties of smaller pores24,42 sufficiently exerts an influence across the larger opening, even when the electrical DL is much shorter than the opening $D_s$. This effect has also been attributed to biphasic pulse behaviour in TRPS.49

The reported setup allows for the easy modification of the pore wall, and by using different polymers of different thickness it may be possible to further tune these larger pores to be ion selective.

**Modification of the pore walls with ssDNA**

The main motivation for modifying the pore walls with LbL polymers was to introduce a functionality to which biomolecules could be easily coupled to the pore wall. Similar aspirations have led to the modification of pores with Au allowing thiol-terminated ligands to be placed along the pore wall or the utilisation of the carboxyl groups.43,59 Our method of immobilisation uses EDC chemistry placing the DNA across the $T_s$ and pore surface, shown schematically in Fig. 6a. Fig. 6b shows the $I$–$V$ plots for a pore having gone through the LbL and DNA assembly, a magnified section through the origin is given in Fig. 6c. In the case where DNA was immobilised onto the pore wall, only one bilayer was used. The reason being that one bilayer was sufficient to introduce the carboxyl groups on the surface, and stopping at one bilayer reduced the number of preparation stages. The data in Fig. 6 was recorded at 50 mM KCl. At higher ionic strengths (>50 mM KCl), the DNA-modified pores do not exhibit current rectification; equivalent 5 mM KCl curves are given in Fig. S4.† The reduction in rectification for the DNA surface over the PAAMA layer may be due to the high number of DNA strands on the pore surface and the counter ion condensation shielding the high charge densities; in addition the EDC chemistry may have crosslinked the PAAMA to the underlying PEI causing a reduction in surface charge density.

Incubation of the pore with the target VEGF results in a strong rectification; shown in Fig. 7a (equivalent data for 5 mM KCl are given in Fig. S5†). The decrease in current at positive potentials was shown to be specific to the aptamer-VEGF
interaction in the presence of BSA (Fig. S6†), Fig. 7b, and a mixture of other proteins whose pI values range from 5–7, Fig. S7.†

A similar trend in rectification properties was observed for the lysozyme system in cylindrical pores,59 an explanation for the behaviour was attributed to the high pI of the proteins, and the binding of the protein to the DNA inverting the surface charge. This resulted in a positive surface on one side of the pore and the asymmetric surface charge resulted in current rectification. Our experiment may have produced a similar effect, i.e. the introduction of the protein on one side of the pore produced a different surface charge density from top to bottom. However, if in our study this resulted in a positively charged narrow pore opening, studies into diode-like conical pores would suggest the rectification properties would produce the opposite of the observed effects i.e. an increase in current at positive potentials,60 which is not the observed result.

Our hypothesis for the observed rectification behaviour also relies upon the high pI of VEGF (8.2), and that the protein is positively charged. The inclusion of the protein to the DNA at the pore mouth and $T_s$ increases the cation cloud density at the pore mouth. In addition to this, the folding of the aptamer into a tertiary structure to bind to the VEGF must require an increase in counter ions to stabilise the structure, and these two factors contribute to a decrease in conductivity at the pore mouth for positive potentials, as described elsewhere.24

Fig. 8 shows the concentration dose response of the DNA-modified pore at 50 mM KCl, equivalent 5 mM curves are show in Fig. S8.† As the concentration of VEGF is increased the current measured at 1.6 V decreases, and appears to saturate or remain unchanged beyond 5 nM. The rectification ratios for the 50 mM KCl experiment are given in Table 3. The detection of the VEGF at such low levels, when compared to RPS, may be aided by the inherent nature of the membrane to pre-concentrate cations in the pore mouth.

Conclusions

Presented is a comparison between a resistive pulse and current rectification aptamer assays using the same technology platform. To enable this we present the first LbL modification of PU pores that allows a pH- and ionic strength-controlled current flow. We feel that the tunable pore is unique in this ability to be easily adapted to both, and that the LbL assay reported here offers a simple and reusable method for modifying the surface chemistry of the pores. The RPS assay offers a format that can be easily prepared and multiplexed by pairing each target with a unique particle size/shape. Whilst it offers a wider dynamic range, its sensitivity is limited by the number of proteins required to change the particle surface charge sufficiently to detect a measurable change in translocation velocity. Further improvement to the sensitivity may require the specific design of the particle size and surface to allow a limited and controlled number of aptamers, although this offers a complication in assay time as the flux of material through the pore decreases with smaller particles and pores, or the use of Janus particles to localise the biorecognition.35

The LbL assembly of the pore offers a pH- and ionic strength-dependent rectification behaviour and is the first reported method on such a system. It allows the first observation of strong rectification on pores of this dimension. The strong rectifying properties illustrate that the electric double layer does not need
to significantly extend across the pore opening, although it is acknowledged that further studies are required to assess what the effects of the modification of the top surface of the pore have on the rectification mechanism, and the total contribution of electroosmotic flow in these systems.

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Notes and references

**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 analyze 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.

**Video Link**

The video component of this article can be found at [http://www.jove.com/video/54577](http://www.jove.com/video/54577)

**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 and monitoring DNA-protein interactions. An increasingly common nanoparticle property being utilized in bioassays and in the delivery of therapeutics is superparamagnetism. 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. Particle-by-particle characterization techniques are therefore much more favorable 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). 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 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). 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 characterized individually based on the blockade magnitude, $\Delta I_m$ 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, zeta potential, and concentration measurements simultaneously in a single run and can therefore still differentiate samples of similar, if not the same size by their surface charge, an advantage over alternative sizing techniques.

Zeta potential is defined as the electrostatic potential at the plane of shear, and is calculated from particle velocities as they traverse a pore. Zeta potential measurements of individual particles thus gives insight into the translocation mechanisms and behavior 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

#### 1. Making the Phosphate Buffered Saline with Tween-20 (PBST) Buffer

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 deionized water (18.2 MΩ cm).
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

1. Vortex the calibration particles for 30 sec before sonication for 2 min at 80 watts to create monodispersity of the particles.
2. Dilute the calibration particles 1 in 100 to a concentration of 1x10^10 particles/ml in PBST buffer and vortex for 30 sec.

#### 3. Preparing Streptavidin Coated Particles

1. Vortex the particles for 30 sec before sonication for 2 min at 80 watts to ensure monodispersity.
2. Dilute the streptavidin coated particles 1 in 100 in PBST buffer to achieve a resulting concentration of 1x10^9 particles/ml and vortex for 30 sec.

#### 4. Preparation of Oligonucleotides

1. Reconstitute oligonucleotides with deionized water to a resulting concentration of 100 µM.

#### 5. Addition of Capture Probe (CP) DNA to the Streptavidin Coated Particles

1. Prior to DNA binding, vortex the streptavidin coated particles (200 µl sample volume) for 30 sec followed by a 2 min sonication at 80 watts.
2. Based on the binding capacity provided by the supplier (4,352 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.
3. Vortex the samples for 10 sec and place on a rotary wheel at room temperature for 30 min to allow for the DNA to bind to the particle surfaces via a streptavidin-biotin interaction.
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 magnetic rack for 30 min.
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

1. Add the required amount of target DNA (in excess at 500 nM) to ensure the maximum possible target binding was reached.
2. Vortex the samples for 10 sec and place on a rotary wheel at room temperature for 30 min.
3. Once the hybridization is complete, remove the excess target DNA via magnetic separation by placing the samples onto a magnetic rack for 30 min.
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.
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

1. Plug in the instrument into a computer system with software in place.
2. Calibrate the initial stretch using a caliper.
   1. Measure the distance between the outside of two parallel jaws.
   2. Input into the software by typing the stretch in the 'stretch' field in the 'Instrument Settings' tab and clicking 'Calibrate stretch' underneath the tab.

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. Stretch the jaws between 43 and 48 mm.
   Note: The exact value of the stretch 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 the jaws are stretched.

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.

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.

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.
   Note: The data acquisition is completed at a frequency of 50 kHz with a blockade magnitude lower limit of 0.05 nA, although this can be altered using the software via the 'Analyse Data' tab (under 'Analysis Settings' and 'Resistive Blockades').

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

8. Use a variable pressure module (VPM) to apply a pressure or vacuum to the samples.
   1. To apply an external pressure connect the nozzle to the upper fluid cell, then rotate the pressure arm and click into place (depending on whether a positive pressure (PRE) or a vacuum (VAC) will be applied).
   2. Apply pressure in a 'cm' or 'mm' scale using the pressure stage knob situated on the top of the VPM. Press the knob down to apply pressure on the 'cm' scale and pull it upward to apply pressure on the 'mm' scale.

8. Preparing Samples for TRPS Analysis

1. Vortex samples for 30 sec and sonicate for 2 min at 80 watts prior to TRPS analysis.

9. Calibrating the Nanopore for Zeta Analysis

1. After placing 40 µl calibration particles (1x10^15 particles/ml) into the upper fluid cell, complete a TRPS measurement (setup as in section 7) at 3 applied voltages. Alter the voltage by clicking on the '+' and '-' buttons on the voltage scale in the 'Instrument Settings' tab on the software.

2. Check that the 3 voltages return background currents of approximately 140, 110, and 80 nA. Ensure that at the medium voltage the calibration particles produce an average blockade magnitude of at least 0.3 nA.

3. Apply a pressure so the average full width half maximum (FWHM) durations of the calibration particles are at least 0.15 msec. Do this 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 7.8.2. Once these conditions have been achieved, start the run by clicking 'start' on the software in the 'Data Acquisition' tab.

4. Complete the run by pressing '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 sec (see 'Run Time' also toward the bottom of the screen).

5. Calibrate the system by completing a calibration run as described every time a new nanopore is introduced or for each new day of analysis by completing step 9.1-9.4.

10. Running a Sample

1. Run the samples at the highest or second highest voltage as the calibration samples at ensuring a similar (±10 nA), if not the same, baseline current.
   1. Once the appropriate baseline current is achieved, replace the electrolyte in the upper fluid cell with 40 µl of sample. When a sample is introduced, blockades will be seen 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 sec (see 'Run Time' also situated below the signal trace).
   2. To complete the measurement, click 'stop' in the 'Data Acquisition' tab and save the data file.

2. To save the file, input the file information 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 about the sample or run.

3. 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. Run samples in triplicate with this wash step completed between each repeat sample run as well as between different samples.
**Representative Results**

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. Please click here to view a larger version of this figure.

The removal of any excess DNA that has not bound to the particles surface from the samples is important prior to TRPS analysis, as to not report any 'false-positive' results. The ability to use a magnet to extract and wash the SPPs is a huge benefit for TRPS (Figure 1A). 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. Its ability to complete both size and charge analysis simultaneously in a single measurement 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 functionalized onto the particle's surface.
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.\(^{19}\). Please click here to view a larger version of this figure.

Not only can the technique differentiate between particles unmodified and modified with DNA, TRPS can also differentiate between samples with different concentrations of DNA hybridized 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 hybridized to the streptavidin coated particles. A larger zeta potential value is recorded for particles hybridized with a higher concentration of DNA.

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 hybridized with 210 nM CP DNA and the light green bars/data points represent streptavidin coated particles hybridized with 10 nM CP DNA. Figure adapted from Blundell et al.\(^{19}\). Please click here to view a larger version of this figure.

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.

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 functionalized 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. Please click here to view a larger version of this figure.

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 is the derivative of 1/T (where T is the blockade duration) with respect to voltage, multiplied by the square of the sensing zone length, l. 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 electrokinetic particle velocities of calibration and sample particles, \( \xi_{\text{net Cal}} \) and \( \xi_{\text{net Sample}} \), respectively, are related to their zeta potentials, \( \zeta_{\text{net Cal}} \) and \( \zeta_{\text{net Sample}} \), 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, \( \zeta_{\text{m}} \). The zeta potential of the polyurethane pore was measured using streaming potential techniques12,18 as -11 mV in PBS for this study.

\[
\frac{\zeta_{\text{net Sample}}}{\zeta_{\text{net Cal}}} = \frac{\xi_{\text{net Sample}}}{\xi_{\text{net Cal}}} = \frac{\left( \frac{\xi_{\text{net Sample}}}{\xi_{\text{net Cal}}} \right)}{l} \tag{1}
\]

The zeta potential of each individual particle, \( \zeta_{\text{Sample},i} \), is measured from the respective zeta potentials calculated at various reference points within the pore (equation 2), where \( l_i \) is the position of the particle within the pore after time, \( t=T \times \), \( v_{p,\text{Sample}} \) is the particle velocity of single sample particle i at relative positions \( l_i \), \( v_{\text{c, Cal}} \) and \( v_{\text{c, Sample}} \) 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.

\[
\zeta_{\text{Sample},i} = \frac{\xi_{\text{Sample},i}}{z_{\text{Sample}}} = \frac{\Sigma_{\text{Sample}}(v_{\text{Sample}}-v_{\text{c, Cal}}P)}{z_{\text{Sample}}} \times \frac{(v_{\text{c, Cal}}+v_{\text{c, Sample}})P}{z_{\text{Sample}}} \times \xi_{\text{net Cal}} - \xi_{\text{m}} \tag{2}
\]

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, ΔIΔ, 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 samples.

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 nanopore. Nanopores much larger than the particles being analyzed (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 analyze a range of nanoparticle-based analytes, including the analysis of proteins, DNA, small molecules, aggregation assays, and biologically relevant particles. The versatility of TRPS in characterizing a vast range of analytes shows the techniques potential in a range of areas such as drug delivery, biosensing, and environmental testing.

Disclosures

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