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Applications of Tunable Resistive Pulse Sensing

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Tunable resistive pulse sensing (TRPS) is an experimental technique that has been used to study and

characterise colloidal particles ranging from approximately 50 nm in diameter up to the size of cells.

The primary aim of this Review is to provide a guide to the characteristics and roles of TRPS in recent

applied research. Relevant studies reflect both the maturation of the technique and the growing

importance of submicron colloids in fields such as nanomedicine and biotechnology. TRPS analysis of

extracellular vesicles is expanding particularly swiftly, while TRPS studies also extend to on-bead

assays using DNA and aptamers, drug delivery particles, viruses and bacteria, food and beverages,

and superparamagnetic beads. General protocols for TRPS measurement of particle size,

concentration and charge have been developed, and a summary of TRPS technology and associated

analysis techniques is included in this Review.

Introduction

Tunable resistive pulse sensing (TRPS) is an experimental technique capable of particle-by-particle detection and analysis of submicron colloids and bioparticles.[1-13] The purpose of this Review is to provide the practising researcher with a guide to the characteristics of TRPS, with emphasis on the roles it has played in recent applied research. The Review covers TRPS research into a wide range of particle types, encompassing numerous research fields and research objectives. It draws on information sources that will be of specific relevance to particular TRPS investigations, and also those that promote interdisciplinary understanding of the technique. The Review demonstrates the challenges of colloidal characterization, such as the often complex nature of raw samples (*e.g.* bodily fluids), the importance of understanding the distinct principles behind different measurement techniques, and the sheer variety of interesting particle types.

TRPS is a variant of resistive pulse sensing (RPS), a family of analytic techniques in which particles are suspended in aqueous electrolyte and pass through a single pore in a membrane. An electric potential applied across the membrane drives an ionic current which is disrupted when a particle passes through the pore, generating a resistive pulse (Figure 1(a)). RPS has enabled high throughput particle-by-particle sensing and analysis of cells since the 1950s,[14] submicron particles including viruses since the 1970s,[15, 16] and particles as small as single molecules over the past decade or so, with particularly intense interest in possible nanopore-based DNA sequencing. Recent reviews have covered analysis of nucleic acids using biological nanopores[17] and the broader field of molecular-scale RPS.[18, 19, 20]

TRPS[1-13] is distinctive because the membrane is an elastomer.[11] When the membrane is stretched on macroscopic length scales, the nanoscale dimensions of the sensing pore are mechanically 'tuned'.[12, 13] The most immediate advantages of tuning are pragmatic, such as recovery from a blockage when the membrane is stretched. Also, the signal-to-noise ratio of the resistive pulse signal can be

optimised *in situ*, during an experiment. TRPS therefore has a flexibility unavailable with static pores

used for RPS.

First reported almost a decade ago,[1] TRPS reached essentially its present form by 2010[2] and there

are now established TRPS protocols for measurement of particle size,[3] concentration[2, 4, 5] and

charge.[6-8] Much of this development work has used spherical polystyrene (PS) colloids, commercially

available as size standards. The range of particle sizes measured using TRPS spans from micrometers

down to ~50 nm, covering approximately two orders of magnitude in length scale and bridging the

gap between single molecules and cells.[9] A variety of electrolytes can be used, including those which

replicate physiological conditions, and 100 mM salt is typical. The accessible range of particle

concentrations is approximately $10^5 - 10^{12}$ mL^{-1}.[21] TRPS has sometimes been called scanning ion

occlusion spectroscopy (SIOS), and tunable pores have also been known as resizable (nano)pores.

This Review does not detail RPS work at comparable length scales using (static) solid-state pores.

Such studies have made use of pore materials including silicon-based membranes,[22, 23] carbon

nanotubes,[24, 25] and lithographically moulded[26] and track-etched[20, 27, 28] polymers. Glass pores have

been made by laser machining,[29-31] the pipette pulling technique,[32, 33] and moulding around a sharp

wire tip.[34, 35, 36] Henriquez *et al.*[37] and more recently Luo at el.[38] have reviewed aspects of static RPS

experiments. TRPS has a particular similarity to RPS studies in which the pore geometry is conical.[20,

27, 30, 32, 36, 39, 40]

 The first section of this Review ("Technical aspects") introduces the technology by briefly recounting

descriptions of TRPS and basic sensing characteristics, and then identifying areas of ongoing

technical development. Comparisons with other methods are reviewed, revealing some advantages

and distinct characteristics of TRPS. The second section ("Applications") reviews application of TRPS

to a broad range of particle types. This section summarizes important results and serves as a guide

both to those studies most relevant to each specific experimental area, and to perspectives which

may apply across different fields of research. Studies are broadly classified by application area:

diagnostics and genomics, extracellular vesicles, nanomedicine, phages, viruses and bacteria, and

others. Inevitably, the boundaries between classifications can be indistinct, as in the case of

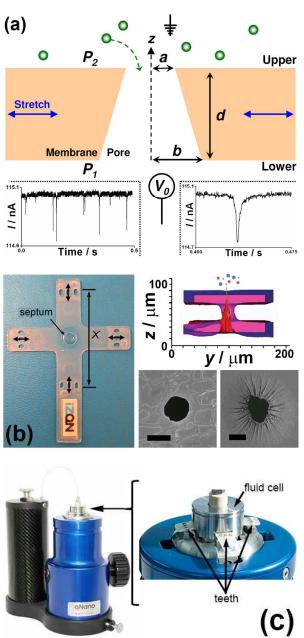structurally-similar EVs, liposomes and emulsions.

Figure 1: TRPS, tunable pore specimens, and typical apparatus. (a) Schematic section through a

tunable pore, indicating experimental variables usually used in TRPS analysis. The conical pore of

length d has openings of radius a and b, and is assumed to be symmetric about the cylindrical z-axis. Potential V_0 and pressure $P_2 - P_1$ are applied across the membrane. Lower left, typical experimental data showing resistive pulses. Lower right, one pulse in greater detail. Adapted from Somerville *et al.*[41] (b) Specimen imaging. Left, a thermoplastic polyurethane specimen. X is ~42 mm for an unstretched specimen, and the pore is located near the centre of the $\sim$200 µm thick circular septum. Reproduced from Willmott *et al.*[13] Right upper, reconstruction of a pore cross-section from confocal microscopy, adapted from Kozak *et al.*[9] Right lower, SEM images of smaller (left) and larger (right) openings of a pore stretched to X = 45 mm (scale bars 1 µm and 20 µm respectively), adapted from Willmott *et al.*[13] (c) The *qNano* (left, Izon Science) TRPS apparatus, and (right) the magnified fluid cell. Ag/AgCl electrodes contact each half of the cell. Actuation is achieved by turning the handle on the side of the *qNano*, which moves teeth placed in the specimen. The black cylindrical VPM connects to the top of the fluid cell via tubing. Adapted from Willmott *et al.*[13] and Weatherall *et al.*[8]

Technical aspects

Specimens, apparatus and stretching

Tunable pore specimens are formed by controlled puncture of a thermoplastic polyurethane (TPU) membrane using a chemically etched tungsten needle.[1] This process is relatively efficient in comparison with the intensive techniques necessary to make pores for detecting single molecules[18] – such as electron or ion beam lithography, or use of biological pores. An approximately conical hole is produced, with larger and smaller pore openings (Figures 1(a) and 1(b)). Depending on the needle[8] and fabrication parameters, commercially-available pores are given a rating indicating their relative size, and therefore the particle size they are most suited to sensing. Scanning electron microscopy (SEM)[2, 3, 7, 11, 12, 41-43] has enabled imaging of a variety of pores at different stretches, and measurement of pore opening sizes, while confocal microscopy can provide three-dimensional information[2, 9, 44] and atomic force microscopy (AFM) has also been used for imaging (Figure 1(b)).[12]

The membrane is the ~200 μm thick central septum of an injection-moulded sample (Figure 1(b)).

Each cross-shaped sample is stretched biaxially and symmetrically by adjusting the separation of

teeth placed in the eyelets on the ends of the arms.[2, 13] Stretching can be quantified using the

distance X between the teeth on opposite arms of the specimen, and X_0, which is the value of X

when no stress is applied (~42 mm at manufacture).[2, 12, 13, 45] TPU (as with all elastomers) is

viscoelastic, but mechanical reproducibility can be maximised by stress-cycling.[11, 13, 46] Macroscopic

membrane actuation changes the micro- or nanoscale pore geometry and ionic resistance, and these

relationships have been modelled both empirically[11-13, 47] and using finite element approaches.[13, 45]

Models for stretching[13, 45, 47] have been used to calculate membrane thickness in experiments.[2, 8, 41, 48]

Membrane thickness can be measured using confocal images[3, 43] or a modified micrometre screw

gauge.[6, 7, 10, 47]

The commercial *qNano* instrument (Izon Science, Figure 1(c))[2] builds on earlier TRPS apparatus.[1, 11, 12]

Precise application of pressure across the membrane controls pressure-driven liquid flows through a

pore, and this is enabled by the gravitational pressure head of water within a variable pressure

manometer (VPM),[8] along with knowledge of the inherent pressure head in the fluid cell.[6, 7] Signal

noise and bandwidth are important,[29] and undersampling occurs if particles are driven too quickly

through a pore, causing the pulse duration to approach the 50 kHz sampling rate.[38] The *qViro*

apparatus (Izon Science) uses the same specimen type, and can withstand the decontamination

processes required for experimentation with viruses. Further bespoke developments (*e.g.* high

frequency actuation[46] and co-ordination with optical techniques[49]) can be anticipated.

Analysis and measurement protocols

Simple, efficient resistive pulse analysis is necessary for high-throughput experimentation. Here we

briefly introduce physical modelling that has been used to develop TRPS protocols for measuring

particle concentration, size, and charge. In the regime relevant to TRPS, models are typically

constructed by calculating resistive pulse magnitude as a function of particle position, and separately

calculating the motion of a particle by considering transport mechanisms acting upon it. Separation

of these calculations is justified by the relatively small timescale of ionic diffusion.[50]

A semi-analytic technique has been used to calculate resistive pulse size as a function of particle

position in TRPS,[51] based on previous RPS work.[9, 11] Early analyses for cylindrical[15, 52] and conical[27]

pores suggest that the pore's electrical resistance to ionic current R_0 can take the form

$$R_0 = \int \frac{\rho}{A(z)} dz, \qquad\qquad (1)$$

where $A(z)$ is the pore's cross sectional area at position z (Figure 1(a)). Here it is assumed that the

electrolyte resistivity ρ is homogeneous, because the size of the electrical double layer (EDL, *e.g.*

ranging between 10 to 1 nm for 1 to 100 mM KCl) is small relative to usual TRPS geometry. The

access resistance beyond the ends of the pore[53] ($R_{end} = \rho / 4a$ for opening radius a) is usually

included.[2] If a particle (an insulating sphere) is introduced, the new pore resistance can be

numerically calculated using the same approach. By implementing this technique, models[43, 51] have

reproduced experimental resistive pulse asymmetry, enabled resistance calculations when a particle

is partly or fully outside of the pore, and found that pulse size should be maximised when the

particle is not entirely within a conical pore.[44, 53]

The particle position as a function of time can be calculated using the Nernst-Planck approach.[54]

Transport mechanisms give a vector sum for the particle flux **J** (units: particles m^{-2} s^{-1}), taking the
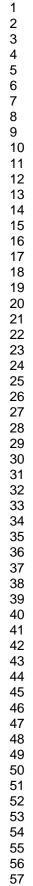
form

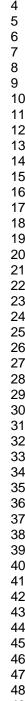$$\mathbf{J} = \left(\mathbf{J}_{ep} + \mathbf{J}_{eo} \right) + \mathbf{J}_{pdf} + \cdots$$
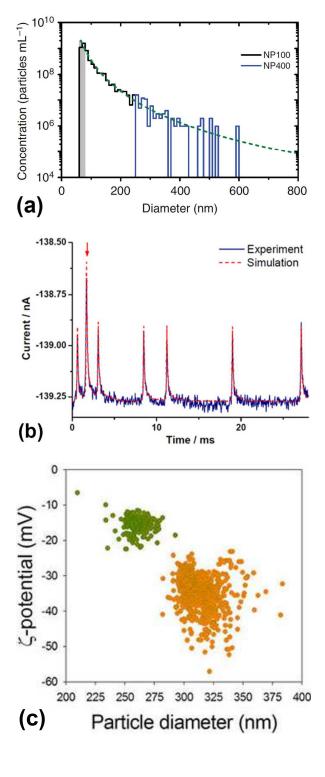
$$= \frac{C\varepsilon}{\eta}\left(\zeta_{particle} - \zeta_{pore}\right)\mathbf{E} + \frac{C\mathbf{Q}}{A} + \cdots. \qquad (2)$$

The specified terms represent fluxes driven by the three most important mechanisms for TRPS: electrophoresis ($\mathbf{J}_{ep}$), electro-osmosis ($\mathbf{J}_{eo}$) and pressure-driven flow ($\mathbf{J}_{pdf}$). C, ε and η are respectively particle concentration, solution permittivity and viscosity. ζ is the zeta potential of the subscripted surface, $\mathbf{E}$ is the electric field and $\mathbf{Q}$ is the volume flow rate. The explicit expressions for electrophoresis and electro-osmosis are appropriate for a thin EDL, and all three terms require that the pore wall has a low gradient, approximating a cylinder.[7, 54] Development of transport modelling for TRPS was recently summarized, with the relative importance of transport mechanisms compared over a range of geometries.[55] Together, Eqs. 1 and 2 can be used to construct resistive pulses.[6, 43, 48] The model developed for TRPS has been used elsewhere,[56] and there are relevant RPS studies of transport mechanisms.[22, 34, 57] Finite element modelling of TRPS[51, 58] has used comparable methodologies to conical-pore RPS simulations,[32, 40, 50, 59] and has been compared with the semi-analytic model.[51]

Concentration. When transport is dominated by pressure-driven flow, the pulse rate is proportional to the flow rate and independent of chemical and physical differences between particles (Eq. 2). Concentration of an unknown particle set can be calculated by calibrating the pressure-driven flow using particles of known concentration. Using standard PS particles, the pulse rate has been demonstrated to vary linearly with pressure up to ~1.8 kPa[2, 5] and with concentration between approximately 1×10^8 and 5×10^{10} mL^{-1}.[2, 4, 44] Independence from particle type has been confirmed by verifying these relationships with particles such as virions and bacteria,[4, 44, 60] and liposomes.[61] Alternative concentration methods include a calibration-free method using geometric parameters[4, 44] and an internal calibration technique[62] which avoids the requirement for separate sample and calibration measurements and could therefore be advantageous for complex biological media.
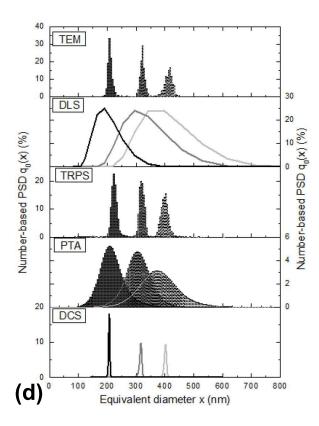
(a)

(b)

(c)

(d)

Figure 2: Analysis and measurement. (a) Size distributions for urinary EVs obtained using two tunable pores of different size (black and blue data) are combined to give a wide-ranging distribution, fitted by power law (green dashes). Reproduced from van der Pol et al.[63] (b) Resistive pulses for 1 μm spheres from an experiment (blue) and the semi-analytic model (red). The pulse indicated by the red arrow is modelled as a dimer of spheres, whereas other pulses correspond to individual particles. Reproduced from Willmott et al.[48] (c) Two populations of 300 nm carboxylated PS particles are distinguished from a mixture. The scatter plot includes particle-by-particle measurements of size and $\zeta_{particle}$, the latter calculated from FWHM durations. Reprinted with permission from Kozak et al., *ACS Nano*, 2012, **6**, 6990.[6] Copyright 2012 American Chemical Society. (d) Comparison of TRPS with other particle characterization techniques (PTA is equivalent to NTA) showing PS particle size distributions for three solutions. Reproduced from Anderson et al.[64]

Size. The principle that particle volume is proportional to the corresponding pulse magnitude was established using cylindrical pores[15] and first demonstrated with TRPS by Vogel et al.,[3] who also

showed that results were consistent with the semi-analytic model (based on Eq. 1) for conical pores. TRPS size measurements have been carried out at a range of pore stretch settings,[47] using multi-modal distributions,[44, 62-64] and by combining size distributions obtained using pores of different sizes (Figure 2(a)).[63] Arjmandi *et al.* recently used TRPS apparatus to demonstrate a sedimentation technique for nanoparticle mass measurement, [65] citing the need for new mass measurement methods for virions in particular.

Particles are usually assumed to be spherical for analysis, and in this Review sizes refer to the equivalent spherical diameter (unless stated). However, the semi-analytic model can be applied to any smooth functions describing pore and particle geometry. TRPS of non-spherical particles is gaining prominence, with recent examples including self-assembled cylinders,[66] viruses,[21] bacterial chains,[60] and specific aggregates.[67] Particle aggregates are especially important for assays, in which a target molecule causes two or more particles to stick together. Two recent assays[68, 69] have attributed pulses to dimers, trimers, and tetramers when the pulse height has been an integer multiple of the height for individual particles, as initially modelled for superparamagnetic particle aggregates (Figure 2(b)).[48, 67]

Charge. TRPS can be used to find a particle's zeta potential ($\zeta_{particle}$), the most widely used measure of colloidal electronic charge. Considering Eq. 2, the electrophoretic mobility can be calculated by measuring the particle velocity, then finding the electric field and pressure-driven flow using the semi-analytic model. To enable calculation of $\zeta_{particle}$, the zeta potential of TPU (ζ_{pore}) has been measured.[7, 42] TRPS measurement of $\zeta_{particle}$ was first achieved[7] by finding the pressure at which **J** = 0 in Eq. 2. Pressure can be varied either continuously or in discrete steps, and **J** = 0 can be identified using either the greatest value of the full-width half maximum (FWHM) duration or the minimum pulse rate.[42] When **J** is non-zero, velocity profiles can be built for individual pulses,[47] yielding particle-by-particle $\zeta_{particle}$ values using either geometric inputs[6] (Figure 2(c)) or calibration particles.[8]

Comparable RPS charge measurements include a method based on transit time alone[24] as well as more detailed calculations.[22, 38] TRPS has been used to find $\zeta_{particle}$ for an emulsion,[41] lipsomes[70, 71] and DNA conjugated nanoparticles.[72] Pulse rates and durations can also be used to infer changes in surface functionalization without explicit calculation of charge.[2, 44, 73-76] For example, injectable drug carriers often use poly(ethylene glycol) (PEG) to avoid adhesion to biological material before reaching a tumour, and TRPS can be used to observe the addition of PEG to particle surfaces.[77]

Ongoing technical development

At present, the greatest measurement uncertainties when using the semi-analytic model usually relate to pore geometry, and interactions driven by steric factors or surface chemistry.[78] Brownian motion can generate complicated pulse shapes when particles are slow-moving[10] or comparable in size to the pore opening.[79] The importance of particle trajectory has become evident due to spatial variations both along[42, 51] and perpendicular to[55] the pore axis. The effects and potential uses of additional transport mechanisms could be studied, including dielectrophoresis,[55] diffusion-osmosis[80] and thermophoresis.[81] Zeta-potential measurements will become more widely applicable following collection of more ζ_{pore} and calibration $\zeta_{particle}$ data under different experimental conditions. Ionic distributions can cause resistive pulse distortions in relatively low molarity electrolyte,[50, 82, 83] and could be explored in more detail using the Poisson-Nernst-Planck (PNP)[50] or space-charge[54, 82, 84] continuum models.

Comparison with other techniques

Colloidal characterization techniques such as TRPS, dynamic light scattering (DLS)[85] and scanning and transmission electron microscopy (SEM and TEM) each employ different physical principles. They are often used together to study the same sample, with each experiment providing distinct information. Two quantitative studies[64, 86] have compared the same emerging techniques (TRPS, nanoparticle tracking analysis (NTA), and differential centrifugal sedimentation (DCS)) with more established

techniques (TEM and DLS). Anderson et al.[64] measured particle size distributions for monodisperse

and multiple population solutions of 220-410 nm PS particles (Figure 2(d)), whereas Bell et al.,[86] who

also used scanning mobility particle sizing (SMPS), measured monodisperse samples of five Stöber

silica particle sets with diameters of 100-400 nm. Both studies produced TRPS size distributions

consistent with TEM. Another quantitative comparison[63] included multimodal size distributions of PS

beads measured alongside urinary EVs, and compared TRPS with TEM, NTA, and conventional and

dedicated flow cytometry (FC). For the PS beads (> 100 nm), TRPS had the lowest sizing error after

TEM and dedicated FC, with the latter technique performing well due the refractive index uniformity

of PS particles. In concentration measurements (approximate range 10^9-10^{10} mL^{-1}), TRPS gave good

results for PS standards, and results for EVs agreed with values obtained using NTA, with FC giving a

lower result and TEM suffering from losses during sample preparation. Recently, Varga et al.[78] used

EVs to compare TRPS, NTA, electron microscopy, small angle X-ray scattering (SAXS), size exclusion

chromatography and DLS. Modal values were consistent (~130 nm) across the techniques, but the

size distributions varied. There are similar comparative studies that have not involved TRPS.[87, 88]

Eldridge et al.[10] made a qualitative comparison of TRPS with DLS, NTA, DCS, FC, suspended

microchannel resonators (SMR) and electron microscopy in the context of drug delivery applications.

The physical nature of each technique was summarized, and measureable ranges of sample size,

concentration and volume were tabulated. Similar qualitative comparisons have specifically referred

to measurement of EVs[89] and solid lipid nanoparticles.[90] Heider and Metzner's review of virion

measurement methods[21] considered TRPS, NTA, advanced field flow fractionation (FFF), and a virus

counter method involving fluorescent labelling, along with time-consuming biological techniques.

Advantages of TRPS for virions include efficient size measurement to determine aggregation state,

the ability to measure low particle concentrations by applying pressure, and (along with NTA and

FFF) charge measurement capability. Virus shape is not a great issue for TRPS, while the small

instrument footprint and the possibility for specificity are advantages. On the other hand, some

viruses are currently beyond the lower size capabilities of TRPS, and refinement of raw biological samples is important for all techniques.

Other experimental comparisons involving TRPS have typically been restricted to a few measurements, the relative merits of which may not extend beyond each set of experimental conditions. Perceived advantages of TRPS have included the accessible ranges of particles,[86] electrolytes[86, 91] and measured quantities.[63, 91, 92] Advantageous sample volume (tens of μL),[60, 93] cost and portability[91] and accuracy[61] have also been noted. The accessible size range is not unlimited,[62, 63, 86, 94 78] but can be extended by the use of multiple pores. Discussions of measurement speed (a few minutes per measurement, following training)[60, 63] and associated pore clogging[63] require experimental context. For example, sample preparation[95] and classification is an issue for complex biological media regardless of the measurement technique.[93] In concentration measurements, TRPS has been more accurate than flow cytometry and phase contrast microscopy,[4, 92] and better than than optical density measurements for matching the plating method for bacteria.[60] However, inconsistencies have been demonstrated in a comparative study of ~200 nm EVs and liposomes[71] and studies affected by sample contamination.[21, 60, 96] There is relatively little comparative work involving charge measurement. Like TRPS, commonly used DLS-based techniques derive $\zeta_{particle}$ from electrophoretic mobility (Eq. 2). In a recent comparison using ~200 nm PS particles,[42] the typical difference between zeta potentials obtained using TRPS and DLS was 15% (<5 mV), with an experimental error of ~10% for each technique.

Distinct characteristics. TRPS avoids difficult sample preparation and experimental artefacts associated with electron microscopy,[38] although the electrolyte can cause unwanted aggregation.[90] Measurements are independent of optical properties[78] such as particle labelling, knowledge of refractive index, or refractive index contrast. TRPS analyses assume that the particle is an ideal insulator, and measurement protocols can require calibration.[63] Experimental parameters can be

varied to optimize TRPS data resolution, and two recent studies have done this systematically.[71, 97]

When particles fall outside the size detection thresholds for a particular pore, summary statistics[97] and concentration measurements[71, 97] are necessarily affected. This highlights the advantage of using the mode to describe particle size distributions, and the benefits of TRPS comparisons under identical experimental conditions. In terms of data handling, TRPS provides particle-by-particle data which is beneficial for resolving multi-modal[44, 62-64] and high dispersity[63, 90, 98] distributions (especially in comparison with DLS), can be used to calculate any central value or spread statistic, and can be transformed for direct comparison with ensemble average data (see Supplementary Information).[41]

Many considerations apply more generally when selecting a colloidal characterization technique. For example, the accessible ranges of particle concentration and size is important, as well as the volume and type of solution (for TRPS, see the Introduction). The overall importance of user knowledge regarding instrument settings and data handling has been noted.[71, 78] Care is required regarding the specific type of size measurement[86] and theoretical differences in size distributions.[63, 87] For biological solutions, sample preparation is especially critical. Lane et al.'s[95] TRPS study, which used liposomes to systematically compare isolation protocols based on ultracentrifugation, sedimentation reagents and density gradient, is important in this respect. There are opportunities to develop traceable uncertainty analyses for most emerging techniques, including TRPS.[78] Coumans et al.[97] recently used TRPS to obtain 102 repeat size distribution measurements for urinary vesicles, highlighting the importance of studying reproducibility. Finally, the experimental design should always be considered relative to the specific research question.

Table 1. Summary of TRPS studies in which particle aggregation has been detected. PEG = poly(ethylene glycol), PDGF = platelet-derived growth factor, PNA = peptide nucleic acid, SPBs = superparamagnetic beads, RBCs = red blood cells.

Ref.	Particle Material	Surface Function	Indicative Size[1] / nm	Particle Concentration / x 10^9 mL^{-1}	Analyte	Analyte Concentration[2]	Detection
Assays							
67	Au/Ni rods	Avidin (Ni) and PEG (Au)	1230 long x 300 diameter	0.24	Biotin	~100 fM	Mean ΔI, FWHM
		Aptamer (Ni) and PEG (Au)	1100 long x 300 diameter	0.09	PDGF	~100 fM	Mean ΔI, FWHM
68	SPBs	Avidin	3000	0.5	Biotin	~1 pM – 1 nM	Mean ΔI, mono- and multimer fractions
		Avidin	1000	6	Biotin	~1 nM	Monomer fraction
		Streptavidin	300	10	Biotin	~1 nM	Monomer fraction
69	Au	DNA[3]	25	60	DNA	5.0 pM	Pulses observed
73	Au	Citrate	50	45	PNA	5 nM	Size-duration scatter plot
99	Au	(i) Avidin (ii) DNA	(i) 30 (ii) 55	(i) 4.2 (ii) 13	DNA	530 copies	Size threshold
100	Magnetic beads	Protein[4]	1000	6 x 10^{-4}	RBCs	~10^7 mL^{-1}	Mean size, size distribution
Other				(Specified units)	(Mechanism)		
41	Soy bean oil	β-lactoglobulin	150	10 wt% oil	~120 days		Size mode, mean
		β-lactoglobulin	150	10 wt% oil	Salt		Size mean, median
48	SPBs	Carboxylate	1000	0.4 x 10^9 mL^{-1}	Bar magnet		Size distribution, pulse rate, time between pulses[5]
91	Carbon Nanohorns		1-2	0.5-50 µg mL^{-1}	In cell media		Size distribution
	Carbon black		14	0.5-50 µg mL^{-1}	In cell media		Size distribution
	CeO$_2$		7-25	0.5-50 µg mL^{-1}	In cell media		Size distribution
	Ni		60	0.5-50 µg mL^{-1}	In cell media		Size distribution
101	Liposomes	EPC-3-based[6]	250	5 mM lipids	~1 hr		Size distribution
102	Wine proteins		200 – 4000[7]	100 mg L^{-1}	Heating		Size distribution, concentration

[1] As available: mode, mean or manufacturer's specification.

[2] Order of magnitude estimate indicated by ~ unless limit of detection claimed in original publication.

[3] Two types of bead with different DNA ends (18 and 100 bp) matching the ends of the target.

[4] The protein annexin-V binds to phosphatidylserine found on the surface of RBCs after eryptosis.

[5] This study pointed out that the mean particle size gives higher sensitivity to aggregation than the mode when most beads are not aggregated.

[6] Hydrated egg phosphatidylcholine in lipolysis medium.

[7] Range of aggregate sizes formed from molecular protein.

Applications

Diagnostics and Genomics

This section focusses on the use of TRPS to study DNA, aptamers and other molecules which support

specific binding interactions. The goal is often a diagnostic, sensing or monitoring assay at low

concentrations of target. Bead aggregation is one common method for detecting the presence of a

target, and understanding particle aggregation is of wider importance for TRPS. TRPS studies of

particle aggregation have been summarised in Table 1.

DNA. As noted in the Introduction, interest in the wider field of nanopore science has been driven by

potential DNA sensing applications. TRPS is no exception, as the first description of size-tunable

pores[1] involved detection of dsDNA molecules each consisting of 2686 base pairs (bp). Controlled

gating of these molecules was reported (Figure 3(a)), with resistive pulses observed only at relatively

high stretch. The particular geometry of the pore used in this study allowed detection of DNA

molecules, but due to the relative ease of tunable pore fabrication at larger length scales, no

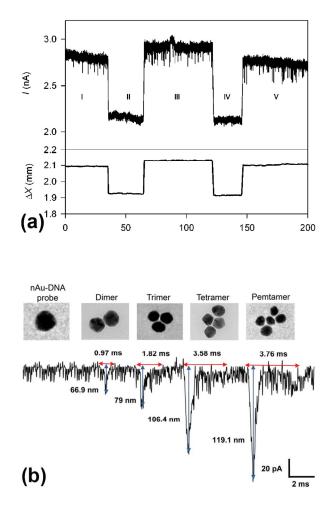subsequent TRPS studies have reported detection of single molecules.

(a)

(b)

Figure 3: TRPS experiments using DNA. (a) Gating of 2.7 kbp dsDNA molecules, reproduced from

Sowerby *et al.*[1] Traces show the stretch applied to a membrane (lower), and ionic current (upper) as

a function of time. Zones labelled I-V are delineated by abrupt changes corresponding to adjustment

of ΔX. (b) Distinguishing multimers of DNA-aggregated 25 nm Au nanoparticles. TEM images (upper)

demonstrate aggregates as observed in the ionic current trace (lower). Arrows and labels indicate

the mean baseline duration and modal magnitude. The latter is scaled to effective spherical

diameter. Reprinted with permission from Ang and Yung, *ACS Nano*, 2012, **6**, 8815.[69] Copyright 2012

American Chemical Society.

Subsequently, TRPS has been used for on-bead DNA sensing in which evidence for molecular

interactions is provided by detecting changes in particle size or surface charge due to

functionalization or aggregation. Firstly, TRPS was used to simply distinguish 220 nm organosilica

nanoparticles modified with λ-DNA (48 kbp dsDNA, ~16 nm long) from unmodified particles.[44] The

modified particles produced an increased pulse duration resulting from reduced surface charge,

without an observable increase in particle size. Low *et al.* studied non-specific aggregation of citrate

capped gold nanoparticles in the presence of a mixed base peptide nucleic acid (PNA, 20 bp

ssDNA).[73] The TRPS size measurement for single nanoparticles (~50 nm) agreed with DLS and TEM

results. On addition of PNA, TRPS indicated aggregates with diameters up to 125 nm in addition to

charge neutralisation (increased pulse duration). The lowest PNA concentration at which

aggregation was inferred was 5 nM, whereas 50 nM was required using DLS. Specific DNA

interactions were first studied with TRPS by Booth *et al.*,[72] who functionalized dextran-based

magnetic beads with 23 bp DNA complementary to a target. There was no significant change in the

modal size recorded before (109 nm) and after (106 nm) the addition of 0.01 nM target DNA to 2 x

10^{11} mL^{-1} beads, but the modal FWHM duration was reduced from 0.95 ms to 0.68 ms. This

indication of increased particle charge was verified using TRPS charge measurements[7] in which the

zeta potential increased from -11 mV to -17 mV.

Two studies[69, 99] have used a TRPS sensing strategy in which two types of gold nanoparticle have

induced aggregation in the presence of specific target DNA. In both cases individual nanoparticles

were too small to be detected, so resistive pulses indicated the presence of the target. Ang and

Yung[69] prepared two sets of 25 nm gold particles with DNA fragments (18 and 100 bp)

complementary to the different ends of a target. With the target present, the observed aggregates

could be classified as dimers, trimers, tetramers and pentamers on the basis of pulse magnitudes

(Figure 3(b), see also[68]). Aggregates were not observed when the target sequence was altered by a

single base. Target concentrations from 5.0 pM to 2.5 nM were near-linearly correlated with

aggregate detection rate, suggesting a method for quantifying target DNA concentration. Yang *et*

al.[99] used 30 nm and 55 nm gold nanoparticles functionalized with avidin and a thiol-DNA probe

respectively. The target DNA, a 340 bp gene from methicillin-resistant *Staphylococcus aureus*

(MRSA), underwent loop-mediated isothermal DNA amplification (LAMP) so that it would bind to both types of nanoparticle, inducing aggregation. Using arbitrary size thresholds, the authors quoted a limit of detection of 530 DNA copies within a processing time of 2 hours. The technique was tested against a negative control, and also showed good specificity against a strain of *S. aureus* which does not carry the same gene.

Most recently,[103] TRPS has been used for label-free counting of individual strands of synthetic *P. aeruginosa* DNA. Padlock probes, which ensured specific capture of the target, were bound to 1 μm magnetic beads and subjected to rolling circle amplification (RCA), producing large coils of ssDNA attached to the beads. When beads were mixed with DNA at a 10:1 ratio, the number of strands could be quantified using the increased baseline pulse duration. This method was demonstrated using a pulse duration detection threshold, with a lower detection limit near to 10 zmol of DNA in 10 μL of electrolyte (*i.e.*, 1 fM). The authors noted that the method is more sensitive than similar fluorescence based methods, with a total assay and analysis time under 1 hour.

Aptamers and other specific interactions. Aptamers are short, single-stranded pieces of DNA or RNA that are developed to have specific binding affinity for a target molecule.[67] The selectivity, stability and cost of aptamers is attractive for sensing applications.[67, 74, 104] In that context, it is unsurprising that TRPS has been used to investigate the use of aptamers more often than other specific interactions. Platt *et al.*[67] were the first to use TRPS to study both an aptamer capture probe and the avidin-biotin interaction. The detection strategy involved aggregation of cylindrical nanorods (diameter 300 nm, length 2-4 μm) in the presence of the target protein, platelet-derived growth factor. Each rod was a 'barcode' of gold and nickel segments. By appropriate functionalization of the barcode, the orientation of aggregates could be controlled, and changes in pulse magnitude and FWHM were specific to the resulting aggregate shape (Figure 4(a)). Detection in the femtomolar range was enabled by the superparamagnetic properties of the Ni-containing rods.

Figure 4: TRPS experiments using aptamers and the avidin-biotin interaction. (a) Nanorod

aggregation in the presence of BBSA. Only the Ni segments of AuNi barcoded rods (dark grey in inset

schematic) were functionalized with avidin, inducing end-to-end aggregates. The changes in resistive

pulse magnitude (i, red) and FWHM duration (ii, blue) are compared with a control analyte (dashed

lines). Reproduced from Platt *et al.*[67] (b) TRPS size histograms with accompanying schematic

diagrams for carboxylated PS nanoparticles in the presence of a target (upper), the same

nanoparticles coupled to aptamers (middle), and modified nanoparticles with the target present

(lower). Black and red dashed lines indicate the modes of the upper and middle distributions

respectively, and green bins indicate the average of DLS size distributions. Adapted from Alsager *et

al.*[74]

Billinge *et al.*[68] subsequently used TRPS to study aggregation of 3 μm superparamagnetic beads

coated with avidin in the presence of picomolar biotinylated bovine serum albumin (BBSA).

Aggregation, as indicated by increased pulse sizes and fewer pulses, reduced at higher analyte

concentrations (~1 nM) due to saturation of the bead surface with analyte. This 'hook' effect, so-

called because of the form of data plots, can produce a false negative result and is a general feature

of aggregation assays. This assay was also studied with variations in bead concentration, bead size,

binding capacity, and with a bar magnet present. The same team reported on detection of the

protein thrombin using beads modified with three different aptamers.[104] In this study, the decrease

in pulse rate due to charge screening was the best indicator of target presence, and pulse rates were

studied in real time (over ~3 minutes) after thrombin (0.1-1000 nM) was added to the beads (3×10^9

mL^{-1}) in the TRPS fluid cell. The change in pulse rate allowed calculation of dissociation constants for

the aptamers, and the values obtained were consistent with literature. Variations in assay

performace between the three aptamers were attributed to conformational changes when binding

to the target.

Alsager *et al.* also used an on-bead aptamer based detection strategy, but without aggregation.[74] An

aptamer for the target 17β-estradiol was tethered to 217 nm PS nanoparticles, and TRPS was used to

study functionalized nanoparticles (5.2×10^{10} mL^{-1}) exposed to target concentrations between 5-150

nM. Resistive pulse sizes increased with the attachment of the aptamer, then decreased on addition

of the target due to conformational change (Figure 4(b)), in qualitative agreement with DLS.

Nanoparticle functionalization resulted in increased pulse duration, which was inconsistent with DLS

charge measurements, and was attributed to increased steric drag in the pore constriction. Pulse

FWHM values also increased when the target was added, in agreement with the DLS trend, and

consistent with charge screening by the target. The assay did not discriminate between the target

and compounds from the same steroid family, but excellent discrimination was observed for

bisphenol compounds. Overall, TRPS assays using aptamers have revealed that changes in particle

size and charge can be detected, but are not always predictable due to the unique three-

dimensional binding conformation between the aptamer and its target.

Further specific interactions studied using TRPS include detection of aggregates formed by 1 μm

beads and red blood cells (RBCs).[100] Aggregation was enabled by the specific affinity between a

protein on the beads and the outer lipid bilayer of RBCs following eryptosis, which was instigated

either by increasing osmolarity or using a potential anti-cancer drug. Lai *et al.*[105] used TRPS size

distributions to observe binding of antibody-loaded protein microparticles to a target molecule.

Protein microparticles were suspended in the fluid cell above a pore, and the mean particle diameter

increased from 1041.5 nm to 1212.6 nm when the target (mouse IgG) was introduced. No size

change was observed on the addition of rabbit IgG.

Extracellular vesicles

TRPS is being applied widely to studies of extracellular vesicles (EVs),[106] which are lipid bilayer

vesicles secreted by most human cells. EVs play a role in inter-cell communication, in gene delivery

and as disease biomarkers.[62, 106, 107] Their concentration and composition can be altered by

pathological conditions,[107] and they can carry microRNA (miRNA).[108] There is currently a need to

classify and understand the roles of particular EVs. By one definition,[107, 109] EVs of endosomal origin

are termed exosomes (generally 30-100 nm), whereas EVs originating from the plasma membrane

are termed microvesicles (100-1000 nm). EVs are also sometimes referred to as microparticles.

However, classification is challenging, because a raw sample of bodily fluid (*e.g.* blood, urine or

lymph) contains a complex, high dispersity mixture of sub-cellular particles and proteins. This

problem has produced high demand for new measurement tools,[87] and consequently TRPS has

recently featured in several reviews and comparisons of EV measurement techniques.[63, 78, 89, 93, 97, 110,

111] EVs have been measured from 60 nm[95, 112, 113] through to 1 μm,[92] and multiple pores have been

used to handle highly disperse distributions,[63, 92] but lower size limits for TRPS do not currently

extend to the smallest EVs[78, 97] as noted in comparisons with electron microscopy (Figure 5(a)).[62, 94, 114] Protocols for EV collection, isolation, handling, and storage for TRPS are in development,[95, 110] and analysis of protein content is not currently enabled.[89] The remainder of this section covers TRPS studies of EVs isolated from particular cell types, demonstrating a wide range of measurement roles and application areas.

Urinary vesicles occur naturally at relatively high concentration with low contamination. In their comparative study, van der Pol *et al.*[63] used urine, centrifuged to remove cells and diluted in phosphate buffered saline (PBS). Size distributions for these vesicles were obtained using pores of two sizes (Figure 2(a)). Coumans et al.[97] also used urinary vesicles for their study of TRPS reproducibility (see Distinct characteristics), while De Vrij *et al.*[62] studied EVs isolated from urine as well as blood plasma and pleural fluid. Their samples all produced TRPS size distributions with more than 95% of particles in the range 150-400 nm. Cheng *et al.* measured size distributions for exosomes derived from urine[115] and blood[114] in studies aiming to develop collection and processing methods for miRNA sequencing. A standardized ultracentrifugation protocol was used to isolate exosomes, and TRPS size distributions provided evidence that the miRNA yield from exosomes isolated from urine and resuspended in PBS was superior to samples from the usual medium, cell-free urine.

TRPS has been used to analyse the size and concentration of microparticles in blood before and after apheresis, a treatment for removing cholesterol.[116] Apheresis did not alter the modal microparticle size, while the concentration dropped when measured using pores of target diameter 200 nm and NTA, but was unchanged when using pores of target diameter 100 nm. These differences illustrate the importance of size thresholds and sample preparation for biological fluids. Burnouf *et al.*'s review of methods for studying platelet microparticles (PMPs),[93] the most abundant microparticles in blood, included a TRPS size distribution for ~400 nm PMPs in platelet-poor plasma. PMPs are 0.1–

1 μm phospholipid-based fragments shed from platelets when they undergo activation, stress, or apoptosis.

Szabó et al.[117] analysed centrifuged supernatant from a culture of leukaemia cells in a study concerned with the effects of EVs on human monocytes in the presence of recombinant human tumour necrosis factor. As well as measuring the EV concentration and size distributions (Figure 5(b)), TRPS played a role confirming that a nominally EV-free medium was indeed free of microvesicles. Patko et al.[94] obtained a size distribution in the range 200-300 nm in a study of leukaemia-derived EVs binding to the extracellular matrix. The role of an enzyme (capase-3) in production of EVs from breast cancer cells has also been investigated.[92] In cells transfected with capase-3, the concentration of EVs increased approximately 5-fold, most significantly in the size range 400-600 nm from an overall measured range of 100-1000 nm. Here, TRPS was preferred over FC as the latter does not give particle size information and underestimated the particle concentration. De Vrij et al. also measured tumor cell-derived EVs between 100 and 200 nm in diameter.[62]

EVs derived from mesenchymal stem cells (MSCs) can be used as a delivery vehicles, and TRPS has been used to measure size distributions for EVs that could deliver enzymes to combat Alzheimer's disease (150-200 nm),[118] and anti-cancer miRNA to tumour cells (60–180 nm, with the major peak at 65–75 nm).[112] In the latter study, TRPS suggested that the number of secreted nanoparticles (including exosomes) increased following transfection of synthetic miRNA into MSCs. Elsewhere, EVs from endometrial epithelial cells were compared with EVs found in uterine fluid and mucus in a study of implantation in the uterine cavity.[119] The similarity of size distributions from different sources (100-500 nm with a mode of ~100 nm) suggested that epithelial EVs are released into the uterine cavity. Liposomes, which are synthetic spherical compartments enclosed by a phospholipid bilayer,[120] can be used as a model for EVs. Lane et al.[95] demonstrated that model liposomes had a

similar size distribution (~100 nm) to exosomes derived from serum-free cell culture media at

concentrations between 1.5 x 10^9 and 3 x 10^{11} mL^{-1}. Maas et al.[71] compared analyses of tumour cell-

derived EVs and 212 nm liposomal EV mimics at 3.3 x 10^{13} mL^{-1} using TRPS, NTA and high-resolution

FC. TRPS results were obtained at various pore geometries, applied voltages, buffers, calibration

particle sets and particle dilutions, and emphasized the importance of reproducible measurement

through understanding and control of experimental settings. Emerging reports on EV research[121]

suggest that there are other TRPS studies in progress concerning EVs derived from fibroblasts,

marrow, white blood cells, and from humans as well as animal models.

(a)

(b)

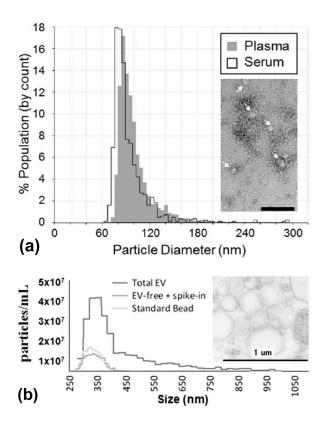Figure 5: TRPS experiments using EVs. (a) Size distributions for exosomes from blood plasma and

serum. Inset, TEM of plasma exosomes (denoted by arrows, scale bar 200 nm). Adapted from Cheng

et al.[114] under a Creative Commons license.[122] (b) TRPS size distributions and TEM (inset) for EVs in

cell-free supernatants derived from acute lymphoblastic leukemia cells. In the TRPS data, exclusion

of EVs in one solution was confirmed by spiking the solution with 400 nm reference beads. Adapted

from Szabó *et al.*[117]

Nanomedicine

Drug delivery. Many nanoparticle formulations are being explored in order to provide new drug

administration methods that are non-invasive, targeted, and provide controlled medication

release.[10, 123] Physical properties (including size, charge and concentration) can affect the circulation

time, localization, cellular uptake, drug release profile and toxicity of nanoparticles *in vivo*.[124]

Therefore accurate characterisation is necessary to ensure effectiveness and quality control.

Regulatory compliance has been highlighted by a recent study[70] which demonstrated TRPS

measurement of reporting statistics for Caelyx, a commercial liposome-based drug treatment. TRPS

yielded a narrower size distribution than DLS, and was also used to study liposomal size differences,

concentration, charge (therefore surface properties), and aggregation during the freeze-thaw

process.

Liposomes, a major class of drug delivery nanoparticles, are usually prepared by extrusion of lipid

through a filter, producing a particle size range close to the diameter of the filter pores. [61, 125]

For the first liposomes studied using TRPS,[125] particle size distributions (~200 nm) and pulse rates

were measured as a function of the jaw width and applied pressure. TRPS size distributions have

been used to monitor the stability of two liposome dispersions, by looking for signs of aggregation in

particle size distributions. In the first case, tumour-targeting polymer-liposome complexes (~150 nm)

were more stable than more standard liposome types when exposed to protein treatment.[75] In the

second, the size distribution of soy phosphatidylcholin (SPC) liposomes (100-160 nm) did not change

significantly following incubation in a medium approximating the gastro-intestinal track, whereas

liposomes based on hydrated egg phosphatidylcholine formed aggregates up to 1 μm in diameter

over 60 minutes.[101] Yang *et al.* also studied SPC liposomes,[61] extruded using filters approximately

100, 200 and 400 nm in size. TRPS was able to take measurements at more than 20 times greater

dilution of phospholipid than DLS. TRPS and DLS yielded similar size measurements for the smaller

two samples, while inconsistencies obtained for the largest sample were attributed to DLS

uncertainties caused by size dispersity. Similarly, a TRPS size distribution for lyophilisomes over the

range 700-1600 nm gave a mean diameter of 1214 nm,[98] in comparison with a higher DLS value of

1695 nm.[126] From SEM, lyophilisome size distributions are known to range in diameter from 100-

3000 nm. Lyophilisomes are biocapsules made from water soluble proteins, with the defining factor

that there is no need for amphiphilicity.[126] A method for calculating extruded liposome

concentrations has been tested using TRPS,[127] yielding ~90% agreement for ~200 nm liposomes at
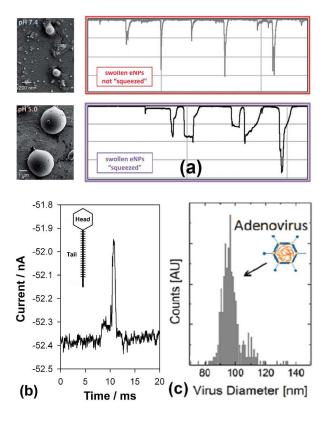
~10^{13} mL^{-1}.

Figure 6: (a) Expansile nanoparticles used for drug delivery are much larger at lower pH (SEM images,

left). TRPS measurements for the expanded particles (right) can 'squeeze' the particles by reducing

the pore size, resulting in longer pulse durations. Adapted from Ref. 74. (b) Resistive pulse for a

lambda phage (length ~230 nm), represented by the inset schematic diagram. The shoulder of the

pulse prior to the maximum resistance change suggests that the phage tail passed through the pore

prior to the head. Reproduced from Willmott et al.[11] (c) TRPS size histogram for adenovirus

particles, with inset schematic of the virus. Adapted with permission from Vogel et al., Anal. Chem.,

2011, **83**, 3499.[3] Copyright 2011 American Chemical Society.

Particles which release their drug in response to a particular chemical or physical stimulus are a

promising development in drug delivery. The drug can be delivered to a tumour specifically, and in

high concentrations. For example, Burgess and Porter[128] used TRPS to study phase-shift

nanoemulsions (PSNEs), which release their drug when an ultrasound stimulus converts

nanoemulsion drops to nanobubbles. The size of PNSEs (mean diameter 178.3 nm and concentration

5.3×10^{11} mL^{-1}) is important as they must be able to move through blood vessel walls into

neighbouring tissue. TRPS has more commonly been used to study particles which release their

payload in the slightly acidic conditions (pH 5.0) found within a cell. This is the case for ECM-

targeting liposomes (above),[75] hydrogel particles of diameter ~1500 nm which degrade below pH

7.4,[129] and polymeric nanoparticles (200-250 nm) which burst at pH 7.4 and in the presence of

gastrointestinal enzymes.[130]

Colby et al.[79] used TRPS to study 'expansile' nanoparticles, which SEM suggests are 20-200 nm in

diameter near pH 7.4, but expand to 200-2000 nm at pH 5.0 as their polymer matrix breaks down.

The proportion of particles measured by the larger of two pores increased from <1% to 90% over 5

days, consistent with an increase of measured average diameter from 876 ± 259 nm to 1339 ± 516

nm between days 3 and 5. Pulse durations for expanded nanoparticles became larger and more

irregular when the applied stretch was decreased (Figure 6(a)), suggesting that the soft expanded

nanoparticles squeeze through the pore. This experiment represents an initial foray towards the use

of tunable pores to mechanically interact with soft nanoparticles, as also suggested in recent RPS reports using static membranes.[38, 131]

Magnetically loaded particles also show promise for drug delivery because they can be directed to specific sites using an external magnet.[132] TRPS has been used to measure the size distributions of lipid particles that were small enough for administration by inhalation (< 5 μm), and contained superparamagnetic iron-oxide nanoparticles (SPIONs) along with a model drug, budesonide.[133] These particles had a mean diameter of 2.2 μm (mode 1.6 μm) without SPIONs, rising to 2.8 μm (mode 1.8 μm) when SPIONs were included. Results obtained using DLS (3.2 μm and 2.9 μm respectively) were again larger due to a small population of large aggregates. The range of drug delivery systems analysed using TRPS now includes solid lipid nanoparticles[90] and fluorocarbon droplets which could simultaneously act as contrast agents.[134]

Nanotoxicology. The potential for TRPS in nanotoxicology has been demonstrated by Pal *et al.*,[91] who measured aggregates of engineered nanomaterials (ENMs) - carbon nanohorns, carbon black, CeO_2 and Ni nanoparticles. Size and concentration measurements were carried out in fetal bovine serum, and although TRPS and DLS particle size distributions were similar, TRPS was able to distinguish two modes in the size distribution. A key outcome for *in vitro* toxicity assessment was that particle characterization uncertainties were less significant than the uncertainty in relating an administered dose to the dose delivered to a cell.

Phages, viruses and bacteria

Bacteriophages and viruses, which range in size from tens to hundreds of nanometers, and bacteria, typically a few micrometers in size, can all be cultured in solutions suitable for TRPS. In these fields, TRPS has mostly been used for basic characterization of size and concentration. There is a particular need to complement or replace plating, the laborious gold standard for bacterial concentration

measurements.[60] The potential for TRPS in this area was first demonstrated using lambda phage,[11] which infects *Escherichia coli*. The shapes of resistive pulses appeared to be consistent with the head-tail geometry of the phages (Figure 6(b)). Another phage measurement[135] concerned the *Serratia entomophila* anti-feeding prophage (Afp), which causes amber disease in New Zealand grass grub. A particle concentration of 4.1 x 10^7 mL^{-1} within the size range 140-165 nm was deemed to correspond to the Afp.

The first TRPS measurement of a virus[3] used purified samples of the spherical adenovirus virion, suspended in PBS, yielding a size distribution with a modal value of 96.5 $\pm$ 15 nm, compared with a literature value of 70-90 nm. The narrow histogram peak (Figure 6(c)) suggested that the virions did not aggregate, an important trait for potential gene therapy applications. Farkas *et al.*[96] used TRPS to study rotavirus (diameter ~75 nm), the most common intestinal virus. A size exclusion chromatography method for sample purification was paired with TRPS to prevent virus count overestimation, a common problem for rotavirus. On the other hand, measurements based on protein and DNA content caused concentration overestimates (> 10^{12} mL^{-1}) for lentivirus (80-100 nm) due to contaminating molecules outside the virions.[21] The comparative TRPS value (8 x 10^{10} mL^{-1}) was compared with an infectivity titer, and indicated that only 0.001% of particles were infectious. Elsewhere, Arjmandi *et al.*'s mass measurement technique[65] was applied to inactivated virions in 75 mM KCl. Human immunodeficiency virus was measured to be 198 $\pm$ 15 nm in size with density 1.2 $\pm$ 0.1 g cm^{-3}, and Epstein–Barr virions were 170 $\pm$ 13 nm at a density of 1.7 $\pm$ 0.2 g cm^{-3}. These results were comparable with measurements made using rigid etched silicon nanopores and other methods.

TRPS size and concentration measurements were first applied to bacteria by Roberts *et al.*,[4] who studied the marine cyanobacterium *Prochlorococcus,* and *Baculovirus* occlusion bodies. TRPS size distributions for *Prochlorococcus* (range 300-1200 nm with a mean of ~650 nm) and *Baculovirus* (~1

μm) agreed with previous reported values. Bacterial concentrations (6.0 x 10^8 mL^{-1} and 9.9 x 10^7 mL^{-1}

respectively) were consistent for different users on different days, and lower (by 6% and 17%

respectively) than measurements using a FC haemocytometer and phase contrast microscopy.

Accurate determination of *Baculovirus* concentration is vital for insecticide applications. Allen *et al.*[60]

measured cell size and concentration simultaneously for strains of *Bacillus subtilis* and *Escherichia*

coli during colony growth. TRPS concentrations correlated more closely with colony plating than

optical density (OD) measurements. Particle volumes between 1.5-10 fL were obtained for each

sample, consistent with previous work and microscopy, and the cultures incubated with more

glucose present produced slightly larger values. Bennett *et al.*[136] found that the bacteria in probiotics

used in four dairy feed products had similar sizes, in the range 800-2000 nm, with concentrations

between 4.4 x 10^9-1.2 x 10^{10} mL^{-1}. In studies of water-borne pathogens which can be harmful to

humans, Chung *et al.*[76] compared a wild type strain of *Francisella tularensis* with a mutant, while

Pang *et al.*[137] investigated potential surrogates for *Cryptosporidium parvum*. In the former study, the

mutants (~750 nm) were slightly larger than wild type bacteria, with longer pulse durations

suggesting that they also have less negative charge. In the latter, TRPS confirmed that the size of

protein-modified PS microspheres (~4.9 μm, and concentration 2 x 10^6 mL^{-1}) was similar to *C. parvum*

oocysts (reproductive cysts containing a zygote).

Other

Food and beverage emulsions can be structurally similar to particles such as EVs and liposomes,

consisting of small capsules stabilized by surfactant molecules. TRPS has been used to characterise

soy bean oil droplets stabilised by β-lactoglobulin,[41] an emulsion model for milk. The emulsion was

refrigerated and monitored over four months, during which the modal droplet size increased from

150 nm to more than 200 nm. The size distribution dynamics inconclusively suggested that the

dominant growth mechanism involved migration of oil molecules, as in Ostwald ripening, whereas

aggregation induced by addition of salt was more consistent with flocculation and coalescence. The

surface charge of the emulsion droplets was also measured using the variable pressure method.[7]

Average zeta potentials were calculated using both resistive pulse rate (-18.9 mV) and duration (-21.8 mV) data, and were compared with a DLS measurement (-27.6 mV). Beyond emulsions, Gazzola *et al.* studied protein haze,[102] a wine quality defect. Aggregation of five wine proteins was analysed following different treatments containing phenolics and/or polysaccharide, by measuring aggregate sizes from approximately 200 nm to 4000 nm, and concentrations from 2.0×10^5 to 7.5×10^6 mL^{-1}.

Superparamagnetic beads (SPBs) are widely used in biotechnology, because a magnetic field can transport SPBs independently of other interactions.[138] This is useful for separation, concentration and aggregation, and as discussed above SPBs have been used to assist drug delivery,[132] separation in DNA assays,[72, 103] and efficient separation and aggregation in other targeted assays.[67, 68] Two studies have focussed exclusively on understanding magnetic particle transport in TRPS.[48, 55] In the first, 1 μm SPBs were used to show how TRPS can be used to detect aggregation.[48] Upon application of a magnetic field, frequently observed larger pulses could be modelled to determine aggregate size (Figure 2(b)). An observation of many pulses in close succession suggested that columnar aggregates (aligned by their dipole moments) were hydrodynamically separated as they moved through the pore. In a more quantitative study of 1.1 μm SPBs,[55] a bar magnet generated a magnetic field of up to 15 mT at the tunable pore. Beads in the nearer half of the fluid cell were attracted away from the pore, reducing the pulse rate and increasing the FWHM duration as the magnet was moved closer. Size measurements suggested a lack of aggregation, and it was noted that the particles were at relatively low concentration, that aggregates could be sterically excluded from the pore, and that hydrodynamic forces in the pore constriction were strong enough to overcome dipole interactions.

Buchs *et al.*[139] measured the size of selenium (Se) nanoparticles formed by conversion of dissolved Se into insoluble, high purity Se by aquatic organisms. The size distribution for these biogenic nanoparticles (~360 nm) suggests a mean settling velocity of 2.93 cm per day. Such information

could aid the efficient removal of Se nanoparticles from suspension, with positive environmental

effects and potential benefits due to demand for Se in dietary supplements and industry, particularly

photovoltaics. Yoon *et al.*[140] used TRPS to investigate novel amphiphilic Janus particles synthesized

using electrohydrodynamic cojetting, with proposed applications related to self-assembly at

interfaces. One population was found to have an average diameter of 2 μm, while the concentration

of a second population (average diameter 300 nm determined by DLS) was found to be 5 x 10^7 mL^{-1}.

TRPS has been used to characterize cylindrical micelles which self-assemble from elastin-like

polypeptides by genetically fusing an assembly domain to one end. The equivalent diameter (100-

150 nm) was broadly in agreement with DLS.[66] Elsewhere, [141] TRPS was an efficient high-throughput

method for analysing raspberry-like particles consisting of 4.5 μm PS particles covered by various,

smaller microgel spheres in 10 mM formate buffer at pH 3.3.

Conclusion

Application of TRPS can be summarized by considering the functions served by TRPS in published

studies. For particles in complex raw (*e.g.* bodily) fluids, especially EVs, the challenge is to provide

useful information while accounting for particles of unknown classification or origin. Key issues for

such research include clear specification of the research question, standardized solution

preparation, and dealing with highly disperse populations by using multiple pores and considering

measurement thresholds. For more controlled environments associated with drug delivery agents

and food emulsions (for example), TRPS is usually useful for quality control and could be extended to

regulatory compliance. Development of high-quality traceable measurement standards is a

challenge for colloidal characterization techniques, and continued comparisons will be of benefit,

particularly between physically distinct techniques and particle types. Another broad function of

TRPS is monitoring of on-bead chemistry, which is less closely linked to measurement protocols.

Development in this area is likely to focus on best practice methods for specific samples and assay

targets, with possible diagnostic applications.

Among many opportunities for new TRPS developments, perhaps the most exciting involve the use

of actuation to directly interact with particles - trapping, gating[1, 44] or squeezing.[79] So far, actuation

has been of most practical benefit for flexible, efficient experimentation, but the effects of

stretching on measurement have begun to be quantified.[47, 63, 125, 142] Other opportunities include

exploration of the fundamental link between electrophoretic mobility measurements and the

corresponding colloidal surface chemistry. Extraction of particle shape and orientation information

from TRPS would be of broad interest. Co-ordination of TRPS with optical and plasmonic

technologies seems likely, as does the eventual application of the technology at molecular length

scales.

Acknowledgements

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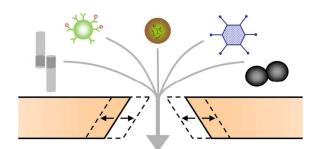
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Table of contents entry

This Review focusses on the recent surge in applied research using tunable resistive pulse sensing, a

technique used to analyse submicron colloids in aqueous solutions on a particle-by-particle basis.

Biographical notes

Eva Weatherall completed an honours degree in chemistry at Victoria University of Wellington in 2011. Following a period of employment at Izon Science, the manufacturer of tunable resistive pulse sensing systems, she commenced PhD studies based at Callaghan Innovation (Lower Hutt, New Zealand) and affiliated to Victoria University in 2013. Her research focuses on protocols for charge measurement using tunable resistive pulse sensing.

Geoff Willmott is a Senior Lecturer in the Departments of Physics and Chemistry at the University of Auckland, New Zealand, and a Principal Investigator with the MacDiarmid Institute for Advanced Materials and Nanotechnology. He started working on tunable resistive pulse sensing more than five years ago, while based at Industrial Research Limited (now Callaghan Innovation, Lower Hutt, New Zealand). His interests range from nanofluidics and soft condensed matter through to dynamic microfluidics and high-speed photography.