Detection of extracellular vesicles: size does matter
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CHAPTER 7

Reproducible extracellular vesicle size and concentration determination with tunable resistive pulse sensing

Abstract

*Introduction:* The size of extracellular vesicles (EVs) can be determined with a tunable resistive pulse sensor (TRPS). Because the diameter of the sensing pore varies from pore to pore, the minimum detectable diameter also varies. The aim of this chapter is to determine and improve the reproducibility of TRPS measurements. *Methods:* Experiments were performed with the qNano system (Izon) using beads and a standard urine vesicle sample. With a combination of voltage and stretch that yields a high blockade height, we investigate whether the minimum detected diameter is more reproducible when we configure the instrument targeting (1) fixed stretch and voltage, or (2) fixed blockade height. *Results:* Daily measurements with a fixed stretch and voltage (n = 102) on a standard urine sample show a minimum detected vesicle diameter of 128 ± 19 nm [mean ± standard deviation; coefficient of variation (CV) 14.8%]. The vesicle concentration was 2.4 ± 3.8 · 10⁹ vesicles mL⁻¹ (range 1.4 · 10⁸ – 1.8 · 10¹⁰). When we compared setting a fixed stretch and voltage to setting a fixed blockade height on 3 different pores, we found a minimum detected vesicle diameter of 118 nm (CV 15.5%, stretch), and 123 nm (CV 4.5%, blockade height). The detected vesicle concentration was 3.2 – 8.2 · 10⁸ vesicles mL⁻¹ with fixed stretch and 6.4 – 7.8 · 10⁸ vesicles mL⁻¹ with fixed blockade height. *Conclusion:* Pore-to-pore variability is the cause of the variation in minimum detected size when setting a fixed stretch and voltage. The reproducibility of the minimum detectable diameter is much improved by setting a fixed blockade height.

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7.1 Introduction

To establish whether there is any clinical value in the particle size distribution (PSD) of extracellular vesicles (EVs), reproducible measurements are a prerequisite. In Chapter 4, we have determined the PSD of urinary vesicles by transmission electron microscopy, flow cytometry, nanoparticle tracking analysis, and tunable resistive pulse sensing (TRPS) [230]. We found that TRPS, commercialized as the qNano (Izon, Christchurch, New Zealand), is the most accurate technique to determine the PSD of vesicles in suspension. However, we did not investigate the reproducibility of TRPS measurements. Moreover, standard procedures for determining the PSD of vesicles by TRPS are absent.

7.1.1 TRPS operating principle

The operating principle of TRPS is illustrated in Fig. 7.1. In TRPS, the resistance of a small pore filled with conductive medium is continuously measured [144, 82]. To measure the pore resistance, a voltage is applied across the pore, and the electric current through the pore is measured. The resistance \( R \) of a cylindrical pore is given in eqn. 7.1 with the conductivity of the medium \( \rho \), the length of the pore \( L \), and the diameter of the pore \( D \):

\[
R_{\text{pore}} = \frac{4\rho L}{\pi D^2} \quad (7.1)
\]

Fig. 7.1B shows a cartoon of the signal from a vesicle moving through the pore. To drive vesicles through the pore, TRPS uses a combination of electrophoretic and convective flow induced by the applied voltage and an external pressure across the pore, respectively [324]. The influence of diffusion and electro-osmosis on the flow rate is typically negligible [324, 171]. While the vesicle is moving through the pore, the pore resistance is increased. This resistive pulse is observed as a transient decrease of the current. The magnitude of the dip in current is called the blockade height in the qNano system. From this dip in current, the resistive pulse is derived, which is proportional to the vesicle volume [171]:

\[
\Delta R = \frac{4\rho}{\pi D^4} d^3 \quad (7.2)
\]

Eqn. 7.2 was derived for a spherical vesicle with diameter \( d \) inside a cylindrical pore. Because \( D \) is unknown, calibration with beads of known diameter is required to find \( 4\rho/\pi D^4 \). If \( D \) changes, the calibration needs to be repeated. The user can adjust \( D \) by stretching the pore, but \( D \) is also affected by factors that are beyond control of the user, such as the Mullins effect (stress induced softening) [312] and accumulation of high-molecular-weight proteins on the pore, such as fibrinogen or von Willebrand Factor. To limit the impact of variation in \( \rho \) and \( D \) throughout an experiment, the change in resistance \( \Delta R \) relative to the resistance of the pore \( R \) is used for analysis (analysis in \( \Delta I/I \) mode). Although the qNano has a conical
7.1. Introduction

![Figure 7.1: Operating principle of tunable resistive pulse sensing. When a non-conductive vesicle in a conductive medium passes through a pore, it causes a brief increase in electrical resistance of the pore. This is measured by monitoring the electrical current through the pore. (A) Schematic representation of a pore with a vesicle passing from position a to d. (B) Current from a particle moving through the pore. The letters a to d correspond to vesicle positions a to d in (A).](image)

instead of cylindrical pore geometry \[171\], eqn. 7.1 and 7.2 are sufficient to describe the principle of sizing with TRPS.

The vesicle concentration is derived from the count rate, which is also calibrated with reference beads. The diameter of the pore used in the qNano system can be tuned by changing the stretch of the pore holder, hence the name tunable resistive pulse sensing. Fig. 7.2 shows a picture of the qNano system.

7.1.2 Size limits of TRPS

The diameter of cell-derived vesicles in body fluids such as urine typically ranges from 30 to 1,000 nm. Since TRPS cannot detect the smallest vesicles, it is important to know what the smallest detectable size by TRPS is, especially because the smallest detectable vesicle size strongly affects the main parameters of a PSD, such as mean size, median size, standard deviation and concentration \[230\]. From eqn. 7.1 and 7.2 it becomes clear that the smallest detectable size of TRPS is limited by the pore size, conductivity of the medium, stability of the baseline current and noise of the detection electronics \[82, 131\]. A smaller pore will increase the magnitude of the resistive pulse and therefore decrease the smallest detectable vesicle size. A change in pore size also affects the stability of the baseline current. Thus, a reproducible and stable pore size is required to have reproducible measurements. To distinguish resistive pulses from noise and eliminate the impact of noise, a detection threshold is set below the noise by the analysis software (see Fig. 7.1B).

Particles larger than the pore cannot be measured with TRPS, because these
particles cause pore clogging (described as pore blockages in other papers). If pore clogging occurs, the measurement has to be paused to unclog the pore, which may alter the dimensions of the pore.

7.1.3 Goals

Our goals are to determine and improve the reproducibility of TRPS measurements.

7.2 Methods

7.2.1 Approach

We performed 3 experiments to reach our goal. (I) To determine the reproducibility of TRPS, we measured the PSD of a standard population of vesicles 102 times at fixed settings of the voltage and stretch and determine the variation in minimum detectable vesicle size and concentration. (II) To improve sensitivity, we investigated how the set voltage, stretch, and pressure affect the measured baseline current and blockade height. (III) Using insights from experiment I and II, we studied how a fixed blockade height may improve the reproducibility of TRPS.

7.2.2 Standard population of vesicles and reference beads

To obtain a standard population of vesicles with low contamination by lipoproteins, platelets and protein aggregates [233], we selected vesicles from urine as our standard sample. Urine from 1 healthy male individual was collected, pooled, centrifuged twice to remove the cells (10 mL at 180 g, 4 °C, and 20 min at 1,550 g, 4 °C), and filtered with a 0.45 µm filter (MilliPore, Amsterdam, The Netherlands). Aliquots of 150 µL cell-free filtered urine were snap frozen in liquid nitrogen and
7.2. Methods

Figure 7.3: (A) Particle size distribution obtained from 3 measurement of the standard sample. The size distribution is shown as a histogram with bin width 10 nm. The minimum detected size for these 3 distributions is indicated with vertical arrows below the x-axis. (B) Histogram of the minimum detected size of the standard sample for 102 consecutive measurements measured on 102 days. The dashed line shows a fit of a normal distribution with a mean size of 127 nm and standard deviation of 19 nm.

stored at −80°C. Before analysis, samples were thawed in 37°C water to dissolve amorphous salts, and diluted 1:1 in 0.22 μm filtered (Millipore) phosphate buffered saline (PBS; 9.0 g NaCl, 0.22 g Na₂HPO₄·2H₂O and 0.032 g NaH₂PO₄·2H₂O, per L de-ionized water).

To calibrate the size and concentration and to find the relationship between the voltage, stretch, pressure, baseline current, and blockade height, we prepared polystyrene beads with a mode diameter of 203 nm (Izon) and a concentration of 2.0 × 10⁹ particles mL⁻¹. To prevent aggregation of the beads, 0.6 mM sodium dodecyl sulphate (SDS) was added to the bead PBS. Prior to use, the beads were sonicated for 5 min and vortexed for 10 s.

7.2.3 Instrument settings and analysis

Reproducibility at fixed stretch and voltage (I)

To determine the reproducibility of TRPS, we measured the PSD of a standard population of vesicles. A measurement requires selection of electrolyte, nanopore, stretch, voltage and pressure. For the electrolyte, we selected PBS to prevent osmotic effects on vesicle size. Pores with different diameters are available for the qNano. The 3 pore types applicable to the measurement range of 50–1,000 nm are the NP100, NP200 and NP400. The naming of the pore types is not related to the diameter of the pore. Each pore type has a different measurement range with some overlap between the measurement ranges. The NP200 is most frequently
used in our lab because it is the most sensitive pore with an acceptable pore clogging rate on our samples. Pores were stretched 10 times from 43 to 47 mm to eliminate the influence of the Mullins effect [312]. Since standard procedures for measuring vesicles were unavailable at the time we started this experiment, we followed manufacturers’ recommendations at the time and set the stretch at 45 mm, the voltage at 0.34 V, and the pressure at 7.5 mbar.

For each experiment, particles were measured for 10 min or until 1,000 particles were counted. We repeated the measurement if (1) the baseline current drifted by more than 5%, (2) the RMS (root mean square) noise exceeded 10 pA or (3) the $R^2$-correlation of cumulative counts versus time was less than 0.999. The $R^2$-correlation and the baseline current drift are indirect indicators that a change in $D$ may have occurred, which would reduce the size accuracy. Measurement and analysis were performed with Izon Control Suite v2.2.2.44 software. Summary of the data and graphical representation of the data was done in MATLAB 2010b (Mathworks, Natick, MA). Since the detection threshold (Fig. 7.1B) is set by the analysis software at 50 pA from the mean baseline current, the smallest detectable vesicle size can be calculated from the mean blockade height from beads of known size. Variation in the smallest detectable size was expressed as the coefficient of variation (CV), which is defined as the ratio of the standard deviation to the mean.

Optimal settings for detecting small sizes (II)

Experiment I was performed with manufacturer recommended settings. A systematic study of the selected measurement parameters is needed. To find the optimal settings for detecting small sizes, we determined the relationship between stretch, voltage, baseline current, and blockade height by testing combinations of stretch (43, 44, ..., 47 mm) and voltage (0.1, 0.2, ..., 0.8 V). For each experiment, we measured 1,000 beads or for 10 min, whichever came first. We repeated the measurement if (1) the baseline current drifted by more than 5%, (2) the RMS noise exceeded 10 pA or (3) the $R^2$-correlation of cumulative counts versus time was less than 0.999. All data from this experiment were acquired with 3 pores at a constant pressure of 7.5 mbar. Settings that resulted in maximum blockade height, but without frequent pore clogging and without instability in the detection electronics due to excessive current, we appointed the optimal settings for detecting small sizes. These settings were then used to study the influence of pressure during measurement. We measured the bead and the vesicle sample with 3 pores at 0, 2.5, 5.0, ..., 15 mbar applied pressure. For selection of the optimal pressure, we considered the impact of pressure on the blockade height, and on the number of particles measured per minute.

Procedures to improve reproducibility (III)

Based on the optimal settings for detecting small sizes from experiment II, we evaluated whether setting a fixed blockade height improved the reproducibility of TRPS. To set the stretch, we set a voltage and adjusted the pore stretch until the
target current was achieved. According to eqn. 7.1, this is expected to improve the reproducibility of the pore size \((D)\) and particle rate between pores. We did not exceed 47.5 mm stretch to prevent damage to the pore. Next, we adjusted the voltage to achieve a fixed mode blockade height on the calibration beads. This sets the minimum detected size directly, because the detection threshold (Fig. 7.1B) directly relates to the blockade height for a bead of known size to the smallest detected size. This method was compared to fixed stretch and voltage by repeated measurements on a single pore, and single measurements on multiple pores, referred to as inter-pore and intra-pore in the rest of the text. The inter-pore and intra-pore measurements were performed on different days. The pore was loaded onto the qNano, and calibration was performed for each measurement. For the 2 configuration methods, we compared the reproducibility of the particle rate, the minimum detected size and the concentration of the standard vesicle sample.

7.3 Results

7.3.1 Reproducibility at fixed stretch and voltage (I)

We have determined the PSD of a standard population of urinary vesicles 102 times using a fixed stretch and voltage. Three randomly selected PSDs are shown in Fig. 7.3A. While the overall shape of the distributions is similar, the minimum detected diameter was 106, 109 and 138 nm. The distribution of minimum detected sizes on the 102 measurements is shown in Fig. 7.3B. The minimum detected size ranged from 89 to 165 nm (average 128 nm, standard deviation 19 nm). From the shape of the PSD, it is expected that the change in minimum detectable size affects the determined concentration [230]. The mean urinary vesicle concentration is \(2.4 \times 10^9\) vesicles mL\(^{-1}\) with a standard deviation of \(3.8 \times 10^9\) (CV 158 %). Due to the large variability in minimum detected size and the skewed PSD, it is meaningless to describe PSDs with statistical parameters like mean, mode, median, nor to report a concentration without restricting size range.

Since (1) the baseline current is determined by the pore dimensions, (2) different pores have different baseline currents at similar settings, (3) the baseline current fluctuates over time (up to 5% in 1 h, data not shown), and (4) the pore is made from polyurethane, which is a flexible material, we hypothesize that the variations in the minimum detectable vesicle size are caused by variations in the pore dimensions. However, minimizing the impact of the variations in the pore dimensions by optimizing the settings requires a thorough understanding and optimization of the measurement parameters.

7.3.2 Optimal settings for detection of small vesicles (II)

Optimal voltage and stretch

To find the optimal settings for detection of small vesicles, we investigated the relationship between the set voltage, stretch, and pressure, the baseline current
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Figure 7.4: Effect of set voltage and stretch on baseline current and blockade height. Each point represents the average of 3 pores. Lines are linear fits to the data. (A) Relationship between baseline current, stretch, and voltage. (B) Relationship between blockade height and baseline current. The combination of parameters selected for further optimization is indicated with a bold circle.

and blockade height. For these experiments, we have determined the average baseline current and blockade height of 203 nm beads using 3 pores. Fig. 7.4A shows the baseline current versus voltage for different values of the pore stretch. Each data point has a CV between 11 and 22%. The baseline current shows a linear relationship with the voltage, as expected from Ohm’s law. In addition, a linear relationship between baseline current and stretch was found. For all combinations of the voltage and the stretch, the average RMS noise was 7.6 pA (SD 0.8 pA, range 6.0–9.4 pA). This is sufficiently smaller than the 50 pA detection threshold configured in the analysis software to guard against false positive events due to noise.

A higher blockade height for 203 nm beads means that smaller particles can be detected. Fig. 7.4B shows the relationship between the blockade height and the baseline current. The maximum blockade height was found for small stretch and high baseline current. However, a stretch of 43 and 44 mm resulted in frequent pore clogging, and baseline currents in excess of 140 nA occasionally had high RMS noise, making them unsuited for measurements. At 45 mm stretch, the highest blockade was achieved with 0.5 V. Thus, at 7.5 mbar of pressure, settings that are generally applicable to all NP200 pores and optimized for small particle detection are 0.5 V, 45 mm stretch, resulting in a mean current and blockade height of 117 nA and 0.30 nA for 203 nm beads, respectively.

Optimal pressure

The effect of pressure on blockade height and particle rate was determined on 3 pores for beads and urine measured with 45 mm stretch and 0.5 V. An increased
Figure 7.5: Impact of pressure on measurement. The data from 3 pores are shown for beads (black markers) and urine (blue markers). (A) Particle rate versus pressure. (B) Blockade height versus pressure.
pressure resulted in an increased particle rate (Fig. 7.5A) and a reduced blockade height (Fig. 7.5B) for both beads and urine vesicles. Increasing the pressure from 7.5 mbar to 15 mbar approximately resulted in a 2-fold increase in particle rate, a 2-fold decrease in time needed to measure 1,000 particles, a 7% reduction in blockade height, and only a 2% increase of the smallest detectable vesicle. Given this large reduction in measurement time relative to the low loss in sensitivity, we selected 15 mbar as the optimal pressure for urinary vesicles. Consequently, the optimized settings for detecting small size were: 0.5 V, 45 mm stretch, 117 nA baseline current, 15 mbar pressure, and 0.28 nA blockade height for 203 nm beads.

7.3.3 Procedures to improve reproducibility (III)

In experiment I, we found that a fixed stretch and voltage results in poor reproducibility due to variations in the minimal detectable size. In experiment II, we found that 0.5 V, 45 mm stretch, and 15 mbar pressure are optimal settings for urinary vesicle measurements with the NP200 pore. At these optimized settings, however, the current and blockade height for 203 nm beads differ between NP200 pores due to variations in pore dimensions. An alternative approach to fixed voltage and stretch may be to set a fixed blockade height on reference beads, which
7.3. Results

Table 7.1: Particles rate and average minimum and median detected diameter for 2 methods to configure TRPS measurements.

<table>
<thead>
<tr>
<th>Method</th>
<th>Fixed stretch</th>
<th>Fixed blockade height</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inter-pore</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Particle rate (min(^{-1}))</td>
<td>67</td>
<td>136</td>
</tr>
<tr>
<td>Minimum diameter (nm)</td>
<td>96 ± 3.6</td>
<td>121 ± 4.3</td>
</tr>
<tr>
<td>Median diameter (nm)</td>
<td>130 ± 2.8</td>
<td>160 ± 4.7</td>
</tr>
<tr>
<td>Intra-pore</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Particle rate (min(^{-1}))</td>
<td>115</td>
<td>199</td>
</tr>
<tr>
<td>Minimum diameter (nm)</td>
<td>118 ± 15.5</td>
<td>123 ± 4.5</td>
</tr>
<tr>
<td>Median diameter (nm)</td>
<td>156 ± 15.6</td>
<td>166 ± 4.7</td>
</tr>
</tbody>
</table>

Diameter is expressed as mean ± coefficient of variation (CV).

directly determines the minimum detectable size through the detection threshold (Fig. 7.1B). Here, we will evaluate both approaches.

For fixed blockade height, first we set a voltage of 0.5 V, and then adjusted the pore stretch until a baseline current of 117±0.9 nA was achieved. Next, we adjusted the voltage to achieve a fixed mode blockade height of 0.28±0.03 nA on the calibration beads. See Fig. 7.9 for a flow diagram of the fixed blockade method. The target settings were taken from the optimized settings found earlier. The tolerances on current and stretch are achievable in a single iteration of configuring the instrument. The tolerance on the blockade height is expected to contribute up to 3.5 percentage point to the minimum detected size.

We measured the standard vesicle sample in triplicate with a single pore (inter-pore) and with 3 different pores (intra-pore). The results of these measurements are shown in Fig. 7.7 and in Table 7.1. Fig. 7.7A shows the average particle rate, Fig. 7.7B the minimum detectable diameter, and Fig. 7.7C the concentration using a single pore (left; inter-pore) and multiple pores (right; intra-pore) for fixed stretch and voltage, and fixed blockade height. With the intra-pore comparison, the particle rate again was lowest with fixed stretch (115 min\(^{-1}\)) compared to fixed blockade height (199 min\(^{-1}\)). The minimum detected diameter (% CV) was 118 (15.4 %) with fixed stretch, and 123 (4.5 %) with fixed blockade height. The minimum detected diameter CV on fixed blockade height remained comparable between inter- and intra-pore, while the CV with fixed stretch was substantially larger in the intra-pore measurement. This suggests that the fixed blockade height is more effective at standardizing the minimum detected diameter. With the inter-pore comparison, the detected concentration (Fig. 7.7C) has similar spread (range 6.2–8.6·10\(^8\) vesicles mL\(^{-1}\) with fixed stretch, range 5.0–7.2·10\(^8\) vesicles mL\(^{-1}\) with fixed blockade height). For multiple pores, the detected concentration (Fig. 7.7C) is most repeatable for fixed blockade height (range 6.4–7.8·10\(^8\) vesicles mL\(^{-1}\) versus 3.2–8.2·10\(^8\) vesicles mL\(^{-1}\) with fixed stretch and voltage). The 9 % difference in average detected concentration between inter- and intra-pore measurements may be attributable to sample thawing losses, since these experiments were performed on different days.
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Figure 7.7: Inter- and intra-pore reproducibility (both $n = 3$) of particle rate (A), minimum detected diameter (B) and concentration (C) for 2 configuration methods. Labels below (C) refer to the methods fixed stretch and voltage and fixed blockade height.
7.4 Discussion

TRPS allows size determination of non-conducting particles in conducting media [230]. Since vesicles are relatively non-conducting, TRPS is a suitable technique to study vesicles. However, the vesicle size distribution of different samples cannot be compared rigorously if the size distributions cannot be parameterized. Because the PSD of vesicles extends below the minimum detection limit of the qNano system, and because the vesicle concentration increases with decreasing detectable vesicle size in biological fluids, a reproducible minimum detected size is required for this parameterization. The simplest approach to achieve this would be to truncate the data below a certain size. Fig. 7.3 demonstrates that this size cutoff with a NP200 pore would need to be approximately 160–170 nm, which would dramatically reduce the utility of a TRPS measurement because the vast majority of vesicles would go undetected. Therefore, a solution that improves the reproducibility of the minimum detected size is preferred.

7.4.1 Optimal settings for detection of small vesicles

To find settings for detection of the smallest vesicles, we varied voltage to determine the blockade height on beads of known size. We found that low stretch and high current results in high blockade height (Fig. 7.4) and thus improved minimum detected diameter. The minimum applicable stretch is limited by the increased incidence of pore clogs, and the maximum voltage was limited by the instability in the detection electronics above a baseline current of 140 nA. Increasing the pressure reduces the blockade height. However, the number of particles in the urinary samples was so low that we had to select the highest possible pressure of 15 mbar. As optimal settings for detection of small sizes we obtained a stretch of 45 mm, a voltage of 0.5 V, and a pressure of 15 mbar, corresponding to a current of 117 nA and a blockade height of 0.28 nA for 203 nm beads. Much smaller vesicles can be detected if the detection threshold of 50 pA is reduced, for example, by application of digital signal processing techniques which should even allow detection of pulses smaller than the RMS noise.

7.4.2 Procedure to improve reproducibility

The obtained optimal settings for detection of small sizes were close to the settings that we applied to study the reproducibility of the size and concentration of vesicles in 102 urine samples from the same pool. However, in this study we obtained concentrations in a range of 2 orders of magnitude and a minimum detectable vesicles size CV of 15%. We compared the fixed stretch and voltage strategy that led to this to a fixed blockade height strategy. The latter strategy results in a more reproducible pore size, as can be seen in the reduced CV on the particle rate. The most reproducible minimum detected size (CV 4.5%), as well as concentration, is found with the fixed blockade height. These reproducibilities are much better than what was measured on the 102 samples with fixed stretch and voltage; however, a
much larger sample than 3 experiments is needed to determine whether these gains are durable. If not, addition of beads to the sample can improve concentration reproducibility, but will not allow detection of vesicles with the same size as the beads [314].

7.4.3 Minimum detected size would be more reproducible with a tighter tolerance pore

A more reproducible pore could allow a simpler procedure than our proposed solution. Because the tunable pores are made of polyurethane, both the tolerances on the molding process as well as on the fabrication of the pore itself are a challenge. Rejection of pores that deviate from the desired size would reduce this issue, but a very high rejection rate would be cost prohibitive. A cheaper solution would be to produce the pores with, for example, silicon lithography, where tolerances of a few percent on both pore diameter and thickness are feasible. This direction would eliminate the tunable aspect of the TRPS system, which may be convenient as it eliminates the need for setting the stretch parameter.

7.4.4 Applicability to other vesicle types

We used urine vesicles for the selection of the optimal instrument configuration method. We expect the result to be applicable to other vesicle samples as well, provided the preparation of these samples did not induce high levels of large contaminants. Samples with higher vesicle concentrations should be measured at a lower pressure. Isolation by a Sepharose 2B column [42] could be used to minimize the presence of proteins in the sample.

7.5 Summary

Experiments to optimize a TRPS protocol with high sensitivity and reproducibility were performed. We demonstrated that the pore diameter can be controlled by adjusting stretch until a certain baseline current is achieved. The minimum detected size was made reproducible by adjusting the voltage to set the blockade height. With a standard urine vesicle sample, we demonstrated that the variation in detected particle rate, diameters and concentration is primarily caused by variation between pores. Until more reproducible pores become available, we will apply the following steps for the most reproducible results: (1) set the instrument stretch to achieve a fixed current, (2) set the instrument voltage to achieve a fixed blockade height on beads of known size, (3) allow the system to stabilize for 3 min before calibration, (4) measure your samples, repeat the measurement if the baseline current drifts by more than 5%, or the particle rate is not constant ($R^2 < 0.99$), and (6) analyze the data in $\Delta I/I$ mode.