Calibration-Free Electrical Quantification of Single Molecules Using Nanopore Digital Counting

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***Abstract*— Nanopore sensor conceptually represents an ideal single molecule counting device due to its unique partitioning-free, label-free electronic sensing. Existing theories and experiments have shown that sample concentration is proportional to the molecule translocation rate. However, a detailed nanopore geometry and size characterization or a calibration curve of concentration standards are often required for quantifying the unknown sample. In this work, we proposed and validated a calibration-free nanopore single molecule digital counting method for isolated molecule quantification. With the background ions as the in-situ references, the molecule translocation rates can be normalized to the ion translocation rates (baseline current). This in-situ reference alleviates the requirement for knowing the nanopore geometry and size or generating a calibration curve. In recognition of this effect, we developed a quantitative model for molecule quantification without the need for prior knowledge of experimental conditions such as nanopore geometry, size, and applied voltage. This model was experimentally validated for different nanopores and DNA molecules with different sizes. We anticipate this calibration-free digital counting approach would provide a new avenue for nanopore-based molecule sensing.**

Keywords*— digital counting, nanopore, calibration-free, label-free sensing

I. INTRODUCTION

Quantification of isolated biomolecules such as DNAs, RNAs, and proteins is of critical importance for various applications [1, 2]. This process is routinely accomplished by bulk-based optical sensing methods [3, 4]. The resulting analog readout signal is proportional to the bulk sample concentration (Fig. 1a). In contrast to the analog sensing method, digital assays have emerged as a highly sensitive approach for concentration quantification [5-11]. Digital assays rely on physical partitioning and optical detection to generate a binary signal. With Poisson statistics, the sample concentration can be estimated by -ln(1-p), where p is the ratio of the positive partitions over total partitions (Fig. 1b).

Nanopore-based sensors [12-21] allow single molecules to be analyzed electronically without the need for labeling and partitioning. Conceptually, nanopore sensor represents an ideal single molecule counting device due to its unique features of label-free electronic sensing, single-molecule sensitivity, and potential reusability. When a single molecule is driven through the nanopore, a detectable ionic current blockade generates a digital '1' signal, the rate of which is proportional to the sample concentration (Fig. 1c).

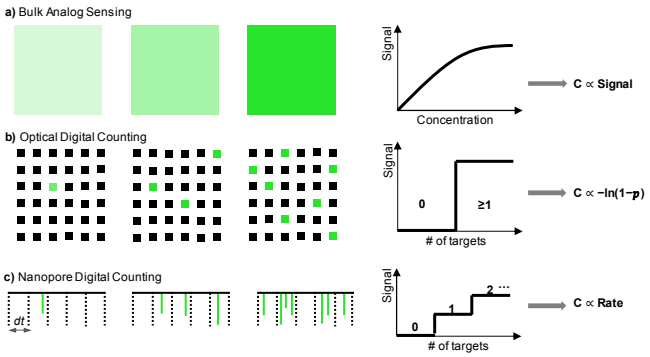

Fig. 1 Particle translocation dynamics. (a) The normalized distribution of bead interarrival time in different channels, with exponential fits to the distributions. (b) Cumulative counted particle numbers versus the elapsed time.

Existing theories [22, 23] and experiments [12, 24] have shown that when interactions between molecules are negligible, the molecule molar concentration (mol/m^3) is linearly related to the translocation rate (s^{-1}) as $R=\alpha N_A C$, where N_A is the Avogadro constant, and α is usually referred to as the capture rate [22, 25]. Wanunu et al. applied this principle to quantify the isolated *miR122a* electronically [26]. Since capture rate α strongly depends on experimental parameters such as nanopore geometry [22, 27], temperature [24, 28], molecule size [15], and applied voltage [29, 30], a calibration curve of the rate versus concentration was necessary to infer the unknown sample concentration [26]. Moreover, the calibration curve must be obtained under the same experimental conditions for comparable capture rate α. Unfortunately, generating this calibration curve is often time-consuming and challenging [31, 32].

In this work, we set out to develop a calibration-free nanopore single molecule counting method for isolated molecule quantification. We first studied the statistics of the molecule translocation rate. In the next step, we developed a quantitative model for molecule quantification without the need for prior knowledge of experimental conditions such as nanopore geometry, size, and applied voltage. This is achieved by using the background ions as the in-situ reference such that the molecule translocation rates can be normalized to the ion translocation rates (baseline current). This model was experimentally validated for different nanopores and DNA molecules with different sizes. While the results presented in this work were from glass nanopores and DNA molecules, the principle could be well extended to other nanopore types and other charged molecules.

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II. Experimental

A reservoir outside of the nanopore was filled with DNA samples, and home-made Ag-AgCl wires were inserted to the nanopore and bulk solution (Fig. 2a). Quartz capillaries with inner and outer diameter of 0.5 and 1 mm were used to fabricate nanopores by a laser pipette puller (P-2000, Sutter Instruments, U.S.A). Glass nanopore characterization was performed by standard I-V measurement (Fig. 2b) and to obtain detailed information about the nanopore geometry and size, SEM (Fig. 2c) and TEM (Fig. 2d) imaging was performed. The nanopipettes were filled with 1M KCl in Tris-EDTA buffer solution. We made the samples by diluting different length of DNA purchased from Thermo Fisher Scientific. We applied a constant voltage across the nanopore with a 6363 DAQ card (National Instruments, U.S.A). Then, we recorded the ionic current traces by an amplifier (Axopatch 200B, Molecular Device, U.S.A). The analog output of the amplifier was sampled with the 6363 DAQ card and a data acquisition software. The sampling rate for the measurement was 100 kHz. A custom-built MATLAB program was developed to analyze the data and measure the duration time of each translocation events, as well as their distribution.

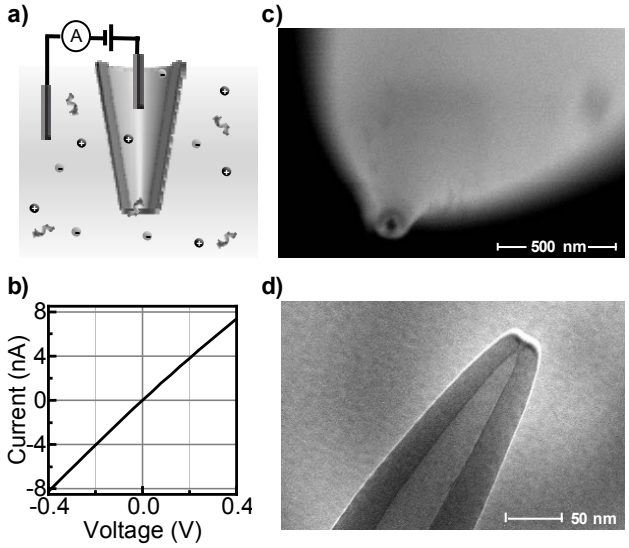

Fig. 2 a) Illustration of the experimental setup (not to scale). b) A typical IV curve for glass nanopore in 1 M KCl with Tris-EDTA-buffer solution. c) SEM image of the glass. d) The TEM image of the nanopore tip showing apparent conical shape.

III. Results and Discussion

It was previously observed that the mean time between single-molecule capture events in solid-state nanopore follows an exponential distribution [27], indicating a Poisson process [27, 33]. To validate if this is also true in our glass nanopore, we performed studies on λ-DNAs with a serial of concentrations ranging from 12-60 pM. A quick eyeball on the current time traces in Fig. 3a shows that translocation occurs more often as the concentration increases. The extracted inter-arrival time distribution also shows a remarkable exponential distribution for each concentration (Fig. 3b). To further confirm the Poisson process, the same raw data sets were used to extract the probability distribution $P(n)$ for observing n events within a fixed time interval (Fig. 3c). Each concentration case is then fitted with a Poisson distribution, $P(n) = e^{-\lambda}\lambda^n/n!$, where λ is the expected occurrence of the events. In a process with the rate of R, $\lambda = Rdt$ where dt is the time interval [27]. As shown in Fig. 3d, both fittings to the exponentially distributed inter-arrival time and fittings to the Poisson distribution yield comparable rate determination at different concentrations.

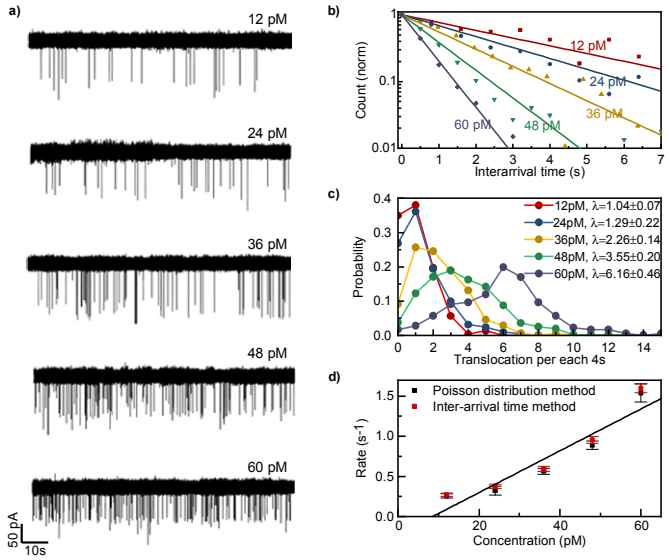

Fig. 3 Translocation recording of λ-DNA through glass nanopore at 1M KCl under 400 mV bias. a) Continuous current readout illustrating the translocation events at different DNA concentrations. b) Normalized distributions of interarrival time for different concentrations with mono-exponential fits to the distributions. c) The probability distribution of the events for different concentrations. d) The translocation rate obtained from both fitting methods versus the λ-DNA concentrations.

With an experimentally efficient approach to determine the rate, the next task is to determine the capture rate α. It is known that the capture rate α could be diffusion limited (molecules spends most of the time diffusing in bulk) or barrier limited (translocation is limited by entropy energy barrier for molecules capture that is associated with the threading of the first few bases) [15]. The glass nanopores used in our experiments are around 10nm in size, which is large enough such that the transport is diffusion-limited [34, 35].

In the diffusion-limited region, the capture rate for the conical-shaped glass nanopore is given by $\alpha=2\pi\mu d\Delta V$, where μ is the free solution electrophoretic mobility, ΔV is the applied electric potential across the pore, and d is the characteristic length of the nanopore. If the nanopore geometry and size is explicitly known for a particular experiment, the capture rate can be directly calculated to determine the unknown sample concentration without calibration. Nevertheless, it is well known that glass nanopore geometry is widely dispersed [36]. TEM characterization of each nanopore is often destructive and is time-, facility-, and expertise-intensive [37]. To properly determine the unknown sample concentration, a calibration curve must be obtained under the same experimental

conditions to extract the capture rate α in that particular experiment [15]. While this could be done, it is often time-consuming and experimentally challenging [31]. To overcome these challenges, we here developed an *in-situ* method for determining the capture rate α without the need for prior knowledge on nanopore experimental conditions. This is achieved by recognizing that the baseline current carries information about the background ion translocation rate (Fig. 4a). Therefore, it is feasible to use the ionic concentration as the internal reference to estimate the unknown capture rate α. The baseline current can be estimated as $I_b=2\pi\Lambda C_{ion}d\Delta V$, where Λ is the molar conductivity which depends on the mobility and valance of the ions as $\Lambda=\Sigma_i N_A e z_i \mu_i$ [38]. The previously inaccessible parameter α can be rewrote as $\alpha=\mu I_b/\Lambda C_{ion}$ which implies that the unknown capture rate can be derived from the experimentally accessible baseline current and the ionic concentration without knowing the nanopore geometry, size and the applied voltage. The molecule mobility μ and molar conductivity Λ can be estimated for a particular molecule and salt. Thus, the molecule translocation rate $R=\alpha N_A C_{mol}$ can be written as:

$$R=\mu N_A I_b C_{mol}/\Lambda C_{ion} \quad (1)$$

To validate Eq. (1), we performed experiments with 10 kbp DNA at 24 pM in the 1M KCl buffer solution. Fig. 4b shows the current time trace at different applied voltages for two glass nanopores pulled from different batches. Two features can be observed. First, higher applied voltage leads to a higher molecule translocation rate. Second, due to the nanopore size variation, the same applied voltage does not generate the same molecule translocation rate. Fortunately, Eq. (1) predicts that the molecule translocation rate scales linearly with the baseline current for a fixed testing molecule and salt concentrations. This is exactly what we observed in Fig. 4c.

After verifying this *in-situ* ionic current reference model, calibration-free quantification of the molecule molar concentration can thus be performed by rewriting the Eq. (1) as:

$$C_{mol}=\Lambda R C_{ion}/\mu N_A I_b \quad (2)$$

Eq. (2) shows that unknown sample concentration can be quantified without explicitly knowing the nanopore geometry, size, and the applied voltage, as long as the parameters on the right-hand side of the equation could be determined. To validate this method, we tested λ-DNA, 5 kbp DNA, and 10 kbp DNA at five known concentrations (12, 24, 36, 48, and 60 pM) in 1M KCl buffer, intentionally using glass nanopores pulled from different batches. Since the free solution electrophoretic mobility of DNA in Tris-EDTA buffer was theoretically [39] and experimentally [40] shown to be independent on the DNA length longer than a few persistence lengths [41], μ of 4.5×10^{-8} $m^2V^{-1}s^{-1}$ was used for all DNA molecules [42]. The buffer solution is dominated by 1 M KCl and thus, the molar conductivity Λ is estimated to be 10.86 $m^{-1}M^{-1}S$ [38]. The baseline current (I_b) and translocation rate (R) was determined from the experiment. Fig. 4d plots the measured versus the input concentration for all tests. All data points falling into a straight line of slope 1, indicating the accuracy of the calibration-free method. It is noteworthy that the molecule concentration determined by Eq. (2) is widely applicable to other kinds of molecules as long as their electrophoretic mobility was known.

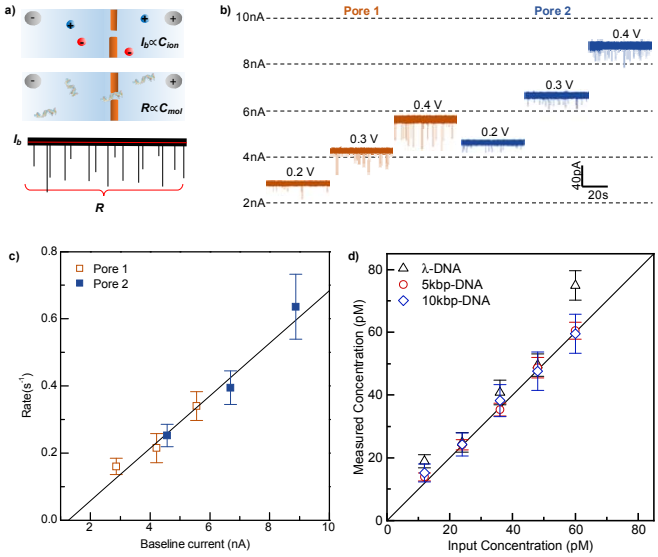

Fig. 4 a) Schematic of ions and molecules translocation through the same nanopore. b) Current time trace of 24 pM 10 kbp DNA in 1M KCl translocating through two distinct nanopores and under different voltages. c) The molecule translocation rate is linearly proportional to the baseline current for the same test sample shown d) Validation of the calibration-free method for concentration determination.

IV. Conclusion

In summary, we presented a nanopore single molecule digital counting method for isolated molecule quantification without the need for prior knowledge of experimental conditions such as nanopore geometry, size, and applied voltage. When single molecules were electrophoretically driven through the 10 *nm* glass nanopore one by one, digital events were registered. We recognized the ionic rates (baseline current) in a particular experiment could be used as an effective *in-situ* reference. We developed a quantitative model for calibration-free quantification of molecule concentration, which was experimentally validated for different nanopores and DNA molecules. While the results presented in this work were from glass nanopores and DNA molecules, the principle could be well extended to other nanopore types and other charged molecules. We anticipate this calibration-free digital counting approach would provide a new avenue for nanopore sensors.

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