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Characterization of vesicular stomatitis virus populations by tunable resistive pulse sensing

Fulya Akpinar*, John Yin

Department of Chemical and Biological Engineering Systems Biology Theme, Wisconsin Institute for Discovery University of Wisconsin-Madison, Madison, WI 53706, USA

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A B S T R A C T

Although transmission electron microscopy (TEM) has historically been the method of choice to estimate concentrations of virus and virus-like particles, these measures can often be time-consuming and labor-intensive to perform. Tunable resistive pulse sensing (TRPS) is an emerging method that applies principles of Coulter counting to nanoscale particles and may provide a simpler and higher-throughput alternative to TEM for the quantitation of virus populations. To assess the performance of TRPS compared to TEM, the samples of polymer spheres at a diameter of 100 nm and vesicular stomatitis virus (VSV) were characterized using both techniques. TRPS was able to quantify concentrations down to 10^7 particles/ml, providing nearly 50-fold larger measurement range, and more reproducible counts than TEM. Total-to-infectious particle ratio of VSV populations as measured by TRPS and plaque assay suggested that each VSV particle is infectious. In addition to particle counts, TRPS successfully measured particle size distributions based on hundreds of particles. Such high throughput sustained by TRPS can assist quantitative characterization of virus populations.

1. Introduction

The total-to-infectious particle ratio is a useful measure of how efficiently virus particles infect cells. The ratio has been estimated traditionally by dividing the total particle concentration measured via transmission electron microscopy (TEM) by the infectious unit concentration, as determined by plaque assay (Galasso and Sharp, 1962; Carpenter et al., 2009). TEM allows for direct visualization and quantitation of virus particles, but it requires significant time and effort for sample preparation, imaging and image analysis (Watson et al., 1963).

As an alternative to electron microscopy, particle quantitation devices based on Coulter principles, known as Coulter counters have been developed, which provide a simple, high-throughput and inexpensive platform for single particle analysis (DeBlois, 1970; Henriquez et al., 2004; Kozak et al., 2011). Typically, a Coulter counter is composed of two fluid reservoirs filled with conductive media and separated by a membrane which has a pore. This pore allows only the particles that are smaller than the pore diameter to pass through. When an electrical field is applied across the pore, the resistance to the resulting ionic current is indirectly proportional to the cross sectional area of the pore. When a non-conducting particle passes through the pore, the increase in resistance is proportional to the particle volume relative to pore size4. This change in resistance is detected as a pulse in ionic current. The pulse frequency is proportional to particle flow rate and thus to particle concentration.

Recently, the usage of tunable pores in Coulter counters, as in tunable resistive pulse sensing (TRPS) technology, has allowed sensitive measurements of a broad size range of particles (Kozak et al., 2011; Vogel et al., 2011). In comparative studies of particle characterization techniques (Bell et al., 2012; Anderson et al., 2013; Heider and Metzner, 2014), TRPS has shown advantages over other techniques due to its ability for simultaneous particle sizing and counting, and its higher accuracy in polydisperse particle sizing as compared to commonly used techniques such as nanoparticle tracking analysis and dynamic light scattering (Anderson et al., 2013; Terejanszky et al., 2014). The success of TRPS in particle quantitation has sparked its applications in the characterization of biological nanoparticles including virus populations (Vogel et al., 2011; Gazzola et al., 2012; Van Bracht et al., 2012; Arjmandi et al., 2014). While the applications of TRPS in the characterization of virus infections continue to expand, its accuracy in the quantitation of virus populations has not been fully tested.

To address the need for the validation of the accuracy of TRPS in quantitative analysis of virus populations, in this study,

* Corresponding author at: 330N. Orchard Street, Madison, WI 53715, USA. Tel.: +1 608 334 9663.
E-mail address: akpinar@uwalumni.com (F. Akpinar).

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the performance of TRPS in characterizing spherical polymer nanoparticles and vesicular stomatitis virus (VSV) populations was compared with that of TEM. As a bullet shaped virus, VSV allowed testing the accuracy of both techniques in the characterization of non-spherical particles with high aspect ratio (length-to-width ratio). The results demonstrated the ability of TRPS to quantify the concentration and size distribution of both spherical nanoparticle and non-spherical VSV populations.

2. Material and methods

2.1. Cell and virus culture

Baby hamster kidney (BHK-21) cells were cultured at 37 °C and 5% CO_2 in Eagle's minimum essential medium (MEM; Mediatech-CellGro, Herndon, VA, USA) with 1% Glutamax I (Life Technologies, Carlsbad, CA, USA) and 10% fetal bovine serum (FBS, Atlanta Biologicals, Norcross, GA, USA). The culture medium was switched to medium with the 2% FBS for all virus infections. A well-defined virus strain based on the Indiana serotype of Vesicular stomatitis virus (VSV), VSV-N1 (Wertz, 1998), was used for infections. VSV is from order *Mononegavirales*, family *Rhabdoviridae*, genus *Vesiculovirus.* To prepare virus stock, BHK-21 cells were infected with plaque purified virus diluted to 0.001 plaque forming units (PFU) per cell in a T-75 flask (Falcon, BD Biosciences, San Jose, CA, USA), incubated for 24 h at 37 °C, filtered with a 0.22 mm filter (Millipore, Bedford, MA, USA), and stored at −80 °C.

2.2. Plaque assay

Virus was quantified by plaque assay. The day prior to infection, cells were removed from T-75 flasks by treating with Trypsin EDTA (CellGro, Herdon, VA, USA). The cells were diluted in 10% FBS media to 10^5 cells/ml. Two ml of the cell solution were pipetted into each well of 6-well tissue culture treated polystyrene plates (Corning, New York, USA). The virus samples were thawed at room temperature and serial 10-fold dilutions were prepared in MEM. The media above the cell monolayer was removed, rinsed with Dulbecco's Phosphate-Buffered Saline (DPBS; Gibco, Life Technologies, New York, USA) and the cells were infected with 200 μl of the virus suspension and incubated for 1 h. The inoculum was removed and the cells were overlaid with 2 ml of 0.6% (w/v) agar (Difco, Becton Dickson, Sparks, MD, USA) diluted in 2% FBS infection medium. After 20 h of incubation, the agar overlay was removed and cells were fixed in 4% (w/v) paraformaldahyde (PFA, MP Biomedicals, Solon, OH, USA) and 5% (w/v) sucrose solution. The fixative remained on the cells for 20 min and then the cells were rinsed twice with DPBS and stained with 2.5% (v/v) crystal violet (CV; PML Microbiological, Wilsonville, USA) diluted in 20% ethanol to aid in visualizing plaques. After the CV dried plaques were counted, virus titers were calculated as PFU/ml.

2.3. Microsphere standards

The particle counts by both TEM and TRPS were calibrated with the dilutions of carboxylated polystyrene microspheres with a nominal diameter of 100 nm (Polybead series, Polysciences, Warrington, PA, USA) and a concentration of 10^{10} particles/ml. Only for the determination of the size distributions by TEM, unmodified polystyrene beads (NIST traceable size standards; Thermo Fisher Scientific, Fremont, CA, USA) with nominal diameters of 102 nm and 10^9 particles/ml was used. Both calibration samples were tested by independent TEM measurements (Smith and Melnick, 1962). To test the size measurements by TRPS, carboxylated polystyrene microspheres with a nominal diameter of 70 nm and 110 nm (BANGS, Fishers, IN, USA) were measured. The counts were calibrated with aforementioned calibration beads.

2.4. Particle quantitation by transmission electron microscopy

The virus samples were mixed with microsphere calibration standards in 1:1 ratio. The prepared mixture was diluted 1:1 with methylamine tungstate (Nanoprobes, Yaphank, NY, USA) and a drop was loaded on a pioloform coated grid. The samples were then imaged using a TEM (CM120, Philips, Netherlands). 15 TEM images were acquired for each sample, and VSV particles and microspheres were counted and averaged. The VSV particle concentration was estimated relative to the concentration of calibration microspheres. The polystyrene microsphere samples were imaged in a similar manner. Instead of calibrating the counts with a reference particle sample, Smith and Melnick method was used to quantify the particle concentrations (Smith and Melnick, 1962). The dimensions of virus particles and microspheres were measured using the software iTEM (Olympus, Münster, Germany).

2.5. TRPS measurements

Virus particles and carboxylated polystyrene microspheres were quantified using a commercial TRPS instrument (qViro, IZON Science, Christchurch, New Zealand). MEM was used as electrolyte. Both microsphere and virus samples were diluted to appropriate levels in MEM with a great care in pipetting and vortexing. A TPU membrane, on which a tunable nanopore was punctured and mounted on TRPS to 100 nm pore size (NP100, IZON Science Christchurch, New Zealand) (Sowerby et al., 2007), was placed on the lower well and stretched to allow particle passage. 75 μl MEM was pipetted into the lower well and the upper well was set on the top of the membrane. 40 μl test sample was added to the upper fluid well. A continuous flow of particles was maintained by adjusting the pore stretch and the voltage. A minimum of 5 particles/min was targeted. A pressure of 10 cm-H_2O was established across the nanopore to minimize the effect of particle surface charge on concentration measurements. To avoid nanopore clogging and serial contamination between sample runs, a blank MEM buffer was run through the pore between the measurements of each sample. Current pulse signals were acquired over 2 min using the IZON Control Suite 2.2. Later the same software was used to process the recorded data. The particle concentration and size distribution was estimated by comparing the particle flow rate and signal magnitudes of the test sample with that of the calibration sample described before. The average size and concentration of calibration sample was entered as the basis of size and concentration calculations.

3. Results

3.1. Quantitation of the concentration and size distributions of microspheres by TRPS

To test the ability of TRPS to measure the size and concentration of nanoparticles and to determine its detection range, the carboxylated polystyrene microspheres were diluted to different concentrations and analyzed by TRPS. Fig. 1 shows a subset of the current signal (yellow line) recorded for three different dilutions of microspheres. Each downward pulse in current signal indicates a single particle passing through the nanopore. The depth and the frequency of the pulse signal are proportional to the particle volume and the particle concentration, respectively (Fig. 1). The depth of the pulse signal varies by approximately 0.5 nA, suggesting the presence of slightly different sized particles.

Based on the current readouts, the flow rates of microspheres were measured and compared with the particle numbers derived

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