Published in final edited form as:

Anal Chem. 2018 June 19; 90(12): 7267–7274. doi:10.1021/acs.analchem.8b00452.

Characterization of Virus Capsids and Their Assembly Intermediates by Multi-Cycle Resistive-Pulse Sensing with Four Pores in Series

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Abstract

Virus self-assembly is a critical step in the virus lifecycle. Understanding how viruses assemble and disassemble provides needed insight into developing antiviral pharmaceuticals. Few tools offer sufficient resolution to study assembly intermediates that differ in size by a few dimers. Our goal is to improve resistive-pulse sensing on nanofluidic devices to offer better particle-size and temporal resolution to study intermediates and capsids generated along the assembly pathway. To increase the particle-size resolution of the resistive-pulse technique, we measured the same, single virus particles up to a thousand times, cycling them back and forth across a series of nanopores by switching the polarity of the applied potential, i.e., virus ping-pong. Multiple pores in series provide a unique multi-pulse signature during each cycle that improves particle tracking and, therefore, identification of a single particle, and reduces the number of cycles needed to make the requisite number of measurements. With $T = 3$ and $T = 4$ Hepatitis B Virus (HBV) capsids, we showed the standard deviation of the particle size distribution decreased with the square root of the number of measurements and approached discriminating particles differing in size by single dimers. We then studied *in vitro* assembly of HBV capsids and observed that the ensemble of intermediates shift to larger sizes over two days of annealing. On the contrary, assembly reactions diluted to lower dimer concentrations an hour after initiation had fewer intermediates that persisted after the two-day incubation and had a higher ratio of $T = 4$ to $T = 3$ capsids. These reactions indicate that labile $T = 4$ intermediates are formed rapidly, and dependent on conditions, intermediates may be trapped as metastable species or progress to yield complete capsids.

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Supporting Information Available. Histograms of the pulse amplitude (i/i) for $T = 4$ capsids measured at each of the four pores in series and variation of normalized pulse amplitude (i/i) over 1246 measurements of a single particle. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Hepatitis B Virus (HBV) is a global public health issue: more than 2 billion people have been infected with the virus and more than 250 million people suffer from chronic infection. HBV leads to liver failure, cirrhosis, and hepatocellular cancer and contributed to 887,000 deaths in 2015.¹ HBV is an enveloped virus with an icosahedral capsid.² HBV capsid assembly has become an important target for antiviral drug development and, more generally, a model system for understanding the virus self-assembly mechanism.³ During HBV capsid assembly, the homodimeric core protein (Cp) forms capsids with T = 4 symmetry (~90% abundance, 120 dimer units, 38 nm diameter) and T = 3 symmetry (~10% abundance, 90 dimer units, 34 nm diameter).⁴ The HBV capsid assembly reaction can be triggered *in vitro* by mixing Cp149 dimer, a truncated version of the 183-residue core protein missing the RNA-binding domain, with an electrolyte, e.g., NaCl, at room temperature without the presence of nucleic acid or polyanion template.⁵ Truncation of the core protein,⁵ core protein concentration, ionic strength,⁶ and degree of dimer oxidation⁴ affect the ratio of T = 3 and T = 4 capsids formed during capsid assembly.

Resistive-pulse sensing^{7–10} has been widely used to analyze biomolecules, such as DNA¹¹ and virus particles.^{12–13} This analytical method, which originated from the Coulter Counter for analyzing red blood cells,¹⁴ features translocation of single particles across an electrically biased pore. The presence of a particle within the pore changes the resistance of the system, resulting in a current change (pulse) for each translocation event. The amplitude of this pulse (Δ) divided by the current baseline (i) is proportional to the particle size; the time between pulses, e.g., pore-to-pore time, is inversely proportional to the particle mobility; and the event frequency is proportional to the particle concentration. Stimulated by the possible application of rapidly analyzing nucleic acids,¹⁵ improvements in fabrication technology¹⁶ have advanced the development of solid-state nanopores¹⁷ with either membrane-based¹³ or in-plane configurations.^{18–19} Over the past decade, resistive-pulse sensing has evolved to be a versatile tool for interrogating a range of biomolecules.^{11,20–21}

One application of resistive-pulse sensing is the study of viruses and virus-like particles.¹² Many virus capsids assemble spontaneously in solution from hundreds of protein subunits through specific assembly pathways and intermediates.^{22–28} The assembly reaction is sensitive to various conditions such as protein concentration, ionic strength, and temperature. Additionally, the reaction can be regulated by small molecule assembly effectors.²⁹ The intermediates, which are partially formed capsids during the assembly reaction, can be analyzed and evolve into complete capsids on a timescale ranging from

seconds to days.⁶ Monitoring capsid formation, in a variety of viruses, and how the assembly process is impacted by different factors can lead to development of new antiviral drugs and synthesis of new nanoscale materials.^{30–34}

We have shown that resistive-pulse sensing with a track-etched conical nanopore easily resolves the T = 3 and T = 4 capsids, which differ by 4 nm in diameter.¹³ We have also developed in-plane architectures, fabricated by electron beam lithography and reactive-ion etching¹⁸ or focused ion beam (FIB) milling,^{6,35} with two pores in series to measure electrophoretic mobilities and to create a unique pulse signature for each event. With this two-pore platform, we studied HBV assembly over a range of dimer concentrations and found that *in vitro* formation of T = 3 and T = 4 capsids goes through different pathways.⁶ Other configurations with multiple pores in series include devices with two membrane-based nanopores stacked vertically,^{36–37} up to 11 micrometer-scale pores in series for node-pore sensing,³⁸ and up to 8 nanopores in series for real-time analysis of virus assembly.³⁹

One fundamental limitation to the accuracy of the measurement of a single particle in solution is Brownian motion.^{40–41} To achieve a better signal-to-noise ratio in resistive-pulse sensing, we fabricate the pore length to be as short as possible to reduce the overall system resistance. As a result, the particle translocates rapidly through the pores (~0.2 ms). If the time becomes too short, thermal fluctuations will affect the current displacement due to the limited bandwidth of the current-sensing electronics. The problem is especially significant for small particles, which have larger diffusion coefficients. However, this error is random in nature and can be minimized by averaging the signals from multiple measurements, and thus, statistics for each particle are compiled.⁴²

One approach to achieve multiple measurements is to fabricate multiple nanopores in series. With an in-plane network of nanochannels and nanopores milled with an FIB instrument, we can easily increase the number of pores in series to measure each particle multiple times. Recently, we compared the performance of devices with up to 8 pores fabricated in series.³⁹ The fabrication of multiple pores in series increases the size resolution due to signal averaging and keeps the acquisition time short allowing real-time analysis of assembly. However, a limitation of this approach arises from an increase in total resistance as a function of the number of pores and the consequent decrease in the pulse amplitude and signal-to-noise ratio. If the total resistance of a device becomes too high, the presence of a particle in one pore does not significantly affect the overall resistance.

Here, we describe an alternative approach to achieve multiple measurements of single HBV capsids by automatically driving individual particles back and forth through four pores in series. Previously, a similar ping-pong technique was used with single nanopores to control and analyze DNA molecules^{42–44} and nanoparticles.^{45–46} This method could provide an unlimited number of measurements for each particle and is easily integrated with our serial array of nanopores. We used the 4-pore configuration to demonstrate as many as 1500 measurements on a single particle. We found the increase in particle-size resolution plateaus after ~60 measurements per particle. For particles with ~60 measurements, we fitted a Gaussian function to the normalized pulse amplitude (i/i_0) distribution to determine the particle size. Fittings with a low R-squared value indicated that multiple particles were

recognized as one and were subsequently excluded from the data analysis. To show the sensitivity of this system, we used the ping-pong technique to monitor an HBV assembly reaction over two days and showed the intermediates shifted from smaller sizes (105 to 113 dimers) to larger sizes (114 to 117 dimers) during this time period.

Experimental Section

Device Fabrication.

Nanofluidic devices were fabricated as described previously.^{35,39} Briefly, the devices consisting of two V-shaped microchannels connected by a series of nanochannels and nanopores were fabricated in D263 glass substrates (Figure 1). Microchannels were patterned into the substrate by standard UV photolithography followed by wet chemical etching in buffered oxide etchant. Access holes at the ends of the microchannels were sandblasted through the substrate with aluminum oxide powder. Nanochannels and nanopores (Figure 1b-c) were directly milled in the 10- μm gap between the V-shaped microchannels with a focused ion beam instrument (Auriga 60, Carl Zeiss AG). The substrate was then bonded with D263 cover glass at 545 °C for 12 h. After bonding, reservoirs were installed over the access holes on the devices with epoxy. Before the resistive-pulse experiments, the channels were wetted with 0.1 M NaOH for 3 min.

Sample Preparation.

T = 3 and T = 4 capsid standards were assembled from Cp149 dimers expressed in *E. coli* and purified by procedures described elsewhere.²⁹ For the HBV assembly experiments, we added ~10 μM Cp149 dimer in 50 mM HEPES buffer (pH 7.5) into a 50 mM HEPES buffer with 2 M NaCl to assemble capsids (5 μM dimer) in 1 M NaCl. The reaction proceeded for 1 h at room temperature, and a portion of the reaction mixture was diluted to 0.05 μM dimer before the resistive-pulse measurements. Both the original assembly reaction at 5 μM dimer and the assembly reaction diluted to 0.05 μM dimer were stored at 4 °C for two days and subsequently analyzed. The assembly reaction at 5 μM dimer stored for 2 days was also diluted to 0.05 μM dimer prior to analysis.

Ping-Pong Experiments.

Four-pore devices were used for the ping-pong experiments. Devices were tested with purified T = 3 and T = 4 capsids in 50 mM HEPES buffer (pH 7.5) with 1 M NaCl. Pure capsids or a 1:1 mixture of T = 3 and T = 4 capsids with a typical concentration of 1 nM were added to one reservoir to fill the microchannels. The potential was applied along the nanochannel, and the current was monitored by an Axopatch 200B (Molecular Devices, LLC) connected through a data acquisition card (PCIe-6353, National Instruments). Current traces were analyzed in real time, and pulses were identified by setting a threshold based on the amplitude of the translocation pulses relative to the baseline current. After the number of pulses detected equaled the numbers of pores, an 8-ms delay was used to allow the capsid to migrate away from the pore entrance, and then the potential was reversed. This delay was necessary because switching the potential caused a current spike due to the capacitance of the sensing electronics. The spike took ~2 ms to decay to baseline. Consequently, these 2 ms of data were ignored in the pulse identification algorithm. With the 8-ms delay before

potential switching, the capsid usually migrated far enough beyond the pore entrance that the capsid did not return to the nanopore within the 2-ms decay time.

The program resumed collecting pulses, and after observing another series of four pulses, the program reversed the potential again. This process was repeated until a specified number of measurements was made or until the particle was lost, e.g., no pulse series was detected within 100 ms of the previous pulse series. In either case, the potential was set to positive for 100 ms before measurements resumed, which marked the beginning of the collection of a new group of pulses. Labels were used to tag the data at different phases of pulse identification (e.g., waiting for a particle to enter the nanopores, monitoring a pulse series, and delaying before potential switching). The program was written in LabVIEW (National Instruments) with an embedded MATLAB script (MathWorks) for real-time data analysis.

Data Processing.

During the ping-pong experiments, current, potential, pulse threshold, and the labels for different phases of pulse identification were all saved to simplify subsequent data analysis. Data were continuously saved to hard disk in TDMS format and analyzed offline with a MATLAB program. The analysis program parsed the data and extracted pulse sequences by the recorded pulse identification phase label.

Pulse sequences that consisted of fewer than 60 pulses (i.e., 7.5 cycles) were discarded. In one cycle, each particle was measured four times at a positive baseline current, and four times at an equal magnitude but negative baseline current. These data were initially added to the entire distribution to verify that the pores and collection algorithm did not discriminate either large or small particles. For pulse sequences that had sufficient counts, i/i was extracted for each pulse and normalized. Histograms of i/i from the same sequence of pulses were fitted with a Gaussian function to ensure only one distribution existed. Distributions with an appropriate goodness of fit (R-squared value) were kept to extract the most probable i/i .

Distributions of the assembly products were fitted with a custom MATLAB program that implemented a Monte Carlo algorithm. A Gaussian distribution was fitted to each subpopulation with different numbers of dimers. Locations of the capsids and intermediates were calculated with the assumption that the two peaks with the highest abundance were from $T = 3$ and $T = 4$ capsids. The standard deviation of each population was assumed to increase linearly as a function of the number of dimers.

Results and Discussion

Characterization of the Ping-Pong Technique.

We used an in-plane nanofluidic platform fabricated in a glass substrate to perform the ping-pong experiments. Figure 1a shows the schematic of two V-shaped microchannels, and Figure 1b-c shows a schematic and scanning electron microscope (SEM) image of a series of four nanopores and nanochannels that were milled with the FIB instrument to connect the microchannels. The nanopores were 60 nm wide, 70 nm deep, and 290 nm long. The pore-to-pore nanochannels were 320 nm wide, 130 nm deep, and 500 nm long, and the side

nanochannels were 500 nm wide and 260 nm deep. Sample and buffer solutions were loaded by adding fluids into the reservoirs and, subsequently, pulling vacuum across the microchannels.

HBV capsids, which have a net negative charge, were driven along the nanochannel through the nanopores under an applied potential. A representative current trace of a ping-pong experiment (Figure 2a) shows that the time interval between sequences of pulses from different capsids is much larger than the time interval between pulses from a single capsid in the nanopores or the time interval to switch between positive and negative currents. Figure 2b shows examples of one forward four-pulse series and one backward four-pulse series, which together constitute one cycle. The time interval of particles traveling between different pores is ~ 1.5 ms with 800 mV applied, and the time to detect a 4-pulse series, including the time delay to switch the potential, is ~ 10 ms. Figure 2b also shows that pulses detected at positive and negative baseline currents have the same absolute amplitude.

To characterize the performance of the ping-pong technique, $T = 3$ and $T = 4$ HBV capsid standards were tested separately on a 4-pore device. During this test, the number of measurements was targeted to be 204 pulses per particle (25 ping-pong cycles plus one half-cycle at positive baseline current to drive the capsid away from the sensing region). If the particle was lost before 204 pulses were recorded, the data were discarded. Approximately 1,000 $T = 3$ and $T = 4$ capsids were counted. Because the pores have slightly different dimensions from the FIB milling, each pore gave a slightly different average pulse amplitude for the same particle. To normalize the data, the average pulse amplitudes from each pore were adjusted with a coefficient extracted from fits to each distribution (see Figure S1 in the Supporting Information).

Each data set for one particle had 204 normalized pulse amplitudes (i/i) where i is the adjusted pulse amplitude and i is the baseline current. The first n values ($n = 1, 2, 3, \dots, 204$) were averaged to study the change in resolution as a function of number of measurements, noted as n pore data. Figure 3a shows examples of pulse-height distributions from 4 pores, 16 pores, and 204 pores. As the number of measurements increases, the distributions for both $T = 3$ and $T = 4$ capsids become narrower and are better resolved. We analyzed all 204 distributions for both $T = 3$ and $T = 4$ capsids and extracted the standard deviation (σ) for each distribution from a Gaussian fit. The standard deviations of the i/i distributions decreased from 3.5×10^{-4} to 8.0×10^{-5} for $T = 3$ capsids and 3.8×10^{-4} to 7.1×10^{-5} for $T = 4$ capsids. Consequently, the resolution (mean/ σ) improved by ~ 5 -fold. Figure 3b shows that the relative standard deviation (RSD) decays as $n^{-0.5}$ where n is the number of measurements. The increased number of measurements dramatically improves the resolution for the first few data points, but after ~ 60 measurements, the resolution plateaus. The $T = 4$ capsid distribution always has a smaller RSD compared to $T = 3$ capsid distribution, and for the $T = 4$ population, the RSD reaches $1/120$ at 85 measurements, i.e., σ equals the size of one dimer. Consequently, particles differing by 4 dimers (4σ) are resolved with the assumption i/i responds linearly with number of dimers.

From the relationship between RSD and the number of measurements, we showed that this ping-pong method achieved single dimer resolution after several tens of measurements. As

the required number of measurements increased, more particles were lost before the target number of pulses (e.g., 204) was reached. To study how many times a particle could be typically cycled and to find an optimum target number of measurements, we performed an experiment with a 1:1 mixture of $T = 3$ and $T = 4$ capsid standards. Measurements of a single particle continued as long as the particle was trapped, and all data were recorded and analyzed. Instead of calculating an average i/i for each particle, the distribution of the dataset was plotted, and a Gaussian function was fitted to extract the i/i values. For each distribution, a goodness-of-fit (R-squared) was also recorded to evaluate the fit.

Figure 4a shows the variation of the R-squared value with the number of measurements. As the number of measurements of a single particle increases, the R-squared for the Gaussian fittings increases. Although the majority of the data points in Figure 4a follow this trend, some points with a large number of measurements have a low R-squared value. Upon a closer look at these individual distributions, we found trapping of two or more particles at the same time was recognized as a single particle by the collection program, which led to a low R-squared. The data collection program relied solely on the particle-to-particle time being much larger than the time between trapping cycles for the same particle. Although these two timescales differ by two orders of magnitude, a small fraction of particles were trapped simultaneously. The number of particles simultaneously trapped becomes larger as the sample concentration increases, which leads to shorter particle-to-particle times. Figure 4b–d shows example distributions for individual particles recognized by the program with different numbers of measurements and R-squared values. Figure 4b shows a distribution for a $T = 3$ capsid with a moderate number of measurements and a good fit. Measurements generating a distribution of i/i for the same particle shows the uncertainty from the thermal fluctuations, which explains why a higher number of measurements results in improved size resolution.

Figure 4c shows the fitting distribution that consists of two particles, one $T = 3$ capsid and one $T = 4$ capsid. A series of 4 pulses was the criterion for the program to start cycling a particle. When two (or more) different particles both entered the nanochannel, each particle passing any of the pores would result in a pulse. Each time four pulses were detected, the potential was automatically reversed. Each time the potential was switched, a random series of four pulses from these two or more capsids were recorded, which resulted in two distinct distributions attributed to a single particle. To minimize the frequency of such events, low sample concentrations were used. At the dimer concentrations used (e.g., $0.05 \mu\text{M}$), simultaneous trapping of particles was rare and very few outliers were observed (Figure 4a).

In the rare case that two (or more) particles occupied the pores simultaneously and if one of them was measured significantly more times, the Gaussian fitting of the corresponding amplitude distributions still gives a correct i/i for this capsid and excludes the signal from the other capsid(s). Figure 4c shows such an example, and the capsid is correctly recognized as a $T = 3$ capsid, which demonstrates the robustness of fitting the data with Gaussian distributions to extract mean i/i values. If a simple average was taken for all data, inconsistent i/i values with intermediate values would be included in the amplitude histograms. In one rare case, both particles were recorded a similar number of times (Figure 4d), and a Gaussian fitting with a single peak gave a i/i value between these two particle

sizes. However, the resulting goodness-of-fit (R-squared) was low, and these instances are easily identified. Specifically, we used an empirical equation to decide if a data point was to be used in the final distribution:

$$R^2 = 1 - \frac{25}{n}, n \geq 60 \quad (1)$$

where n is the number of pulses. We only considered data sets with 60 or more pulses. For each distribution, we calculated the expected R-squared value with the above equation, and only data sets exceeding the R-squared criterion of equation 1 were used for generating the corresponding histograms (i.e., data points between the red lines in Figure 4a). About 15% of the particle data were discarded because they did not meet the required 60 measurements, had a low R-squared value, or both. Only ~7% of the events had enough pulses but with low R-squared values, indicating two different particles were considered as one during the experiment. When measuring capsids with intermediates, similarly sized particles could be treated as the same particle, but the proportion of such events should be much lower than 7%, because under the conditions and timescales studied, the majority of particles measured during virus capsid assembly are easily distinguishable fully formed T = 3 and T = 4 capsids.

Typically, the ping-pong measurements for a single capsid lasted for several seconds, but the acquisition time exceeded 10 s for the collection of one thousand measurements for individual capsids. Figure S2 in the Supporting Information illustrates that i/i values did not change during the time required for cycling the capsid, which showed the capsid did not change size or break apart during the measurement. This particle stability is useful for monitoring single-particle reactions over time.

Assembly of HBV Capsids and Their Intermediates.

So far, we have shown that the ping-pong technique increases the resolution significantly for experiments with T = 3 and T = 4 HBV capsids and have established procedures to improve data analysis. To demonstrate the power of the ping-pong approach for studying virus assembly, we analyzed an assembly reaction of 5 μ M Cp149 dimer in 50 mM HEPES buffer (pH 7.5) with 1 M NaCl. Under these conditions, the assembly reaction occurs on a timescale ranging from seconds to days and produces a complex ensemble of late-stage, kinetically trapped intermediates that can re-arrange to form virus capsids on the timescale of minutes and days.⁶ The reaction was conducted at room temperature for 1 h before being diluted to 0.05 μ M dimer for the ping-pong resistive-pulse measurements. This dilution step slows the reaction and minimizes the number of events where two particles are considered as one by the collection program. The typical time for collecting a set of data with ~1000 particles was ~1.5 h. The data were then analyzed as described for the capsid standards. Solutions containing the original reaction mixture with 5 μ M dimer and the reaction mixture diluted to 0.05 μ M dimer were stored for two days at 4 °C. The lower temperature weakens Cp-Cp interactions and can promote annealing. After two days, the reaction mixture with 5 μ M dimer was diluted to 0.05 μ M dimer and measured whereas the reaction mixture with 0.05 μ M dimer was analyzed as is.

Figure 5a shows the i/i distribution plotted against the pore-to-pore time distribution for the particles formed during the 1 h reaction time. The overall resolution increases significantly with the ping-pong technique, and both the T = 3 and T = 4 capsid distributions are much narrower compared to the four-pulse data. The corresponding i/i distributions are shown as histograms in Figure 5b, and the intermediates are clearly resolved distributions instead of appearing as a shoulder of the T = 4 distribution. Because of the enhanced resolution, the populations of T = 3 capsids, T = 4 capsids, and pre-T = 4 intermediates (everything between the two major peaks) are easily extracted.

The pore-to-pore time distributions of the three populations are plotted separately in Figure 5c. From these histograms, T = 3 and T = 4 capsids had pore-to-pore times of 1.59 ± 0.11 ms and 1.55 ± 0.08 ms, respectively, i.e., T = 4 capsids have a higher electrophoretic mobility, which is consistent with a previous study.³⁸ More interestingly, the pore-to-pore times of the pre-T=4 intermediates (1.61 ± 0.10 ms) were longer than the pore-to-pore times of both the T = 3 and T = 4 capsids. The lower electrophoretic mobility of the intermediates might be because the particles have a similar hydrodynamic volume as T = 4 capsids but fewer charges distributed on the surface due to missing dimers. As a comparison, the pore-to-pore time distributions for T = 3 and T = 4 capsids with only data from the first four pulses do not show these differences as resolved because of the much lower resolution.

Figure 6a-b compares the assembly reaction after 1 hour and 2 days. We found that the ratio of T = 3 and T = 4 capsids did not change significantly; however, the intermediate distributions shifted to a larger mass, closer to the T = 4 capsid distribution. This shift in mass suggests that over the two-day period kinetically trapped intermediates slowly annealed into larger particles, which implies recruitment of the few remaining free subunits or that some particles dissociated to provide material to further assembly of other particles.

To quantify this shift in intermediates, we fitted the distributions from the three reactions with Gaussian functions for each dimer with a program written in MATLAB that implemented a Monte Carlo algorithm (Figure 6). The large peak on the left is the Gaussian distribution fitted to the T = 3 capsid distribution with a mean of 90 dimers. The standard deviation was extracted by fitting the T = 3 capsid distribution alone to have the heights of each Gaussian distribution as the only fitting parameters. For all other peaks, the standard deviation was assumed to be a function of the number of dimers.

Figure 6 shows the fitted curves and the heights for each fitted peak, which represent each species with a different number of dimers. From the Monte Carlo fitting, we observed that the intermediates ranged from 105 to 113 dimers after 1 h (Figure 6a). After two days at 4 °C, these smaller intermediates shifted to larger intermediates from 114 to 117 dimers (Figure 6b), but were still a few dimers from the full-sized T = 4 capsids composed of 120 dimers. During the two-day incubation, the total percentage of intermediates did not change significantly (from 28% to 30%). Of the particles measured, the T = 3 and T = 4 capsids contributed 19% and 53%, respectively, for the 1 h data, and 15% and 54%, respectively, for the 2 d data.

The impact of moving an assembly reaction to a lower dimer concentration is seen in Figure 6c. The reaction mixture diluted to 0.05 μ M dimer after 1 h for initial ping-pong measurements was incubated at 4 °C for two days. Compared to Figure 6b, the particle distribution from the diluted reaction in Figure 6c shows the percentage of intermediates was considerably lower (11%), the percentage of T = 3 capsids was similar (19%), and the percentage of T = 4 capsids was higher (68%), which suggests the intermediates annealed into T = 4 capsids at the lower dimer concentration over the 2-day incubation. Dilution of the reaction mixture shifted the equilibrium, which, in turn, likely led to release of free dimer in solution that were subsequently incorporated into the incomplete capsids to form complete T = 4 capsids.

Conclusion

We have developed a ping-pong technique to measure single nanoparticles up to a thousand times across multiple nanopores. Multiple measurements increase the resolution of the resistive-pulse platform by ~5-fold and result in amplitude distributions with standard deviations that approach a single 34 kDa dimer in a 4.1 MDa capsid. We defined the relation between the resolution and the number of measurements and developed a procedure to analyze the large data sets generated with this method. The advantages of this technique were realized by monitoring HBV assembly and detailing the shift of late-stage intermediates into T = 4 capsids over time, which is not possible without the multiple measurements. Moreover, multiple pores in series return a unique multi-pulse signature during each cycle that leads to unprecedented control of single particles when coupled with the ping-pong algorithm. Consequently, this versatile technique, which traps single particles for extended periods of time, could be used to monitor the assembly and disassembly of individual particles in real time.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments.

This work was supported in part by NIH R01 GM100071 and NSF CHE-0923064. The authors thank Mi Zhang for acquiring the SEM image in Figure 1 and the Indiana University Nanoscale Characterization Facility for use of its instruments.

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Figure 1.

(a)-(b) Schematics of the nanofluidic device used for the ping-pong experiment. Virus-like particles are brought to the nanochannel entrance by microchannel networks (thick black lines). The potential applied along the nanochannel is controlled by a LabVIEW program, and potential switching is triggered when the number of detected pulses equals the number of pores. The particle is driven back and forth (i.e., ping pong) until either a specified number of cycles is achieved or the particle is lost. Only particles cycled 7.5 or more times (60 measurements with 32 forward pulses and 28 backward pulses) were included in the subsequent analysis. (c) Scanning electron microscope (SEM) image of the nanochannels and four nanopores in series.

Figure 2.

(a) Representative current trace of five particles trapped during the ping-pong experiment. Particles are driven back and forth automatically by switching the applied potential. In one cycle, each particle is measured four times at a positive baseline current and four times at an equal and opposite negative baseline current. The particle-to-particle time (~ 1 s) is much longer than the timescale for cycling a single particle back and forth (~ 10 ms). (b) A forward and backward series of four pulses from a single particle cycled through four pores in series.

Figure 3.

(a) Histograms of the normalized pulse amplitude ($\Delta i/i$) of T = 3 and T = 4 HBV capsids for 4, 16, and 204 pulses (i.e., 0.5, 2, and 25.5 cycles). (b) Variation of the relative standard deviation (RSD) with number of measurements for T = 3 and T = 4 capsids. These data were extracted from measurements with four pores in series and 25.5 cycles, i.e., 204 measurements, for each T = 3 and T = 4 capsid.

Figure 4.

(a) Variation of R-squared (goodness-of-fit) with number of measurements (n) for Gaussian fittings of the normalized pulse amplitude ($\Delta i/i$) distributions for individual particles. Distributions with 60 or more measurements and a large R-squared ($R^2 = 1 - 25/n$) are considered a valid point and fall between the red lines. A relatively poor fit is caused by either too few measurements or two particles in the series of four pores simultaneously. (b-d) Three examples of $\Delta i/i$ distributions of $T = 3$ capsids. Both $n = 100$ and $n = 900$ give correct $\Delta i/i$ values (panels (b) and (c), respectively). When a $T = 3$ capsid and $T = 4$ capsid are seen as one particle by the program, the fitting returns a correct particle size if one particle has a significantly higher n than the other particle (panel (c)). However, the fitting gives an incorrect particle size if both particles have similar n (panel (d)). In this case, the fitting R-squared is low, and the data are not considered further.

Figure 5.

(a) Distributions of the normalized pulse amplitude ($\Delta i/i$) and pore-to-pore time for an HBV assembly reaction at 5 μM dimer in 1 M NaCl after a 1 h incubation. (b) Histograms of the normalized pulse amplitude ($\Delta i/i$) that compare data from the first 4 pulses of each data set for a single particle and data from the Gaussian fitting of ≥ 60 pulses for each particle with an R-squared between the red lines in Figure 4(a). (c) Pore-to-pore time distributions for T = 3 capsids, T = 4 capsids, and pre-T = 4 intermediates taken from the first 4 pulses and ≥ 60 pulses. With ≥ 60 pulses, the pore-to-pore times for the T = 3, T = 4, and pre-T = 4 particles are sufficiently resolved.

Figure 6.

Histograms of the Monte Carlo fitting of the particle distributions for an HBV capsid assembly reaction with 5 μM dimer in 1 M NaCl after (a) 1 hour, (b) 2 days, and (c) dilution to 0.05 μM dimer at 1 hour and measured after 2 days. (a) After 1 hour, the majority of the intermediates are isolated as 105 to 113 dimers. (b) After 2 days at 4 $^{\circ}\text{C}$, intermediates shifted to 114 to 117 dimers, but were still a few dimers from T = 4 capsids (120 dimers). (c) After dilution from 5 μM to 0.05 μM dimer at 1 hour and incubation at 4 $^{\circ}\text{C}$ for two days, most of the intermediates disappeared and shifted toward T = 4 capsids, and the ratio of T = 4 to T = 3 capsids increased. The black lines, blue symbols, and red bars represent the fitted curves, position of the capsids and intermediates, and heights for each fitted peak, respectively.