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Single-Molecule Enzymology Based On The Principle Of The Millikan Oil Drop Experiment

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Abstract

The ability to monitor the progress of single molecule enzyme reactions is often limited by the need to use fluorogenic substrates. A method based on the principle of the Millikan Oil Drop Experiment was developed to monitor the change in charge of substrates bound to a nanoparticle and offers a means of detecting single enzyme reactions without fluorescence detection. As a proof of principle of the ability to monitor reactions which result in a change in substrate charge, polymerization on a single DNA template was detected. A custom oligonucleotide was synthesized which allowed for the attachment of single DNA templates to gold nanoparticles with a single polymer tether. The nanoparticles were then tethered to the surface of a microfluidic channel where the positions of the nanoparticles, subjected to an oscillating electric field, were monitored using darkfield microscopy. With short averaging times, the signal-to-noise level was low enough to discriminate changes in charge of less than 1.2%. Polymerization of a long DNA template demonstrated the ability to use the system to monitor single molecule enzymatic activity. Finally, nanoparticle surfaces were modified with thiolated moieties in order to reduce and/or shield the number of unproductive charges and allow for improved sensitivity.

Keywords

nanoparticle; DNA polymerase; microfluidic; single-enzyme

Introduction

Enzymes catalyze most biochemical reactions: therefore understanding the underlying mechanisms of enzymatic reactions is of great importance in understanding cellular processes. However, ensemble enzyme kinetics often miss reaction details such as the production and consumption of unstable intermediates and an understanding of dynamics. The development of assays capable of monitoring reactions catalyzed by single enzyme molecules has greatly advanced the field of enzymology [1], yet the development of new assays remains a challenge to the field. Many assays depend on fluorescent detection [2] which can be versatile, but can involve complicated reagents, expensive instrumentation,

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and susceptibility to photobleaching. The development of novel methods to monitor single molecule enzymology without fluorescent labels is of great use to the field.

Here, we propose to use charge-based detection based on the Millikan Oil Drop Experiment for single molecule enzymology. In 1911, Millikan was able to calculate the charge of a single electron by suspending oil droplets of differing charge under a uniform electric field and observing the velocity at which these drops fell under gravity [3]. With a simple microscope and a stopwatch, Millikan was able to calculate the total charge on the drops based on the balance of gravitational, viscous, and electrical forces with high sensitivity and accuracy.

In a similar manner, by observing the kinematic response of a particle subjected to opposing external forces, changes in the charge of functionalized nanospheres in an aqueous solution can be applied to monitor enzymatic activity. An example of the successful application of this concept to monitor ensemble enzymatic activity focused on the enzyme PLC- γ , which catalyzes a reaction to convert negatively charged lipids to neutral lipids [4]. To monitor this activity in real time, Galneder et. al. held lipid-coated beads in an optical trap. When the beads were oscillated by an external electrical field, the positional amplitude of the beads, which was sensitive to bead charge, was monitored as a function of time. In their experiment, a balance of optical, hydrodynamic, and electrical forces determined the bead motion. As the reaction proceeded and the charged lipids were converted to neutral ones, the positional amplitude of the beads decreased. The time dependence of this reaction matched the expected kinetics, illustrating that Millikan detection could be used to monitor enzymatic activity in aqueous environments. It is important to note that in contrast to the original Millikan experiment in which the charge of the oil droplet is simply the charge at the oil-air interface, in an aqueous medium the effective charge of the particle is determined by the diffuse double-layer extending into the aqueous medium and is dependent on the surface charge and moieties on the particle, as well as the ionic strength of the suspending medium.

This study presents a similar, bead-based, detection method with two major differences. First, the reagents were designed to be capable of monitoring single molecule activity. Second, we chose to localize the beads by use of polymer tethers in a microfluidic channel, rather than with an optical trap. Using polymer tethers simplified the required instrumentation and allows for higher parallelization. An oscillating electric field was applied across the channel while bead position was monitored by dark field microscopy. The relevant forces are the entropic restoring force exerted by the tether, hydrodynamic, and electric forces. Gold nanoparticles, rather than polymer microspheres, were chosen as the base bead to decrease the number of excess surface charges by decreasing the surface area. In addition, gold scatters light much more efficiently enabling the use of dark field microscopy as a detection method for submicron particles [5]. Finally, the nanoparticle surfaces could be modified readily using well-understood thiol chemistry [6-8].

DNA polymerase activity was selected as an enzyme system where activity results in a change in charge of the substrate. This system has been well studied in single molecule enzymology experiments [9-10]. As the polymerase reaction proceeds, dNTPs are incorporated into a growing DNA template. For each dNTP addition a phosphate group is added to the backbone, which increases the charge of the DNA molecule. A key challenge for this study was to come up with an approach to ensure that for every tethered gold nanoparticle, there was one and only one copy of the substrate for polymerization.

A custom oligonucleotide was designed with three functionalities to ensure that if a bead was tethered, it contained exactly one DNA template copy. The three functionalities of the capture oligonucleotide included 1) a 20-mer capture sequence that could be used to

hybridize longer, single stranded templates, 2) a disulfide group for attachment to the gold particle, and 3) an amine group which could be reacted with the polymer tether. An 80 kDa poly(ethylene glycol) (PEG) molecule with heterobifunctional ends was utilized as the tether. PEG was chosen due to its neutral charge, low affinity for surface interactions with the gold nanoparticle and microfluidic channel, and the availability of custom synthesis of hetero-bifunctional, monodisperse, high molecular-weight fractions. One end of the 80 kDa PEG molecule contained an NHS ester that would react with the primary amine of the capture oligonucleotide, while the second end contained a biotin group.

Full functionality of this single molecule enzymology detection approach was demonstrated by attaching the capture oligonucleotide to gold nanoparticles and tethering the nanoparticles to the surface of a microfluidic channel. Tethered bead kinematics and system sensitivity was characterized by changing the electric field as a proxy for changing bead charge. Error analysis demonstrated that this platform could be sensitive to 1% changes in charge with sufficient averaging time. Finally, biochemical activity was demonstrated by polymerization of a 425-nucleotide long DNA template.

Materials and Methods

Oligonucleotides

Four custom oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA). The capture oligonucleotide, oligo A, contained three functional groups: a disulfide (DTPA, for gold bead attachment), a primary amine (NH₂T, for polymer tether attachment), and a single stranded 20-mer oligonucleotide (for hybridization of DNA template) (Figure 1a). A 6-monomer long (18 bond) PEG spacer between the disulfide group and the rest of the oligonucleotide separated the DNA template from the particle surface to reduce potential steric hindrance of a polymerase to attach to the double-stranded region of the template.

Two additional oligonucleotides were used as PCR primers to create a 425 base template from human genomic DNA. One primer, oligo A', with a sequence identical to A, contained a biotin at the 5' end for purification purposes. The second primer, oligo B, contained a fluorescein (FI) group at the 5' end although this functionality was not utilized for these experiments.

Finally, oligo C, was designed as a polymerization template with a 20mer region at the 3' end complementary to oligo A.

Oligo A: 5'/DTPA/PEGspacer/NH₂T/GAAGAGCCAAGGACAGGTAC3'

Oligo A': 5'/bio/GAAGAGCCAAGGACAGGTAC3'

Oligo B: 5'/FI/GGAAAATAGACCAATAGGCAG3'

Oligo C: 5'CTGCCTATTGGTCTATTTTCCAAGTACCTGTCCTTGGCTCTTC3'

Gold nanoparticles

Gold nanoparticles have a number of desirable properties for use as the base particle. First, compared to micron-sized polystyrene beads, the smaller surface area reduces the number of excess, non-substrate charges. In addition, gold scatters light much more effectively than polymer beads, making the small nanoparticles detectable by dark field microscopy [5]. Finally, the ability to modify the surface with well understood thiol chemistry allowed the particle surface coatings to be easily manipulated [6-8]. Nonionic surfactant stabilized 60

and 70 nm diameter gold nanoparticles were purchased from Cytodiagnostics (Ontario, Canada). Size and zeta potential characterization of similarly synthesized particles has been reported by Perrault and Chan [14]. These nanoparticles as characterized by transmission electron microscopy are relatively monodisperse and spherical, with geometric standard deviation (size) of 1.09, and the longest-to-shortest axis length ratio of 1.13 with coefficient of variation of 0.08. In the Cytodiagnostics website, the size dispersity for 60 and 70 nm gold nanoparticles are specified to be ± 10 nm (www.cytodiagnostics.com/gold_nanoparticles_properties.php).

Particles were imaged in microfluidic chips by darkfield microscopy with a Nikon Diaphot (Nikon Instruments, Melville, NY) equipped with a PH3 phase ring and a 10x objective. A mercury arc lamp with a 100 W bulb housed in Oriel housing (Oriel Instruments, Stratford, CT) was utilized as an illumination source. A CMOS camera (EO-0413M, Edmund Optics, Barrington, NJ) captured bead images. MATLAB (Mathworks, Natick, MA) code was written to convert the images into the bead position using a center-of-mass calculation.

The position of non-diffusing, stuck beads was analyzed to explore errors in determining bead position related to bead brightness. Images of a bead were collected at frame capture rates of 20 and 100 frames/sec while the error in position was analyzed. Later, frame capture rate was optimized to balance data acquisition rates with high bead intensity.

Capture oligo-PEG tether coupling

All reactions were performed at room temperature unless otherwise specified. The custom synthesized tether consisted of an 80 kDa hetero-bi-functional poly(ethylene glycol) with NHS-ester and biotin groups (Jenkem Technology USA, Allen, TX). The use of uncharged PEG as the tether was ideal because it did not add unproductive charge to the system, thus preserving sensitivity to substrate charge. For most experiments, the capture oligonucleotide was coupled to the PEG tether by incubating in 8.5 mM phosphate buffer (pH 7.5) with the bi-functional 80 kDa PEG tether at room temperature for two hours with PEG tether added in excess at molar ratios of 10:1 PEG:oligonucleotide. The high ratio of PEG is used to ensure that most of the trifunctional oligonucleotides (>99.995% by Poisson statistics) has a tether.

Before attempts were made to tether nanoparticles to microfluidic channels, a Bioanalyzer 2100 microelectrophoresis system (Agilent, Santa Clara, CA) was utilized to confirm that the reaction between the capture oligo, A, and the PEG tether was successful. First, oligo C was hybridized with oligo A by incubating solutions of 5 μ M oligo A and 10 μ M oligo C at 95°C for 2 minutes and allowing to cool slowly to room temperature. Following hybridization of the template and the capture oligo, the sample was split and the PEG tether was added to one aliquot following the procedure described in the previous paragraph. The hybridized oligonucleotide with and without PEG tether were analyzed using a DNA 1000 assay with the Bioanalyzer. Only double stranded DNA is visible in this assay: any excess, unhybridized oligo C was not detectable.

Creation of nanoparticle-tether assemblies

Capture oligonucleotides with tethers attached were then reduced by incubation with a gel containing immobilized tris(2-carboxyethyl)phosphine (TCEP, Thermo Scientific, Rockford, IL) according to manufacturer's instructions. This ensured all sulfhydryl moieties were in the reduced form. Following reduction, the PEG-coupled oligo A was added to gold nanoparticles at a ratio of 0.5:1. The reaction was incubated under a helium atmosphere on a shaker overnight. Poisson statistics were relied upon to produce mostly (>90%) of beads

with either zero or one oligonucleotide and tether (60.65% with zero and 30.32% with one tether, only 9.02% has 2 or more tethers).

Before use, small aliquots of nanoparticle:oligonucleotide:tether reaction mixtures were incubated with excess streptavidin in 5 mM Tris for 30 minutes. Nanoparticles were washed three times in 5 mM Tris to remove unbound streptavidin and other unreacted reagents. Meanwhile, bovine serum albumin (BSA) was incubated for two hours in 8.5 mM phosphate buffer with the 80 kDa PEG tether. Ratios of 1:1 PEG:BSA were used to produce biotin-PEG-BSA. After two hours, this biotin-PEG-BSA was incubated with the nanoparticle-oligonucleotide-PEG-avidin construct. This resulted in a gold nanoparticle with a double, or 160 kDa, PEG tether attached to BSA (Figure 1b). Increasing the contour length of the polymer tether increased the positional amplitude at the same electrical force, thus improving the signal-to-noise ratio of the nanoparticle positional measurement.

Tethering particles to microfluidic channel

Custom glass microfluidic chips with a $6\ \mu\text{m} \times 312\ \mu\text{m} \times 12.6\ \text{mm}$ channel were manufactured by Caliper (Alameda, CA). Channels were washed with 1 N NaOH for 30 minutes, then rinsed with a solution of 100 mM Tris (pH 8.0) followed by deionized water. Nanoparticle-PEG-BSA assemblies in 5 mM Tris, pH 8.0, and 2% DNA 5k gel (DNA 5k Bioanalyzer kit, Agilent, Santa Clara, CA) were introduced into the chip and allowed to incubate for 30-60 minutes to allow sufficient time for BSA to nonspecifically attach to the channel surface. In addition, long incubations with the DNA 5000 gel, which contains a proprietary gel, allowed sufficient time for the polymer to adsorb to the glass surface and prevent electroosmotic flow [11]. Following incubation, chips were rinsed with slow, pressure-driven flow of 1:50 dilution of DNA 5000 gel in nuclease free water. The solution was incubated in the channel for 2 or more hours, which appeared to minimize non-specific bead sticking as it coated the channel surface. Before use, the diluted gel solution in the microfluidic channel was exchanged with the desired run buffer using a gentle pressure flow.

Sensitivity and error analysis

An Agilent 2100 Bioanalyzer was utilized as a programmable voltage source using a custom electrode block which carried the voltage from output channels in the Bioanalyzer to specific wells in a caddy attached to the microfluidic chip. Electric fields oscillating at varying frequencies and strengths were applied to beads tethered in the channel. Videos of bead motion were captured and a program written in MATLAB was used to convert images to bead position in time. Altering the strength of the electric field was used as a proxy for changing the charge on the bead, thus analytical sensitivity and error analysis could be performed without the addition of biochemical reactions. Positional amplitude was utilized to monitor changes in electric force. The electric field oscillation frequency was kept at a low enough rate that the bead reached the steady state position and diffused for two or more frames before the field polarity was reversed. When the bead was at the end of its tether the position was a result of the balance between the electric force and the restoring force of the polymer tether. When the electric force increased, either by an increase in bead charge or an increase in electric field, the positional amplitude also increased.

Production of a primed DNA template

Two oligonucleotides, A' and B, were utilized as PCR primers to amplify a portion of the beta-globin gene of human DNA. CEPH control DNA was used as a template (Applied Biosystems, Carlsbad, CA), to create a double stranded, 425bp product with a biotin at the 5' end of one strand. A PCR kit (Takara Bio Inc., Japan) was utilized according to manufacturer's instructions. Reactions were 50 μL with 0.2 μM primer concentrations. A

negative control with no template was also performed. Following PCR, products were purified with a PCR clean up kit (Qiagen, Valencia, CA). The concentration and size of the products were confirmed by electrophoresis with an Agilent Bioanalyzer.

A 10 uL aliquot of streptavidin coated magnetic beads (Promega, Madison, WI) was washed three times in 20 uL of 2 M NaCl/10 mM Tris, pH 8.0. After resuspending in 15 uL of the same buffer, 15 uL of 150 nM double stranded PCR product was added to the magnetic bead suspension and incubated for 15 minutes to allow the biotinylated PCR product to attach to the beads. The bead suspension was rinsed one time with 15 uL 1M NaCl/5 mM Tris to remove unbound oligonucleotides, then resuspended in 15 uL of 0.1 M NaOH to denature the DNA. After 10 minutes, the NaOH supernatant, which should contain free single stranded PCR product, was removed and 12 uL 0.4 M HCl and 3 uL 1 M Tris, pH 7.0, were added to the NaOH solution for a final pH less than 7.5 [12]. Next, 0.45 uL of 10 uM oligonucleotide A (the sulfhydryl-containing capture oligonucleotide, which has the same sequence as the biotinylated primer, A') was added to the single stranded solution. The solution was heated to 95°C and allowed to cool slowly to room temperature. This created a primed piece of single stranded DNA 425 bases long, where the primer contained the active sulfhydryl and tether attachment areas, enabling the assembly of a nanoparticle-tether-BSA construct.

The primed template was purified with a Qiagen PCR cleanup kit to remove unhybridized capture oligonucleotides. The resulting primed, single stranded template was eluted in 25 uL of elution buffer. Polymerization buffer (NEB2 at 0.5x, New England Biosciences, Ipswich, MA), 22 uM dNTPs, and Mako polymerase (Enzymatics Inc., Beverly, MA) were added to a small aliquot of the primed template and incubated at room temperature for 20 minutes. Analysis with a Bioanalyzer demonstrated that no double stranded product was present without the addition of polymerase. With the addition of dNTPs and Mako a peak corresponding to 425 base pairs was present, which confirmed the production of a primed template. The primed template was reacted with the PEG tether at a ratio of 2:1 PEG:template in 10 mM phosphate buffer, pH 7.5. The disulfide was reduced and the PEGylated primed template was attached to gold nanoparticles as described above.

Polymerase Assay

A polymerization assay was developed to confirm activity of the DNA template. Klenow exo- DNA polymerase was purchased from Enzymatics (Beverly, MA). The polymerization buffer was made with a 1:20 dilution of NEBuffer 2 (New England Biolabs, Ipswich, MA), 1:20 dilution of Klenow and 1:100 dilution of DNA 5000 gel. The processivity of Klenow is around 1-100 bases, with a replication rate of about 15 bases/second [13].

For the assay, nanoparticles were tethered as described previously. The fluid in the channel was replaced with the polymerase solution, while 8 uL of the same solution were pipetted into one well. A 12 uL aliquot of polymerase solution plus 100 uM dNTPs was placed in the opposite well. This volume mismatch created a pressure flow to introduce dNTPs into the microfluidic channel. Meanwhile, an electric field of 336 V/cm oscillating at 5 Hz was applied to the channel. Positional amplitude of the bead was monitored for up to 20 minutes. Enzyme free and dNTP free negative controls were performed to ensure that any changes in bead positional amplitude were a result of polymerization.

Data Analysis

For each bead the positional amplitude versus time data was reduced with a moving average. Outliers were rejected under the following criteria: any data point greater or less than 10% of the average for the surrounding 200 data points. The reduced data was averaged for 23

experimental beads (both Klenow and dNTPs were present) and 25 control beads (Klenow was present but not dNTPs). Any natural drift in the time course of the data was assumed to be linear. The data was fit with two functions: a linear function, and the combination of a first order product growth curve and linear function as follows.

$$y=b+m \times t+a \times \left(1-e^{-\left(\frac{t-t_1}{t_c}\right)}\right) \quad (1)$$

Here t represents time, b is the intercept of the linear function, and a is the amplitude of product growth. The start time (t_1), slope (m), and time constant (t_c) of the growth function were constrained within reasonable values to speed convergence. A reduced sum of squares analyzed the error for both fits, the function with the lowest reduced sum of squares was chosen as a best fit.

Nanoparticle surface modification

The nanoparticles were purchased with a proprietary, physically-adsorbed coating of nonionic surfactants for stabilization. Thiolated polyethylene glycol (PEG-SH, molecular weight 1 kDa, Sigma-Aldrich, St. Louis, MO) and (11-mercaptoundecyl) triethyleneglycol alkane (thiolated-alkane-PEG, Sensopath, Bozeman, MT) were purchased to modify the nanoparticle surfaces and displace and/or shield existing negative surface charges. PEG-SH was reduced with TCEP immobilized gel as described previously. PEG-SH and thiolated-alkane-PEG groups were incubated with nanoparticles in excess of a maximum anticipated surface coverage, $10/\text{nm}^2$ [14], although the actual surface density is not known. Following overnight incubation, nanoparticles were washed three times in deionized water. Zeta potential measurements were performed to confirm changes in effective particle charge. The surface coatings of gold nanoparticles were not altered in other experiments.

Zeta potential measurements

Microfluidic channels were prepared as previously described, except following the wash with deionized water, a solution of 5 mM Tris with a 1:50 dilution of Agilent DNA 5000 gel was allowed to flow through the channel for 15 minutes. Free solution electrophoretic mobility was measured in 10 mM Tris HCl, pH 8.0, (Ambion, Austin, TX) by applying a voltage gradient across a linear microfluidic channel. Electrophoretic mobility, μ , was calculated from particle velocity and field strength. Zeta potential, ξ , was calculated from the Smoluchowski equation (2).

$$\mu=\frac{\varepsilon_r\varepsilon_o}{\eta}\xi \quad (2)$$

Here ε_r and ε_o are the relative permittivity and the permittivity of a vacuum, respectively, and η is the solution viscosity. The zeta potential can be converted to effective surface charge density by Guoy-Chapman theory (3).

$$\sinh\left(\frac{e\xi}{2k_BT}\right)=\frac{\sigma}{(8N\varepsilon_r\varepsilon_0k_BT)^{\frac{1}{2}}} \quad (3)$$

Here, e is the charge of an electron and σ is the surface charge density. N represents the number of ions per unit volume of the bulk solution, k_B is the Boltzmann constant and T is temperature [4, 15].

Results

Optimizing particle imaging

Although gold nanoparticles scatter light efficiently, there was some risk that the bead scattering would be insufficient for detection via dark field microscopy. This assay relies on the nanospheres as optical labels that enable monitoring of the enzyme-catalyzed reaction. System error and sensitivity will depend on the ability to accurately determine the nanoparticle position in time. To explore the relationship between positional accuracy and bead brightness, the position of stationary beads stuck to the microfluidic channel surface was analyzed. Figure 2 demonstrates the same stuck bead analyzed for 500 frames at a capture rate of 20 frames/sec (a) and 100 frames/sec (b). The position of each bead is centered at the origin, but the standard deviation was much lower at 20 frames/sec ($0.03\ \mu\text{m}$) compared to 100 frames/sec ($0.10\ \mu\text{m}$). Note that the center of mass algorithm used to identify bead position was able to do so with sub-pixel accuracy (one pixel was equivalent to $0.21\ \mu\text{m}$). This experiment confirmed that the 70 nm gold nanoparticles scattered enough light to be used as a base bead. However, a balance between low frame capture rates/high positional accuracy and high frame capture rates/low positional accuracy must be obtained.

Capture oligo PEG tether coupling

Figure 3 demonstrates the electropherogram of the hybridized oligonucleotides A and C with and without attachment of the PEG tether. The elution time of the oligos with the PEG tether was slower than the bare oligonucleotides due to the increase in hydrodynamic drag. This increase in drag resulted in a 3 base pair difference between the two materials, as called by the Bioanalyzer analysis software. The reaction occurred at high efficiency: the PEG tether appeared to have coupled with 100% of the capture oligonucleotide demonstrating that every capture oligonucleotide should contain a PEG tether. Likewise, since a single PEG tether can only react with a single capture oligo, any gold nanoparticle that is tethered in the microfluidic channel must have only a single copy of a DNA template

Tether behavior & analytical sensitivity determination

Figure 4 shows the position of a tethered bead as a function of time with the electric field oscillating at a frequency of 5 Hz and a frame capture rate of 100 frames per second. When the electric field was reversed, the gold nanoparticle reached the end of its tether within one or two frames and diffused for about nine frames. In order to obtain enough data for accurate velocity measurements faster frame acquisition rates would be required. With the current optical setup, faster frame rates are impractical because the scattering of the beads was not strong enough to obtain good positional accuracy (see Figure 2). Instead, positional amplitude was used to monitor changes in charge on the nanoparticles. As the number of charges on the bead increased, so did the electrical force, and consequently the positional amplitude.

For future experiments, the frame rate was decreased to 40 frames per second. At this capture rate the bead remained at the end of tether for multiple frames, allowing position averaging, while the brighter bead images improved the accuracy obtained by the center of mass algorithm.

Next, it was necessary to explore the analytical sensitivity and error rates of the current system. Figure 5a shows normalized positional amplitude and bead velocity versus electric field averaged for 7 beads. The positional amplitude at a given field strength varied between beads, however, if the amplitudes of each bead were normalized to the maximum value at field strength of 676 V/cm, the individual beads collapsed onto one curve. Although the plot

was not linear for the full range tested, it was linear for small field changes. Thus if the change in bead charge is small, the resultant change in positional amplitude should be linear.

The final test of the tether was whether the sensitivity could be high enough and the error small enough such that small changes in charge, 1% or less, could be detected accurately. Figure 5b shows an example of a 1.2% increase in electric field where the positional amplitude at each field strength was averaged over 1000 cycles. The average amplitude plus or minus one standard error ($\sigma/(N)^{0.5}$, where σ is the standard deviation and N is the number of cycles) are shown. The points are separated by at least 2 standard errors, 2SE, which demonstrates that with sufficient averaging time, less than a 1.2% change in field can be detected with a 95% confidence level.

DNA polymerase activity: single molecule versus averaged behavior

The sensitivity and error analysis demonstrated that single molecule enzymology experiments were feasible. The next step was to demonstrate that the system could be used to monitor enzymatic activity. To begin, we chose to monitor a polymerization experiment that would result in a large change in charge. This simplified detection of the polymerization signal by making it less sensitive to drift.

Figure 6a demonstrates the average behavior of more than 20 nanoparticles in the presence of Klenow DNA polymerase, with and without dNTPs. Polymerization results varied on a per-particle basis, thus initially the time course behavior was averaged over multiple nanoparticles to ensure that ensemble behavior was reasonable. When dNTPs were introduced at about 300 seconds, the positional amplitude of the nanoparticles began to increase. The amplitude of the first order product growth fit was 0.036, or a 3.6% increase in positional amplitude after polymerization. When dNTPs were not introduced into the channel, the average bead behavior was best described by a linear fit. These results demonstrated that polymerization of the DNA template had occurred.

Representative behavior of single beads is shown in Figure 6b. These three beads were in the same field of view and subject to the same polymerization conditions. Note that the increase the positional amplitude was quite variable for the three beads. The amplitude of bead 1 increased by about 18%, bead 2 increased by nearly 10% and bead 3 showed no significant change in amplitude. In addition, the traces for beads 1 and 2 were not smooth, first order product growth curves. Instead, the polymerization appeared to pause for up to 200 seconds at unique times. These pauses may be a result of periods of inactivity by the enzyme, or the enzyme coming off and on the DNA template [13].

Gold surface modification

On average, the sensitivity of the system was rather low: an increase of 425 charges increased the positional amplitude by 3.6%. To better understand these results, zeta potential measurements were first utilized to estimate the number of charges on unmodified nanoparticles. As shown in Table 1, the zeta potentials of the unmodified, surfactant stabilized nanoparticles were -33.7 and -37.8 mV for 60 and 70 nm diameter particles, which equated to about 600 and 900 effective charges per particle in 10 mM Tris. The standard deviation for these measurements was consistently about 10% of the mean, thus the population was not uniform. The 60 nm diameter particles were explored in addition to 70 nm particles because, as expected, the reduced surface area reduced the number charges on the particles. However, smaller particles may be more susceptible to positional error due to the decrease in scattering and increased diffusional noise.

Polymerization of the 425 base pair template should have increased the number of charges from 1350 (bead plus 425mer and primer) to 1750, or an increase in total charge of about

30%. Based on the extrapolation of the calibration shown in Figure 5a, a 30% change in charge should have resulted in a 15% increase in positional amplitude. Although the increase in charge of the averaged polymerization experiments shown in Figure 6a was lower than expected, it was within the correct order of magnitude.

Next, to explore a method to increase the sensitivity of the system, nanoparticle surfaces were modified to decrease the number of effective charges on the beads. If the adsorbed nonionic surfactant was replaced with 1 kDa thiolated PEG molecules, the zeta potential and number of effective charges was reduced by approximately a factor of two, or 260 and 560 charges/particle for the 60 and 70 nm diameter particles, respectively. The alkane-PEG-thiol was even more successful at reducing effective bead charge; the zeta potential was reduced by a factor of four compared to the nonionic surfactant. In the case of the 60 nm diameter nanoparticle, the number of effective charges was reduced to less than 200 per particle.

Discussion

This work presents a novel detection method capable of monitoring single molecule enzymology without the use of fluorescence. While optimization is still required to improve sensitivity, this study demonstrates a path forward for charged-based monitoring of single molecule enzymatic reactions.

Following verification of bead scattering and tether behavior, the polymerization experiments demonstrated that the DNA template was intact, and that enzymatic reactions could be performed and monitored with the tethered nanoparticles. The change in positional amplitude after polymerization of a 425 bp template, 3.6%, was smaller than the expected 15% increase in positional amplitude. Factors which may contribute to the smaller than expected amplitude change include shielding of the DNA template by the adsorbed surfactant (which will be discussed shortly), and underestimation of effective particle charge. The representative single molecule traces presented in Figure 6b demonstrated a large variance in single bead behavior. Single molecule behavior is stochastic in nature, with individual polymerase molecules being subject to pauses and unbinding events [13]. For example, monitoring the time course of two successive replication runs on the same template showed pauses at different locations which lasted different lengths of time before resuming replication, and such stochastic behavior was observed for two different polymerases (Klenow and Sequenase). In our case, Averaging averaging the behavior of 25 individual polymerization experiments may be insufficient to obtain truly representative ensemble behavior. Klenow, in particular, is known to have relatively low processivity and is subject to long pauses, which may account for the apparent absence of polymerization of some beads such as bead 3 (Figure 6b). The polymerization rate of partially active beads, incorporated into the ensemble averaging, is likely to account for the decrease in the measured positional amplitude (Figure 6a) as compared to expectation. In fact, the bead with the highest polymerization rate reaches the expected percent increase (Figure 6b). Another potential challenge with charge-based monitoring of single molecule enzymology is the charge associated with the enzyme itself, as polymerase molecules may come on and off the template of the course of an experiment. Most DNA polymerase molecules are negatively charged at pH 8.0. In order improve interpretation of future experiments, it may be beneficial to utilize more processive enzymes such as T7 DNA polymerase [13] or an exonuclease deficient version of Phi29 [9, 16].

Another aspect of the method that will require optimization to improve sensitivity is modification of the gold nanoparticle surface coatings. Surface charges on the gold nanoparticles are primarily due to adsorbed ions (often citrate or hydroxyl groups) from the synthesis process [6]. However, as mentioned in the Introduction section, the effective

charge of a particle in response to an electric field is not simply dictated by the surface charge alone. The electrokinetic mobility (μ) of a colloidal particle is a result of the diffuse double-layer which extends into the aqueous phase and is a function of the ionic strength and hydrodynamic drag of the suspending medium as well as the surface charge [17]. Moreover, when the particle is coated with a polymer, μ also depends on the molecular weight, charge, and surface density of the polymer. In this study, the surface coatings PEG-thiol and thiolated-alkane-PEG were neutral in charge. Neutral coatings lower the particle mobility because of increased hydrodynamic drag and a decrease in the effective charge behind the shear surface [18]. In other words, the neutral polymer coating can act as a shield for the surface charge of the bare particle, depressing the effective charge when the double-layer thickness is less than the average coating thickness.

Using the following equation developed by Doane et al. [19] based on Ohshima's mobility formula,

$$\xi = \frac{\xi_c}{1 - (\kappa l_p)^{-2}} \left[\frac{1}{\cos\left(\frac{L}{l_p}\right)} - \frac{e^{-\kappa L}}{\kappa l_p} \left(\frac{1}{\kappa l_p} + \tanh\left(\frac{L}{l_p}\right) \right) \right], \quad (4)$$

the effective particle zeta potential (ξ , calculated from the measured particle mobility μ using Eq. (2)), can be utilized to estimate the core zeta potential, ξ_c . Thus, the effective charge of the polymer coated sphere as tabulated in Table 1, is modulated by the zeta potential of the particle core ξ_c , polymer layer thickness L , hydrodynamic permeability l_p , and Debye length κ^{-1} . For 1 kDa PEG on gold nanoparticles, L was calculated to be 8.2 nm and l_p to be 2.2 nm [19]. In a solution of Debye length 0.4 nm^{-1} , the approximate Debye length of polymerization conditions, this resulted in a conversion factor from effective zeta potential to core zeta potential of about 7. In other words, the addition of thiolated 1 kDa PEG molecules to the nanoparticles provided sufficient shielding such that the effective charge was one seventh the core charge.

The unmodified gold nanoparticles were coated with physically adsorbed nonionic surfactants that added approximately 10 nm to the particle diameter according to the manufacturer, yet replacing this adsorbed surfactant with thiolated compounds successfully reduced the number of effective negative charges on the surface of gold nanoparticles by a factor of 2-4. This may, in part, be due to reduction of nanoparticle core surface charge by physical replacement of surface ions with thiol groups. In addition, the hydrodynamic permeability in the PEG and alkane-PEG groups may be lower than the adsorbed surfactant, which would provide better charge shielding. Adding thiolated alkane-PEG molecules to the particle surfaces was most effective at reducing the apparent nanoparticle charge. Because of their ability to form self-assembled monolayers, alkane-thiol groups may be able to pack at a higher surface density compared to 1 kDa PEG, thus displacing a greater number of surface-adsorbed ions [19, 20]. In general, as can be seen in Eq. (4), further increasing the polymer layer thickness, L , or reducing the hydrodynamic permeability, l_p , will both aid in reducing the effective particle charge. While optimizing surface coatings, care must be taken to ensure that the enzyme substrate remains outside the corona of the surface coating. If the enzyme substrate lies within the surface coating during an experiment, the changes in charge of the substrate due to enzymatic reactions will also be subject to shielding, resulting in reduced system sensitivity.

Another challenge is to avoid colloidal particle aggregation as the effective charge is decreased. Due to reduced ionic stabilization, nanoparticles coated 1 kDa PEG were less tolerant to sodium chloride than nanoparticles coated with the adsorbed surfactant. Particles coated with PEG-alkane-thiols were found to be the least salt-tolerant. Future experiments

may benefit from utilizing molecules with larger PEG groups to provide more effective steric stabilization of the particles [21].

For the particular application of monitoring charge as a DNA molecule is synthesized, this method could be further developed for DNA sequencing. The sequencing field is rapidly evolving as it has the potential to revolutionize basic research and personalized medicine [22, 23]. In particular, the ability to ascertain the sequence of a single DNA molecule is advantageous in applications such as diagnostics, where often only a small amount of DNA, such as DNA from a single cell, can be obtained. Single molecule sequencing also offers practical improvements compared to clonal sequencing techniques including simplified sample preparation, eliminating bias from amplification steps, and avoiding noise from dephasing [10, 24]. While the sensitivity requirements of single molecule sequencing by synthesis would be challenging to achieve, i.e., monitoring single charge changes, this method could be adapted for use with sequencing by hybridization [25] or with charge-labeled nucleotides.

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Figure 1. System Schematic. a) The tri-functional capture oligonucleotide includes a single stranded oligonucleotide sequence, a disulfide group and a primary amine. B) A schematic of a tethered gold nanoparticle where two 80kDa PEG moieties are linked by streptavidin to create an uncharged 160kDa tether.

Figure 2.
Calculated location of a stuck bead captured at 20 frames per second (a) and 100 frames per second (b).

Figure 3.

Electropherogram showing the elution time of the capture oligo A hybridized to oligo C alone (black) and reacted with the PEG tether (grey). The PEG tether added hydrodynamic drag which decreased the electrophoretic mobility of the oligo. Note that after the reaction, there appeared to be no tether-less oligos.

Figure 4.
Position of a tethered gold nanoparticle in time with a frame rate of 100 frames/s and electric field of 5 Hz.

Figure 5.

Positional sensitivity. a) The average normalized positional amplitude versus field strength for 7 beads. The amplitude of each bead has been normalized by the maximum amplitude at 676 V/cm. b) The positional amplitude for a 1.2% change in field is shown. The values were averaged over 1000 cycles and the error bars represent one standard error.

Figure 6.

DNA polymerization. a) Polymerization of the 425 base template, averaged over 26 beads, resulted in an increase in bead amplitude while amplitude of the dNTP-free controls did not change. b) Polymerization curves of three individual beads from the same field of view. Note the increase in positional amplitude varied between 0-18% for the three beads.

Table 1

Zeta potential and effective number of surface charges for 60 and 70 nm gold nanoparticles. Three surface coatings were tested: physically adsorbed nonionic surfactant, 1 kDa PEG-thiol, and an alkane-PEG-thiol.

	60 nm		70 nm	
Nanoparticle Coating	ζ (mV)	Effective Charges	ζ (mV)	Effective charges
Nonionic surfactant	-33.7 ± 3.0	590	-37.8 ± 4.6	920
1 kDa PEG-thiol	-15.8 ± 2.1	260	-24.2 ± 2.2	560
Thiolated-alkane-PEG	-10.1 ± 2.2	170	-9.8 ± 1.0	220