

Gold nanoparticle aggregation for quantification of oligonucleotides: Optimization and increased dynamic range

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ABSTRACT

A variety of assays have been proposed to detect small quantities of nucleic acids at the point of care. One approach relies on target-induced aggregation of gold nanoparticles functionalized with oligonucleotide sequences complementary to adjacent regions on the targeted sequence. In the presence of the target sequence, the gold nanoparticles aggregate, producing an easily detectable shift in the optical scattering properties of the solution. The major limitations of this assay are that it requires heating and that long incubation times are needed to produce a result. This study aimed to optimize the assay conditions and optical readout, with the goals of eliminating the need for heating and reducing the time to result without sacrificing sensitivity or dynamic range. By optimizing assay conditions and measuring the spectrum of scattered light at the end point of incubation, we found that the assay is capable of producing quantifiable results at room temperature in 30 min with a linear dynamic range spanning 150 amol to 15 fmol of target. If changes in light scattering are measured dynamically during the incubation process, the linear range can be expanded 2-fold, spanning 50 amol to 500 fmol, while decreasing the time to result to 10 min.

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There is a growing interest in developing new nucleic acid-based tests to detect and quantify infectious diseases at the point of care (POC)¹ in low-resource settings [1,2]. To be effective in these settings, tests should have a fast run time, deliver unambiguous results, require minimal infrastructure, be extremely sensitive and specific to small quantities of the targeted nucleic acid sequence, and ideally quantify the amount of target present [3]. A number of approaches are being pursued to achieve these goals [4,5]. These techniques often face two major challenges: (i) the amplification of small quantities of target and (ii) the detection and quantification of the amplified target.

Metallic nanoparticles conjugated to nucleic acid probe sequences have been identified as a promising method to address the challenge of target detection and quantification of very small quantities of nucleic acids due to their unique optical properties [6–10]. In this approach, two different DNA probe sequences (1 and 2) are conjugated to gold nanoparticles (AuNPs), which are complementary to adjacent regions of a target sequence (1'2'). When the target is added to a solution containing a mixture of the oligo–AuNP conjugates, the AuNPs aggregate, causing a strong shift to the red in the scattering spectrum of the solution due to the plasmon resonance of the closely linked AuNPs

[11–17] (Fig. 1). This scattering shift can be easily visualized using a simple glass waveguide system that illuminates scattering by directing light through the plane of a standard microscope slide [11,15]. The aggregation assay is specific to the targeted nucleic acid sequence, with an ability to detect single nucleotide polymorphisms, and produces results that are detectable by the naked eye [18,19]. Although the assay has been used with a variety of hybridization buffers, heating to 37 °C during incubation, and a wide variation in incubation times (from 1 to 24 h), it is not clear which conditions lead to an optimal limit of detection (LOD) or whether results can be read quantitatively across a meaningful dynamic range [11,12,20–22]. Previous studies investigating the dynamic range of other DNA-based AuNP aggregation assays have found them to be linear over a range of approximately 1 to 2 orders of magnitude of target concentration [22,23].

This article reports a series of studies designed to optimize the aggregation assay for decreased LOD, increased dynamic range, and minimal run time and to eliminate the need for external heating by varying reaction conditions known to affect metallic nanoparticle aggregation such as incubation temperature, incubation duration, and salt content of the buffer [21,24–26]. We compared the LOD and dynamic range of the assay using three different readout methods: photographic assessment of color, spectrophotometric measurement of light scattering at a single time point, and temporal variations in the spectrum of scattered light. We also present a new method for dynamic measurement of the aggregation process

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¹ Abbreviations used: POC, point of care; AuNP, gold nanoparticle; LOD, limit of detection; PBS, phosphate-buffered saline.

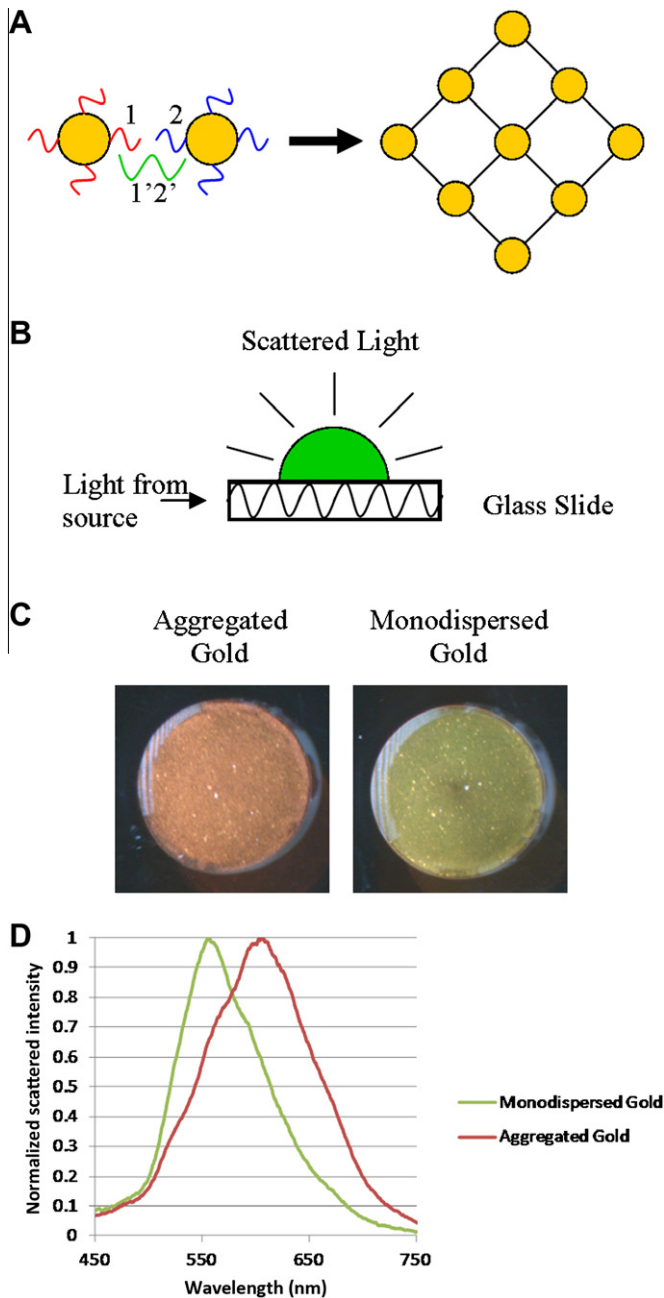


Fig.1. (A) Schematic showing the basic process of the aggregation reaction. Oligo-AuNP conjugates 1 and 2 are bound together into a large aggregated network by the target sequence 1'2'. (B) Schematic of the principles of the side illumination waveguide system used to illuminate the scattering of the samples. (C) Photograph of representative samples on the side illumination system showing the visible red shift of aggregated versus monodispersed AuNPs. (D) This shift can also be observed in the spectroscopy of the samples.

that allows for quantification of the targeted nucleic acids. We found that monitoring dynamic changes in light scattering increases the quantifiable dynamic range of the assay by 2 orders of magnitude without compromising the LOD and also decreases the time before the assay returns a result by 3-fold.

Materials and methods

Materials

DNA probe and target sequences were ordered from Integrated DNA Technologies (Coralville, IA, USA) and resuspended in water at

100 μ M. AuNPs (50 nm diameter) were obtained from Ted Pella (Redding, CA, USA). Formamide, dextran sulfate, sodium chloride, DNase-free water, phosphate-buffered saline (PBS), mineral oil, and magnesium chloride all were obtained from Sigma-Aldrich (St. Louis, MO, USA). Quant-iT OliGreen was obtained from Invitrogen (Grand Island, NY, USA). Aldehyde-coated glass slides were obtained from Arrayit (Sunnyvale, CA, USA).

Methods

Conjugating AuNPs and oligonucleotide sequences

As an example of a clinically relevant target, we selected the eukaryotic parasite genus *Plasmodium* that causes malaria. There is a growing interest in developing new nucleic acid-based diagnostics for malaria in order to target markers of resistance and to increase the sensitivity and specificity over current diagnostic methods. Oligonucleotide probe sequences were designed with the National Center for Biotechnology Information's (NCBI) Basic Local Alignment Search Tools (BLAST) to be specific to the genus *Plasmodium* and to not cross-react with human DNA. A poly-A tail and polyethylene glycol (PEG) spacer were used to increase the flexibility of the sequence. A thiol group was attached to the 5' end of each DNA probe to allow for conjugation with the AuNP. Sequences for the two probes and target were as follows:

Probe 1: 5'-ThioMC6-A₁₅-PEG18-CAT CAA AAG CTG ATA GGT CA-3';

Probe 2: 5'-ThioMC6-A₁₅-PEG18-GAA ACT CGA TTG ATA CAC ACT A-3';

Target (from *Plasmodium* 18S gene): 5'-TAG TGT GTA TCA ATC GAG TTT CTG ACC TAT CAG CTT TTG ATG-3'.

Oligonucleotide sequences were attached to the gold particles using methods adapted from the literature [12]. In brief, 200 μ l of 15 μ M disulfide-oligonucleotide sequences were mixed with 5 μ l of 1 M tris(2-carboxyethyl)phosphine (TCEP) for 30 min at room temperature to reduce the disulfide bond. Then 800 μ l of 75 pM 50-nm gold colloid was added, and the mixture was allowed to sit overnight. Over the next 48 h, the salt concentration of the solution was raised, in steps of 0.1 \times , to 0.5 \times PBS in order to increase the loading of oligonucleotides onto the surface of the AuNPs. To remove excess oligo sequences, the oligo-AuNP conjugates were then washed three times by centrifuging at 4000g for 10 min and resuspending in 1 \times PBS. After each batch of probe conjugation, the transmission spectrum of the oligo-AuNP conjugates was measured using a Cary 50 spectrophotometer; a single extinction peak at 535 nm was taken as evidence that no aggregation occurred during the conjugation step. For this study, 50-nm AuNPs were chosen because they have been shown to offer a compromise between a strong optical signature and stability of the colloids in solution [11,19]. When characterizing probe creation, a series of experiments were carried out to determine the average number of oligonucleotide sequences on each gold particle. This was accomplished by mixing 1.3 M β -mercaptoethanol with the AuNP-oligo probes overnight to release the DNA from the gold. The amount of DNA in the supernatant was then quantified using a commercial fluorescent oligonucleotide quantification system (Quant-iT OliGreen). We found that, on average, 460 copies of probe 1 and 530 copies of probe 2 attached to each of their respective AuNPs.

Aggregation assay

To test the performance of the assay, a set of target concentrations were prepared by serial dilution ranging from 50 pmol to 15 amol of target in steps of half a log. Target amounts are expressed as the total number of target DNA strands added to

the final reaction mixture. In 5 μl of target added to the final mixture, this works out to a concentration range of 10 μM to 3 pM. A hybridization buffer consisting of 20% formamide, 16% dextran sulfate, and 3.75 mM MgCl_2 was prepared. For each experiment, a working solution was created by mixing 60 μl of each probe and 80 μl of the hybridization buffer. This working solution (10 μl) was mixed with 5 μl of a target solution to give a final reaction mixture. The standard assay conditions reported most commonly in the literature were defined to be a 2-h incubation in a 37 °C water bath using the hybridization buffer described above [11,18,20,21].

To optimize reaction conditions for decreased time, eliminating heating, and maintaining or perhaps improving the LOD and dynamic range, we varied the assay runtime, temperature, and salt concentration in the buffer. A master solution was prepared for each target concentration and then split into aliquots for each condition. The temperature of the incubation step was varied using a programmable water bath, and the solutions were incubated for 2 h with the hybridization buffer described above. The time of the incubation was varied by creating a master solution for each target concentration and then splitting it into separate aliquots for each time point. All of these aliquots were then placed simultaneously in a 37 °C water bath and then removed after the chosen incubation time (from 10 min to 3 h). To vary the salt concentration of the assay, different hybridization buffers were created with varying added volumes of a 5-M stock of NaCl to achieve NaCl concentrations in the final solution of 0.3, 0.6, and 1 M. These samples were incubated for 2 h at 37 °C. The need for heating was investigated by running the aggregation assay with a 2-h incubation at 37 °C and a 2-h incubation at room temperature. Under each set of conditions, we compared the LOD and contrast between positive (aggregated) and negative (monodispersed) samples by recording the color of the spots with photography and spectrophotometry.

Measurement

Assay results were quantified by spotting 1 μl of the final reaction mixture onto an aldehyde-coated glass slide and capturing a digital photograph of scattered white light and by measuring the spectrum of scattered light across the visible spectrum. Illumina-

tion of the scattering spectrum for both measurements was accomplished by placing the slide with the samples on it into a side-illuminated illumination system consisting of an Edmund Optics MI-150 light source, a fiber-optic light spreader, and a custom-built mount to couple the light into the edge of the slide, using it as a waveguide [15]. To record image data, a dissection microscope (Olympus SZ61) and a Zeiss Axiovision MRc5 camera were used with the Zeiss Axiovision software with an exposure time of 1.25 s for each image. To record spectra, an Ocean Optics USB 4000 portable spectrophotometer was used with a fiber-optic probe (400 μm core diameter), and data were recorded with an exposure time of 100 ms and a boxcar averaging width of 5. The fiber was clamped into a mount to keep it a uniform distance from the slide and normal to the surface of the slide during and between experiments. The fiber was positioned under the center of each spot for data collection. Preliminary experiments testing the repeatability of the system found less than 5% difference between repeated measurements of the same spots. Images and spectra were recorded immediately after spotting each sample on the slide. Spectral data were processed by subtracting the spectra of a solvent blank and normalizing the subtracted spectra to a peak height of 1. To measure light scattering images and spectra dynamically, a 1.5- μl drop of mineral oil was layered over the 1- μl spot of sample to retard drying, and images and spectra were taken immediately after spotting and then every 2.5 min up to 10 min.

Results and discussion

Using the standard conditions, the aggregation assay was run five times for a wide range of target concentrations. Using visual inspection of scattered light photographs to determine when the color of the spot changed from green (negative) to orange (positive), the LOD of the assay was between 150 and 500 amol of target in the final reaction mixture (Fig. 2A). To quantify spectrophotometric data, the ratio of red to green scattered light at 605 nm to that at 555 nm was calculated; these wavelengths were selected because they correspond to the peak scattering wavelengths of monodispersed and aggregated oligo-AuNPs, respectively. Spectroscopy ratios below 0.8 were considered as negative (containing

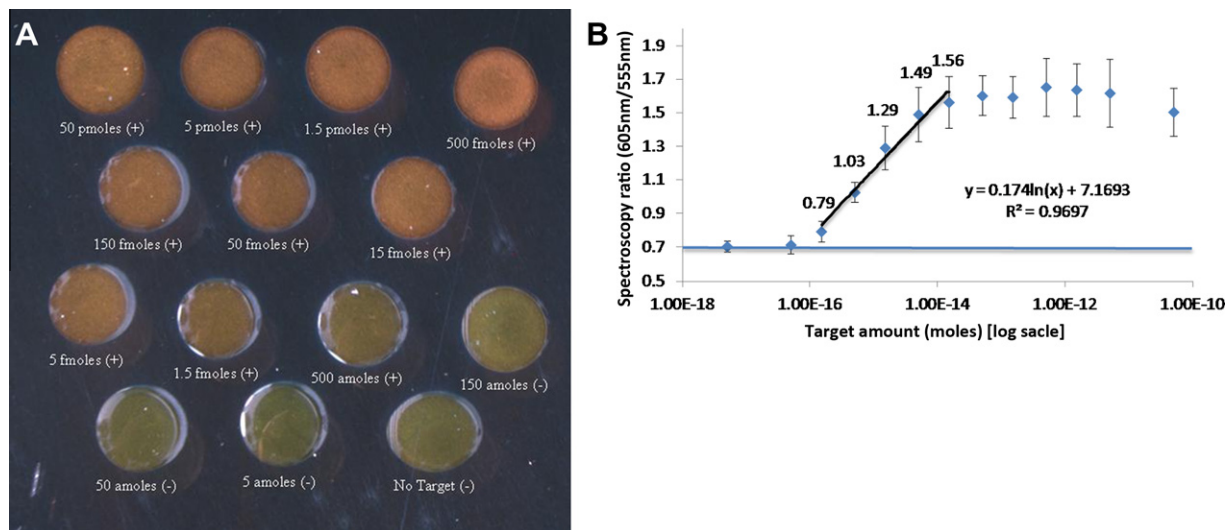


Fig. 2. (A) Photograph of one run of the aggregation assay using standard reaction conditions. Each sample is marked with whether it would be considered as positive (aggregated) or negative (monodispersed) by visual inspection. (B) Averaged peak spectroscopy ratios ($n = 5$) as a function of target concentration. The spectroscopy ratio is the ratio between the amount of scattered light recorded at 605 nm and that recorded at 555 nm after both of these spectra have been normalized to their respective peak values. The spectroscopy ratios have been labeled for samples in the linear range of this assay. Note that the 150-amol sample can be determined as positive by spectroscopy but not by visual inspection. A horizontal line has been plotted on the graph to show the average red/green ratio of the no-target control sample. Error bars represent 1 standard deviation.

mostly monodispersed AuNPs), whereas samples with spectroscopy ratios equal to or greater than this were considered as positive (containing aggregated gold). The assay red/green ratio was linearly related ($R^2 > 0.95$) to the log₁₀ of the amount of target with a dynamic range spanning 150 amol to 15 fmol of target (Fig. 2B). Together, these results suggest that the oligo–AuNP conjugation method and the aggregation process are repeatable and that using the spectroscopy ratio of the two peak wavelengths is a reliable way of quantifying the amount of target.

We then aimed to optimize the reaction conditions of the assay (temperature, time, and buffer composition) to improve its sensitivity and yield an assay more amenable for POC use. These conditions were chosen because of their well-known effects on AuNP aggregation. During these optimization experiments, we used three different target amounts: 50 fmol, which was found to consistently cause complete aggregation; 500 amol, an intermediate amount that was found to consistently lead to partial aggregation of the oligo–AuNPs; and a negative control containing no target to show the effects of the varied conditions on unaggregated samples.

We first tested the effect of temperature to determine the effect of carrying out the aggregation assay at room temperature. Other studies have used room temperature incubation in this assay format, but in this study we sought to specifically isolate its effect on the LOD of the assay [18,21]. Increased temperature (a 45 °C incubation) was also investigated and was found to cause aggregation in all samples, including the no-target control (data not shown). We hypothesize that this is caused by non-DNA-mediated aggregation of the AuNPs. At 37 °C incubation, the assay had an LOD of 500 amol of target, as determined by both spectroscopy and visual inspection (Fig. 3A). Under room temperature incubation conditions, the color difference between aggregated and monodispersed AuNPs is too subtle for visual detection, but spectroscopy maintains an LOD of 500 amol. By using spectroscopy, it is possible to maintain a low LOD even when no heating apparatus is used.

We then tested the effect of incubation time on oligo–AuNP aggregation in order to determine the minimum aggregation time because a short time to result is critical in POC settings. The aggregation assay begins producing results after a relatively short time frame (Fig. 3B). The 50-fmol sample became positive by spectroscopy and visual inspection after 10 min. The 500-amol sample became positive after 20 to 30 min. Up to 180 min of incubation, the no-target sample remained mostly monodispersed. These data suggest that aggregation can happen on a short time scale and that the assay can produce results at the full LOD in less than 1 h compared with the 2 h or longer often reported for this assay.

Finally, we changed the buffer composition, increasing the concentration of salt ions to favor the electrostatic interactions that drive oligonucleotide annealing and to decrease the surface charge repulsion experienced by the AuNPs [21]. Using 1 M NaCl in the final mixture was found to cause aggregation in all target concentrations, including the no-target control (Fig. 3C). The use of 0.3 and 0.6 M NaCl was found to improve the contrast between positive (aggregated) and negative (monodispersed) samples as well as to improve the LOD over the standard salt concentration by making it easier to classify the 500-amol sample as positive by visual inspection.

Combining the findings of the previous experiments, the optimal conditions for carrying out the aggregation assay were found to be a 30-min incubation at room temperature in a reaction solution containing 0.6 M NaCl. A shortened time was used because the higher salt concentration was found to increase the speed at which samples aggregated. These optimized conditions were tested five times on the same range of sample dilutions as the standard condition experiments (Fig. 4). The spectroscopy ratio had a strong linear relationship with the log₁₀ of the target concentration over

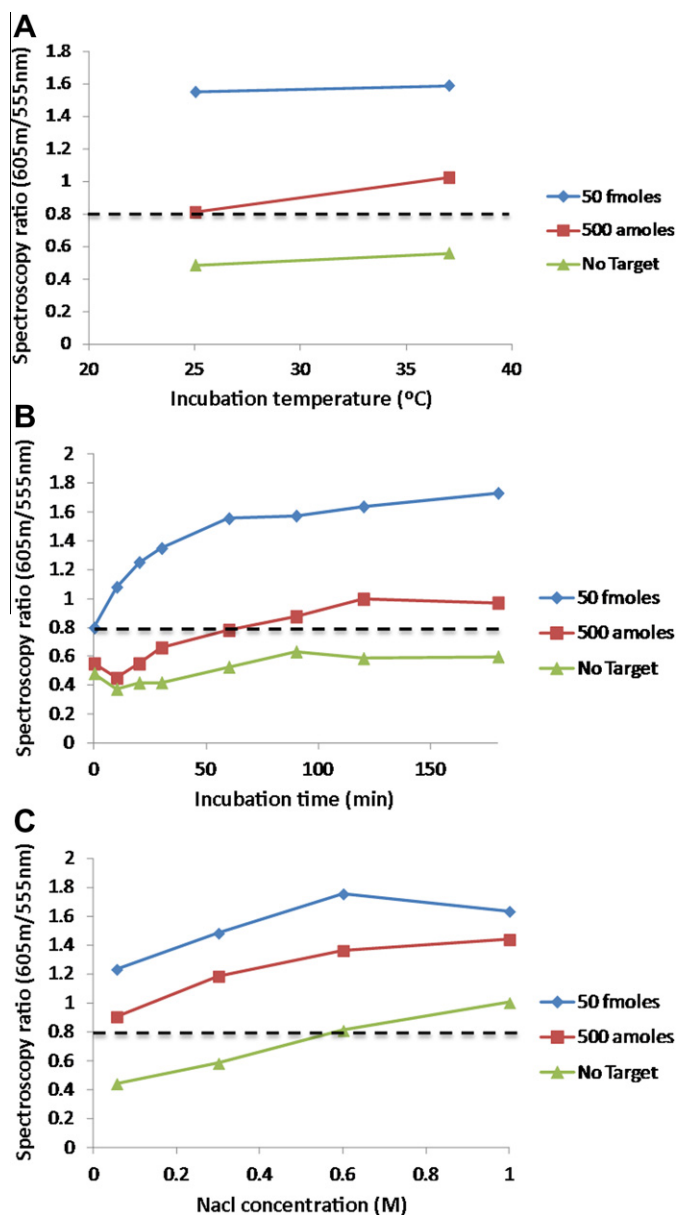


Fig. 3. (A) Effect of incubation temperature on the spectroscopy results of the aggregation assay. (B) Effect of different incubation durations. (C) Effect of the salt concentration of the reaction buffer. A dashed line has been drawn for red/green ratio = 0.8, above which spectral data were considered as positive. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

a dynamic range from 50 amol to 15 fmol ($R^2 = 0.95$). A nontargeted DNA sequence was also tested with the optimized conditions over a range of 500 fmol to 5 amol. The assay was found to be negative at all concentrations of the nontargeted sequence, demonstrating the selectivity of the process (data not shown).

Compared with the standard conditions, the optimized conditions decreased the LOD, maintained a similar dynamic range, and decreased the contrast between positive and negative samples. The increased spectroscopy ratio of the baseline is thought to be due to the increased salt in the reaction causing some non-DNA-mediated aggregation. The decreased spectroscopy ratio of the high target samples is thought to be due to the decreased reaction time not allowing for as much aggregation to occur as in the standard assay. Both sets of conditions have similar linear ranges. Given 5 μ l of target input to the reaction, the optimized reaction

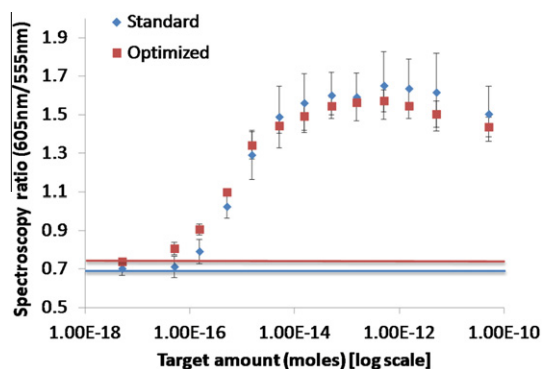


Fig. 4. Comparison of the spectroscopy ratios of the standard and optimized reaction conditions as a function of the amount of target. Horizontal lines have been plotted on the graph to show the average red/green ratio of the no-target control samples. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

has an LOD of 10 pM. The optimized conditions maintain the performance and repeatability of the standard aggregation assay, while being much more useful as a POC diagnostic technique, because results can be obtained in a quarter of the time and with no heating apparatus required.

We hypothesized that monitoring dynamic changes in light scattering would offer faster results and a greater dynamic range. Samples spotted onto a microscope slide dry out after approximately 7 min, causing irreversible aggregation. Using a drop of mineral oil to cover the sample droplet on the surface of the slide was found to delay this drying by up to 15 min. An aliquot of the reaction mixture was spotted on a slide immediately after preparation and covered with oil; spectra were acquired immediately after spotting and at 2.5, 5, 7.5, and 10 min after spotting (Fig. 5A). The red/green ratio was plotted versus time, and the time at which the ratio reached 1 was estimated using linear interpolation (Fig. 5B). A variety of red/green ratio thresholds were investigated, from 0.8 to 1.3, and a threshold of 1 was found to give the most reliable relationship between the target concentration and the time to cross the threshold (data not shown). The experiment was carried out five times, and the ratios at each concentration were averaged. There was found to be a strong linear region ($R^2 > 0.95$) for target concentrations with a dynamic range of 50 amol to 500 fmol (Fig. 5C). As samples dried on the slide, they were found to aggregate, including the no-target controls that achieved a red/green ratio of 1 after 9 min. Although the no-target controls aggregated, samples containing target were found to aggregate significantly faster, allowing for measurement of the amount of target in the solution. This offers an alternative measurement method for the aggregation assay, which not only decreases the time to result

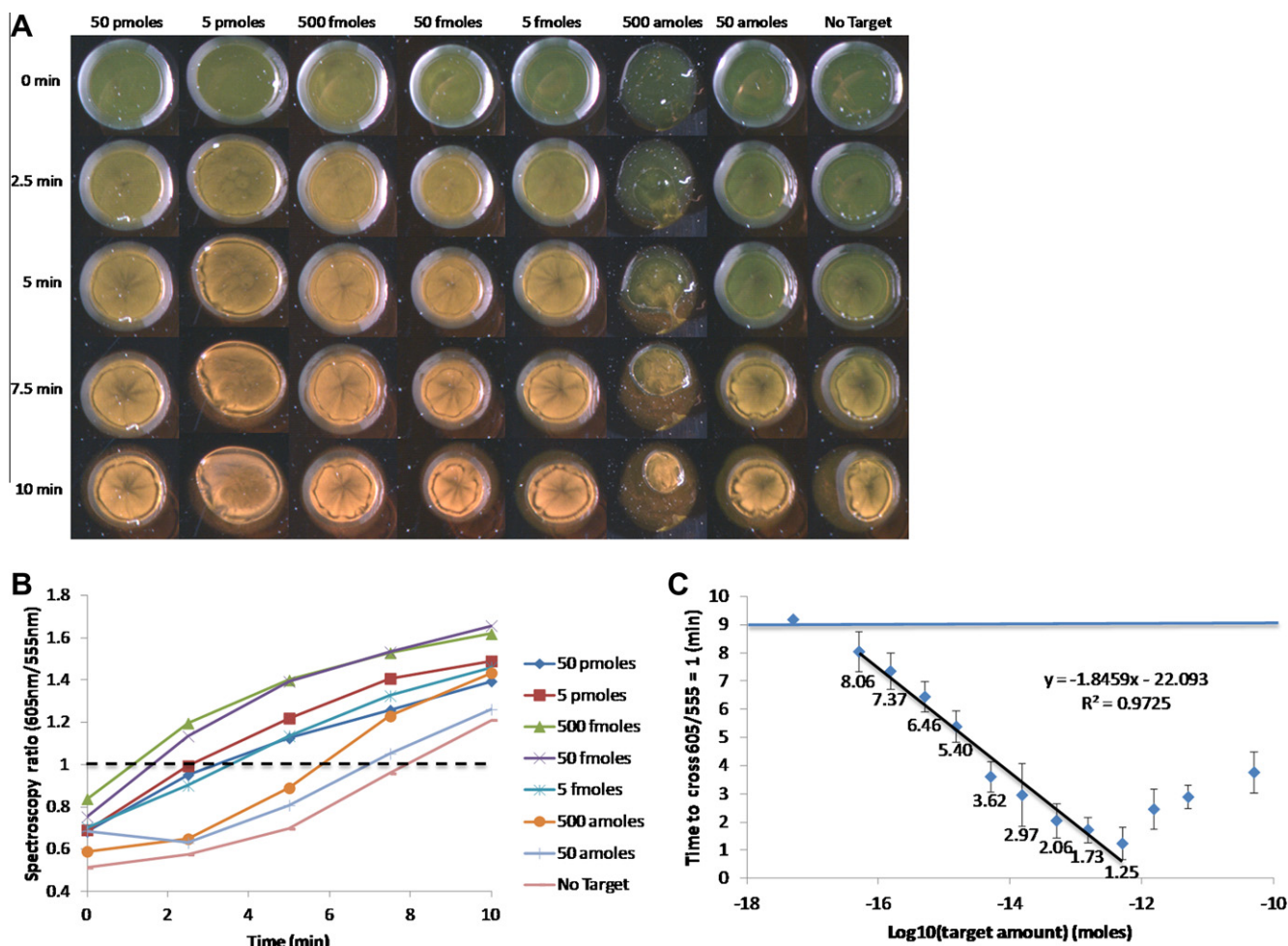


Fig. 5. (A) Visual results of monitoring aggregation kinetics on the side illumination system using oil coverings to prevent sample drying. (B) Spectroscopy ratios calculated as a function of time. A dashed line has been drawn at 605 nm/555 nm = 1, and the time each sample took to cross this threshold was calculated. (C) Average of five samples of the kinetic experiment on the side illumination system. Error bars represent 1 standard deviation. A horizontal line has been plotted to show the average red/green ratio of the no-target control samples. A linear fit line showing the relationship between the log₁₀ of the target amount and the time to cross is shown. The spectroscopic ratios have been labeled for samples in the linear range of this assay.

Table 1
Summary of key experimental conditions and results.

	Standard	Optimized	Oil-protected dynamic measurement
Parameter			
Time to result	2 h	30 min	10 min
Temperature	37 °C	Room temperature	Room temperature
NaCl concentration	0.055 M	0.6 M	0.6 M
Results			
Limit of detection	150–500 amol	50 amol	50 amol
Linear dynamic range	150 amol–15 fmol	50 amol–15 fmol	50 amol–500 fmol

from 30 to 10 min but also increases the range over which the assay is linear by 2 orders of magnitude. The assay conditions and results explored in this study are summarized in Table 1.

In the dynamically measured spectroscopy data, we observed a marked decrease in the red/green ratios of samples containing greater than 500 fmol of target. We believe that above this concentration, the abundance of target sequences are saturating the recognition sequences on the AuNP–oligos, lowering the odds that a given target strand will bind to both recognition sequences in order to bring two AuNPs together. We believe that this effect is more apparent in the dynamically measured data because it operates over a shorter time frame and there is less of an opportunity for the diffusion-limited aggregation reaction to run to completion at higher target concentrations. To resolve the target concentration of an unknown sample, the target could be diluted by powers of 10 to drop the concentration below the 5000-fmol cutoff, which causes decreased time to aggregation of the assay. Using the example of malaria, to which our DNA sequences are specific, as a clinically relevant target, we would expect this assay, with an LOD of 50 amol in 5 μ l, to be able to detect approximately 6×10^6 DNA copies/ μ l. By using an isothermal amplification method suited for the POC such as loop-mediated isothermal PCR (LAMP), which has been shown to yield 10^8 -fold amplification, this assay could potentially detect less than 0.5 parasites/ μ l, the current limit of PCR diagnostics [4,27].

Conclusions

We have demonstrated a method to use the AuNP aggregation assay developed by Mirkin and others to quantify the amount of an oligonucleotide target using the red/green ratio of the samples measured by spectroscopy [12]. We found that using conditions commonly found in the literature, the assay has an LOD of 150 amol of target and a dynamic linear range of 150 amol to 15 fmol. We optimized the parameters of the assay and were able to decrease the runtime of the assay from 2 h to 30 min and eliminate the need for heating. This optimized reaction lowered the LOD to 50 amol while maintaining a similar dynamic range to the standard assay. We also presented a method for dynamic measurement of the aggregation that was found to greatly decrease the assay runtime to 10 min, maintain the 50-amol LOD of the optimized conditions, and double the dynamic range of the assay to cover 50 amol to 500 fmol of target. These findings suggest that, by dynamic measurement of the aggregation assay, it is possible to use it as a relatively simple tool to detect small quantities of nucleic acids and quantify them over a range of 4 logs.

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