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A Plasmonic DNAzyme Strategy for Point-of-Care Genetic Detection of Infectious Pathogens**

Kyryl Zagorovsky, and Warren C. W. Chan*

The development of simple, cost-effective, sensitive, and specific point-of-care (POC) diagnostics represents a major challenge in the 21st century.^[1] The spread of infectious diseases has caused significant losses in global economy and life. A method to control the spread of diseases is to detect the pathogens early at POC, and to administer proper treatment or quarantine.^[2] Detection of DNA as biomarkers is currently a widely used technique for pathogenic detection.^[3] Quantitative PCR (qPCR) is the method of choice, since it includes an enzymatic signal amplification step to achieve highly sensitive and specific detection of genetic targets.^[4] However, qPCR requires expensive equipment and highly trained personnel, limiting the technique use to medical laboratories. There is a strong need to develop cheaper and simpler DNA detection methods.^[5] Here, we combined colorimetric coupling of surface plasmons of gold nanoparticles with DNAzyme signal amplification technology to engineer a fast and simple detection platform for genetic targets with a simple colorimetric readout. The novelty of this study is the integration of these two emerging technologies for point-of-care analysis of infectious disease targets.

DNAzyme is a synthetic DNA enzyme that can catalyze the cleavage of another nucleic acid molecule.^[6-8] Since the catalysis is carried out with multiple turnover, DNAzyme introduces an enzymatic amplification step into the experimental setup.^[9] This amplification is performed without the need for protein components, which are costly and have low thermal and storage stability. While initially designed to detect Pb^{2+} and other divalent cations,^[10-19] a number of modifications have been reported that allow DNAzyme to detect biomolecular targets.^[9,20-27] Two main approaches were used to implement detection of genetic targets. The first method utilizes competitive activation of peroxidase-mimicking DNAzyme, which can catalyze production of colorimetric or chemiluminescent product.^[28] In the second, more sensitive approach, a similar strategy was followed by a number of groups to implement detection of genetic targets by splitting the DNAzyme into inactive components, which could be reactivated by the target binding.^[9,23-25,29] However, these studies used fluorescence as the readout, which

is not an optimal detection modality for POC applications since it requires access to fairly complex fluorometer apparatus. An alternative method using the colorimetric readout of gold nanoparticles (GNPs) has been reported for detection of metal ions, adenosine and cocaine.^[10-14,16-18,20,21] The wavelength at which GNPs absorb light is dependent on whether they are in a monodispersed or aggregated state.^[30] Since the GNP solutions in monodispersed or aggregated states are easily distinguishable by their respective red or purple colors, this approach provides clear colorimetric results that can be visualized by the naked eye.^[31] In this study we combined DNAzyme technology with GNPs to engineer a simple point-of-care diagnostic platform that can be used in remote settings.^[32,33]

Figure 1. **A** – Mechanism of MNzyme catalysis. Adapted with permission from [9]. Copyright 2009 American Chemical Society. **B** – GNP aggregate formation. Linker hybridizes to DNA strands A and B, cross-linking the two sets of GNPs and turning solution purple. **C** – MNzyme assay outline. Target activated MNzyme degrades Linker DNA, preventing GNP aggregate formation. In the absence of the

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target Linkers remain intact and cross-link GNPs (as in B); the solution turns purple. **D** – Schematic depicting how the assay will be conducted at point-of-care to analyze multiple targets in parallel.

Figure 2. A – Optimization of Linker₁ concentration. **B** – Sensitivity of detecting AF-1 genetic target. **A** and **B** - Top: colorimetric readout on TLC plate. Bottom: shift in the peak absorbance. Data based on 3 replicates; error bars are standard errors. **C** – Parallel detection of 5 targets: AF-1, genetic sequences from *N. gonorrhoeae* (Gon) and *T. pallidum* (Syph) bacteria, malarial parasite *P. falciparum* (Mal), and HBV virus. Detection of all 5 targets (top row), 3 out of 5 targets (center row), or no-target control (bottom row). Spot color indicates target presence (red) or absence (purple-black). Images were adjusted for contrast and brightness.

Figure 1 demonstrates the molecular details of experimental setup. Two sets of 13 nm GNPs, GNP-A and GNP-B, are functionalized with 5'-thiol-modified Agg/A (Strand A) or 3'-thiol-modified Agg/B (Strand B) DNA, respectively.^[34] Linker strand is designed to have its 5'-end complementary to 3' of Agg/A, and its 3'-end to complement 5' of Agg/B. Linker can cross-link GNP-A and GNP-B through hybridization, forming GNP aggregate network (Figure 1-B). Close proximity of linked GNPs causes coupling of their individual localized plasmon fields, leading to the shift in their peak absorbance to a longer wavelength, and solution color changes from red to purple. In addition, the Linker strand includes the substrate sequence that can be cleaved by the multicomponent nucleic acid enzyme, MNAzyme, which is one of the reported DNA-responsive DNAzymes. Initially in the inactive state, target's binding to the sensor arms bridges MNAzyme parts A and B, forming the active structure, which can then hybridize to the substrate sequence and catalyze its cleavage (Figure 1-A).^[9] The substrate sequence comprises DNA bases with two RNA nucleotides, which are required for cleavage, imbedded in the middle.^[6] MNAzyme can be made responsive to any genetic target simply by changing the DNA bases of the sensor arms. The sequence of the substrate arms ensures MNAzyme's specificity to particular substrate, so that multiple MNAzyme-substrate pairs can be used in the same assay without cross-reactivity. MNAzyme uses Mg²⁺ as the divalent cation, and has optimal activity at 50°C. Among the three reported DNA-responsive bipartite DNAzymes, we chose to work with the MNAzyme, since it has the highest catalytic rate.^[9,24,25,29]

The genetic sensing assay is implemented as a simple two-step system (Figure 1-C). In the first "amplification" step, a buffered mix of MNAzymes and Linkers is added to the sample solution and incubated at 50°C for 1 hour. If genetic targets are present, they bind and activate the MNAzymes. Each active MNAzyme in turn catalyzes cleavage of multiple Linkers, which effectively translates into signal amplification. In the second "detection" step, a mix of GNP-A and GNP-B is added to the sample solution. In the absence of genetic target intact Linkers interact with the GNPs to form aggregate network, turning solution purple. In contrast, presence of genetic target results in Linker degradation. GNP-A and GNP-B do not get cross-linked, and solution remains red. For readout we use the change in solution color by spotting 3 μ L of solution onto thin layer chromatography (TLC) plates.^[31] TLC plates intensify the color differences allowing for a more sensitive visual detection, and can be stored for later viewing. A measurement of the UV-vis spectroscopic shift of peak absorbance is used for confirmation.^[35]

One critical parameter to optimize for the assay is the amount of Linker strand to use. For a given GNP concentration there is a minimal Linker concentration able to crosslink the GNPs sufficiently to cause visual aggregation. With such a Linker/GNP ratio, even if a small proportion of Linker strands get cleaved by the MNAzymes, this should have an effect on GNP crosslinking state and color of the solution. Therefore, minimizing the Linker concentration effectively maximizes assay sensitivity. Optimization results are presented in Figure 2-A. Assay setup matched the experimental condition of the MNAzyme₁ assay, but lacked the genetic target to prevent Linker₁ degradation. As the amount of Linker₁ is increased from 0 to 100 nM, there is a sigmoidal shift in the peak absorbance, λ_{Peak} , from 527 nm to 546 nm, indicating increase in GNP aggregate size. The TLC spot color follows a similar, but steeper sigmoidal trend. The spots appear red up to 20 nM, then gradually shift to dark purple-black at 50 nM, and remain unchanged for higher concentrations. Based on both color change and peak shift we chose 40 nM as the optimal Linker₁ concentration.

Next, we carried out the MNAzyme assay outlined in Figure 1 to detect AF-1 as the DNA target. As determined previously, the

Linker at 40 nM was used, and we assayed AF-1 from 0 pM to 1 nM (Figure 2-B). Similar to Figure 2-A, spectroscopic data demonstrates a sigmoidal, but decreasing trend in λ_{peak} . Peak absorbance shifts from 533 nm for the sample without AF-1 to 523 nm for 1 nM of target. The color of TLC spots follows similar trend, remaining purple from 0 pM to 25 pM AF-1, then gradually shifting to reach red at 500 pM and 1000 pM. Both spectroscopic and spot color data establish the limit of detection of this assay at 50 pM. This sensitivity is slightly lower than 5 pM reported for the fluorescence readout.^[9]

Interestingly, if the Linker is chosen as the genetic detection target instead of AF-1, the experiment of Figure 2-A matches the original MNAzyme-free direct DNA detection assay reported by Mirkin group (we confirmed that the 50°C, 1 hour incubation step does not affect assay results, Figure S1).^[31] Based on Figure 2-A, our implementation of this assay has detection sensitivity of ~30 nM. Therefore, inclusion of MNAzyme enzymatic signal amplification step increases detection sensitivity by a factor of 600.

We further investigated whether MNAzyme colorimetric assay can detect multiple targets in parallel (Figure 1-D). We chose 4 additional targets corresponding to genetic sequences for gonorrhea (*Gon*)^[36] and syphilis (*Syph*)^[37] bacteria, malaria parasite (*Mal*)^[38] and hepatitis B virus (*HBV*)^[39]. These infectious diseases are prevalent in both the developed and developing world, and can cause severe health problems and death in patients that are not properly diagnosed and treated. To detect these targets, 4 new MNAzyme-Linker-GNP sets were developed, each specific for one of the targets. For example, MNAzyme_{Mal} had substrate arms specific for Linker_{Mal}, and sensor arms complementary to *Mal* target, but not other Linker sequences or targets. Linker_{Mal}, in turn, could aggregate GNP-A_{Mal} and GNP-B_{Mal}, but not other GNP pairs. Another modification introduced was lyophilization of assay components. In this form reagents remain functionally stable during storage and transport, making them well suited for POC testing.^[40]

In the first step of the assay, solution containing genetic targets is added to a reaction tube containing a mixture of 5 sets of MNAzyme-Linker pairs, and incubated at 50°C for 1 hour. Assuming AF-1 and *Gon* targets are present, they activate MNAzyme_{AF-1} and MNAzyme_{Gon}, which in turn degrade Linker_{AF-1} and Linker_{Gon}. The other 3 Linkers remain intact. In the second step, 10 μ L of the MNAzyme-Linker-target solution is transferred to 5 detection tubes, each containing target-specific GNP pair. Any Linkers that remain un-cleaved aggregate the corresponding GNP pairs, turning solution purple. In contrast, Linkers cleaved by target-activated MNAzymes are not able to cross-link associated GNP pairs, and solution remains red. In the example above, only mixtures in AF-1 and *Gon*-specific detection tubes would remain red. Following this protocol we successfully detected all 5 targets simultaneously (Figure 2-C). In addition, the assay with lyophilized components was used to correctly identify AF-1, *Mal* and *HBV* sequences in sample mixture contained 3 out of 5 targets.

In summary, MNAzyme-GNP assay provides a simple and fast colorimetric scheme for detection of genetic targets of bacterial, viral and fungal origins with 50 pM sensitivity without the need for purification and separation steps. Results can be archived when spotted on TLC plates. To our knowledge, this is the first report of combining the multi-component DNA-responsive DNAzyme with gold nanoparticle colorimetric platform. Assay can detect multiple genetic sequences in parallel and is easily translatable to new nucleic acid targets. Color-based readout that does not require any complex equipment, and uses stable and cost-effective reagents make this experimental approach particularly suitable for POC

testing. Furthermore, it is difficult to identify specific requirements for analytical sensitivity of a pathogen as this may vary based on the treatment strategy and whether one analyzing a single or co-infection. For more sensitive analysis, this POC system may require further tuning. Improved sensitivity could potentially be achieved by modifying the nanoparticle size and chemistry, varying the length of MNAzyme/Linker or target sequences,^[41] or by combining the fast catalytic rate of MNAzyme and colorimetric plasmonic readout with the sensitive protein-free autocatalytic DNAzyme approach demonstrated by the Willner group.^[23,42] Furthermore, in a complete point-of-care system, we will likely need to include extra components that can extract the genetic targets of interest. Such technologies have been developed for isolating targets from blood and stool samples.^[43-45] Finally, synthetic genetic targets are commonly used as first step in the assessment of new POC devices. In the next step, further evaluation using clinical samples is required to assess clinical specificity and sensitivity prior to real-world use.

Experimental Section

Experimental details and supplementary figures can be found in the supplement.

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Plasmonic Biosensor

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A Plasmonic DNAzyme Strategy for
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DNAzyme signal amplification is combined with surface plasmon properties of gold nanoparticles to achieve simple and sensitive colorimetric detection of genetic targets. The assay has 50 pM sensitivity without the need for purification steps, can detect multiple targets in parallel, and is easily adaptable to new targets. This technology is capable of rapid detection of genetic targets for gonorrhea, syphilis, malaria, and hepatitis B infections.