VIP DNA Detection

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Ultrasensitive and Selective Colorimetric DNA Detection by Nicking Endonuclease Assisted Nanoparticle Amplification**

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The ability to sense and detect ultralow concentrations of specific DNA sequences by using simple and inexpensive assays is important in clinical diagnostics, mutation detection, and biodefense applications.[1] Conventional methods that use radioactive [^{32}P]-labeled nucleic acid probes or the polymerase chain reaction (PCR) coupled with molecular fluorophore assays offer high sensitivity of detection, but they suffer from several drawbacks that include complex handling procedures, easy contamination, high cost, and lack of portability.[2] In contrast, metal-nanoparticle-based homogeneous colorimetric detection of oligonucleotides holds great promise for low-cost, low-volume, and rapid readout of a target DNA sequence.[3] Despite these attractions, a number of notable challenges associated with this detection system still exist, such as relatively low sensitivity (ca. 10 nM) and the need for stringent control over melting temperatures for the detection of a single-base mismatch in DNA. In addition, this system is generally limited to the detection of short single-stranded oligonucleotides. Herein, we describe homogeneous colorimetric DNA detection by a novel nicking endonuclease-assisted nanoparticle amplification (NEANA) process that is capable of recognizing long single-stranded oligonucleotides with single-base mismatch selectivity and a 10^3-fold improvement in amplification (ca. 10 pM).

A three-component sandwich assay format that includes a target DNA and two sets of oligonucleotide-modified nanoparticle probes is typically used in conventional homogenous nanoparticle-based colorimetric DNA detection. The target DNA also serves as a linker strand that triggers particle aggregation and a concomitant color change. Thus, the colorimetric detection limit is directly associated with the minimum number of the linkers required to initiate particle aggregation that can be visualized with the naked eye. At low linker concentrations (ca. 10 nM), aggregation of 14 nm nanoparticles does not exhibit sharp colorimetric melting transitions (see the Supporting Information). Larger particle probes and reduced oligonucleotide surface coverages can improve assay sensitivities, but sedimentation becomes more dominant with increase in the particle size.[3f]

To increase the sensitivity of homogeneous nanoparticle-based assays, we have developed a detection system that contains an additional oligonucleotide strand as the linker, and a nicking endonuclease (NEase; Figure 1).[4] Unlike

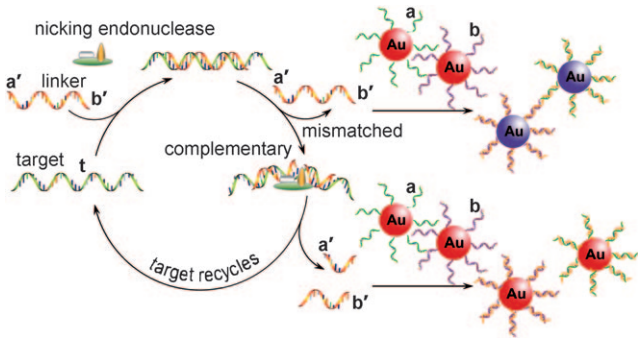

Figure 1. Nicking endonuclease assisted nanoparticle amplification (NEANA) for target DNA detection.

restriction enzymes, the NEase recognizes specific nucleotide sequences in double-stranded DNA and cleaves only one of the two strands.[5] In our detection system, the NEase is specifically designed to cleave only the linker strand. After nicking, the fragments of the linker strand spontaneously dissociate from the target DNA at an elevated temperature. Subsequently, another linker strand hybridizes to the target to continue the strand-scission cycle, which results in the cleavage of a large molar excess of linkers. Upon completion of the strand-scission cycle, two sets of different oligonucleotide-modified gold nanoparticles with sequences complementary to that of the linker strand are added to the solution to detect the presence of a target DNA. If the linker DNA is noncomplementary to the target DNA, particle aggregation will occur.

To test the feasibility of our system for DNA detection, we first prepared various concentrations (1 pmol, 0.1 pmol, 0.01 pmol, 1 fmol, and 0.5 fmol) of a 24-base single-stranded target DNA (**t1**) that contained the recognition sequence (-GATCC-) of a NEase (Nt.AlwI). A single-stranded linker DNA (**a′b′**; 1 pmol), with a sequence complementary to that of the target DNA was then added to each of these solutions. Upon incubation at 58 °C for 5 minutes, the NEase (10 units) was added to the solutions. After standing for 2 hours, the

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resulting mixtures were heated at 80 °C for 20 minutes to deactivate the nicking enzyme, followed by the addition of a mixture that contained 40 μL of two different 15-base oligonucleotide-modified gold nanoparticle probes (**a** and **b**). As anticipated, no apparent particle aggregation was detected for all solution samples even after prolonged (> 30 days) storage (Figure 2), which indicated the cleavage of the linker strand by the NEase through the repeated target-templated strand-scission cycles. In contrast, precipitation of the aggregates was observed in the absence of the target strand in solution (Figure 2).

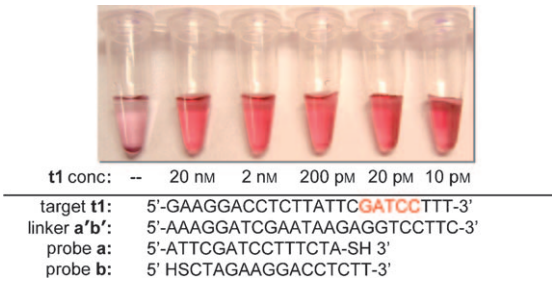

Figure 2. Photograph showing colorimetric responses of a NEANA detection system that comprises a linker strand **a'b'**, Nt.AlwI, and particle probes **a** and **b** in the presence of various concentrations of a single-stranded target DNA (**t1**). The labeled concentrations (20 nM, 2 nM, 200 pM, 20 pM, and 10 pM) are calculated final target concentrations in solutions. The NEase recognition site of the target is highlighted in red.

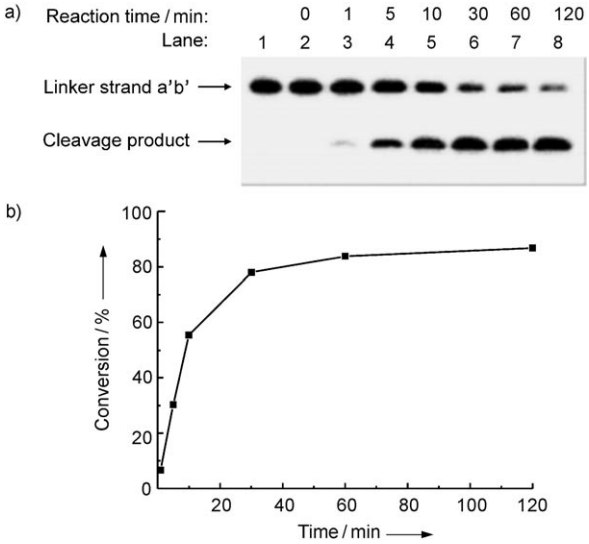

Figure 3. Oligonucleotide cleavage assays by NEase (Nt.AlwI; 10 units). a) Autoradiogram of polyacrylamide gel separated products obtained from a cleavage experiment over time (0–120 min). Lane 1 (control): linker strand (**a'b'**; 1 pmol) with no target DNA added. Lanes 2–8: linker strand in the presence of a target DNA (**t1**; 100 pM). b) Plot of data obtained from counting the radioactivity in the product band in the gel. The data is expressed as the fraction of total radioactivity isolated as the 5′-labeled product.

To validate the sequence-specificity of the detection system, we prepared several different target DNA strands that contained a single-base mismatch at the NEase recognition site. We found that a single mismatch is sufficient to inhibit the cleavage of the linker strand. In all cases, the mismatched target strands were quickly detected, as confirmed by the formation of particle aggregates (Figure 4a). The sequence-specificity of NEANA is attributed to the requirement of full complementarity between the target DNA and the linker strand at the NEase recognition site and sufficient complementarity beyond the NEase recognition site to allow efficient hybridization.

In addition to sensitivity of detection and sequence specificity, the NEANA approach also demonstrates remarkable generality for target DNA with varied base numbers. One of the major challenges for conventional nanoparticle-based three-component sandwich assay lies in the detection of long single-stranded DNA sequences. This technique generally requires an increase in the length of the particle probes as the base number of the target strand increases. For relatively long-stranded oligonucleotide-modified nanoparticles, colloidal instability and cross-hybridization interference between parts of the particle probes become significant problems. In contrast, a fixed set of particle probes and a linker strand in our system can be readily extended to detection of long DNA strands that contain NEase recognition sites. As shown in

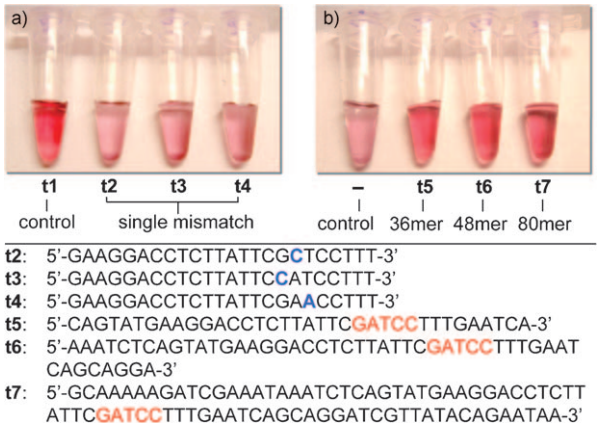

Figure 4. Photographs showing colorimetric detection of various target DNA strands. a) Oligonucleotides (**t2–t4**) with a single mismatch at different positions of the NEase recognition site and b) oligonucleotides (**t5–t7**) of different length containing NEase recognition sites. The mismatch positions and NEase recognition sites are highlighted in blue and red, respectively.

Figure 4b, colorimetric detection of a range of oligomers (36-mer, 48-mer, and 80-mer) was achieved within several hours without the need to modify the particle probes.

In conclusion, we have demonstrated homogeneous, colorimetric DNA detection through the use of NEANA, which utilizes a combination of particle probes, a linker strand, and a NEase. This system offers handling convenience and ultrahigh detection sensitivity and selectivity, while providing additional detection versatility for long stranded DNA sequences. The unoptimized system provides a colorimetric detection limit of 0.5 fmol within several hours for single-stranded oligonucleotides that contain NEase recognition sites. Although only a limited number (ca. 10) of commercially available nicking endonucleases are viable for NEANA applications, this approach may prove particularly useful for rapid, simple, and highly sensitive single-nucleotide polymorphism detection, and point mutation identification. Importantly, the approach can be readily coupled to conventional analytical techniques[6] and applied to a rich variety of different particle probes, which include magnetic, semiconducting, and lanthanide-doped nanoparticles.[7] Upon modification, the approach presented herein could also be extended to detect a broad range of other types of targets including proteins, aptamer-binding small molecules, and metal ions at ultralow concentrations.[8]

Experimental Section

Oligonucleotide sequence detection by NEANA: In a typical procedure, a solution containing 10×NEBuffer 2 (1 μL; New England Biolabs), of a linker strand (1 μL **a′b′**: 5′-AAAGGATCGAATAAGAGGTCCTTC-3′; 1 pmol), and a specified amount of target DNA (1 pmol, 0.1 pmol, 0.01 pmol, 1 fmol, and 0.5 fmol, respectively) were added to a PCR tube. Upon incubation at 58 °C for 5 min, a NEase (1 μL Nt.AlwI; 10 units) was added to the solution (total volume 10 μL). After standing for 2 h, the resulting mixtures were heated at 80 °C for 20 min to deactivate the nicking enzyme, followed by the addition of a mixture containing two different 15-base oligonucleotide-modified gold nanoparticle probes (40 μL; **a**: 5′-ATTCGATCCTTTCTA-SH 3′ and **b**: 5′ HS-CTAGAAGGACCTCTT-3′; 2 nM each). The colorimetric response of the solution was recorded in 30 min and compared in parallel to a control with no added target DNA. Target oligonucleotide detection was repeated by using the NEANA procedure for at least three times.

Polyacrylamide gel electrophoretic analysis: The linker strand-scission process was monitored by denaturing PAGE with autoradiography. In a typical experiment, a linker oligonucleotide strand (**a′b′**: 1 pmol) was first labeled with [γ-^{32}P] ATP at the 5' end in the presence of T4 polynucleotide kinase (T4 PNK), followed by purification by electrophoresis through a denaturing 20% polyacrylamide gel. The slice of band containing the [^{32}P]-labeled linker oligonucleotide strand was cut out of the gel and eluted by soaking in tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl; 10 mM, pH 7) for 3 h, followed by purification by gel filtration chromatography (NAP-25, GE Healthcare) eluting with ultrapure water.[9] The purified [^{32}P]-labeled linker strand (ca. 20 nM) was then added to seven solutions containing a target DNA strand (**t1**: 5′-GAAGGACCTCTTATTCGATCCTTT-3′; 100 pM). Upon incubation at 58 °C for 5 min, a NEase (1 μL Nt.AlwI; 10 units) was added to each of these solutions. Reactions were then stopped at different time intervals (1, 5, 10, 30, 60, 120 min) by the addition of denaturing loading buffer (10 μL, 95% v/v formamide, 18 mM EDTA, 0.025% SDS, 10% w/w xylene FF and bromophenol blue). The reaction products were analyzed by electrophoresis in a 15% polyacrylamide gel. Autoradiographic images were analyzed and quantified using the ImageQuant software.

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