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Enzyme-Based Multi-Component Optical Nanoprobes for Sequence-Specific Detection of DNA Hybridization**

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Gold nanoparticles (AuNPs) coupled with biomolecules have become increasingly important biological nanoprobes owing to the fact that these novel hybrid bio-nanostructures elegantly combine unique physical properties of AuNPs (e.g., localized plasmons) and superior chemical properties of biomolecules (e.g., recognition specificity).^[1–3] Indeed, these nanoprobes have significantly improved sensing performance in a variety of optical and electronic biosensing systems, and have found wide applications in areas including molecular diagnostics, environmental monitoring, and antiterrorism.^[4–10] For example, Willner and co-workers reported the use of AuNPs as nanowires for efficient bioelectronic transduction of redox enzymes.^[11] Mirkin and co-workers developed a series of novel detection strategies for ultrasensitive detection of DNA and proteins by exploiting plasmonic properties of AuNPs.^[12–16] More recently, we reported an AuNP-based chronocoulometric DNA sensor (CDS), enabling sequence-specific detection of femtomolar DNA targets by using cost-effective electrochemical detectors.^[17]

It is well-known that AuNPs possess a very high surface-to-volume ratio. This offers an opportunity to design multifunctional nanoprobes by attaching multiple kinds of biomolecules at the surface of a single particle. An elegant example is the ultrasensitive “bio-barcode” nanoprobe designed for the detection of protein biomarkers.^[18–20] These nanoprobes are AuNPs assembled with both target-specific antibodies and DNA “barcodes” that can be chemically released and ampli-

fied via polymerase chain reaction (PCR). This bio-barcode strategy effectively translates otherwise difficult amplified detection of protein into convenient PCR amplification of barcode DNA strands. In spite of its elegance, we find that the functions of recognition (antibodies) and amplification (DNA) are separated in the bio-barcode nanoprobe. This inspires us to design “molecular machine”-like nanoprobes that can integrate multiple functions.

In this work, we design a multi-component AuNP-based nanoprobe for DNA detection that integrates DNA recognition (DNA detection probe), signal amplification (enzyme), and non-specific blocking. We reason that this multi-component nanoprobe possesses high hybridization specificity as well as other inherited advantages of DNA-AuNPs conjugates, while it obviates additional (separated) signal amplification steps.

We prepared the multi-component nanoprobe by co-assembling thiolated oligonucleotide (detection probe), horseradish peroxidase (HRP, signal amplification), and bovine serum albumin (BSA, non-specific blocker) at the surface of 15 nm AuNPs. HRP was used for signal amplification, which could efficiently catalyze > 10000 substrate turnovers. We employed a standard spectrophotometric assay to determine the activities of free and bound HRP molecules, which were approximately 5 units (per nmol protein) in solution and 35 units (per nmol AuNPs) at the surface of AuNPs. Assuming free and bound HRP molecules possessed similar activities, we estimated that each nanoparticle carried seven HRP molecules. As a rough comparison, one 15 nm nanoparticle could, as a maximum, carry around 19 densely packed HRP molecules (ca. 6 nm in diameter) at its surface. This suggested that our current protocol for nanoprobe preparation led to a sub-monolayer of HRP, leaving sufficient vacant space for the subsequent assembly of thiolated DNA detection probe and BSA.

A “sandwich-type” detection strategy was employed in this work (Fig. 1), which involved a biotinylated capture probe and a thiolated detection probe (Table 1) that flanked the DNA target sequence. Of note, biotinylated capture probes were loaded on avidin-modified magnetic microparticles (MMPs) while thiolated detection probes were assembled on AuNPs via Au–S bonds. In the presence of target DNA, the capture probe brought the target DNA, along with the detection probe, to the proximity of MMPs, and this complex could be magnetically separated for subsequent optical detection. In

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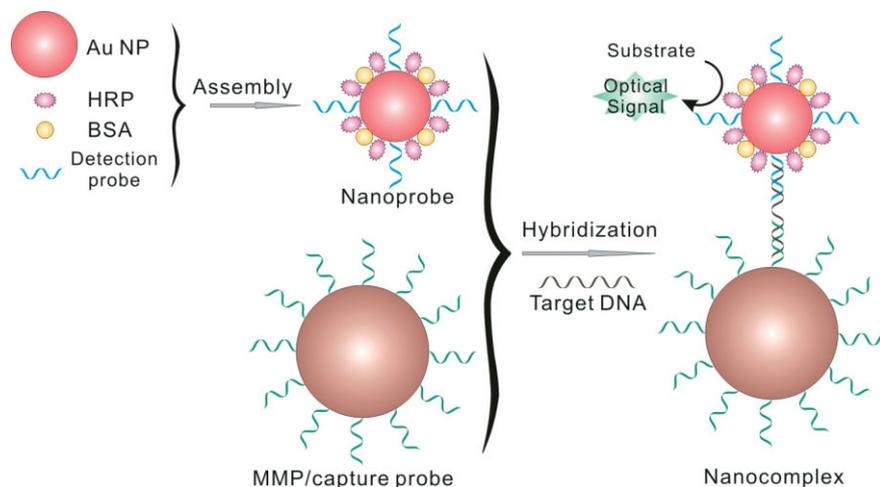


Figure 1. The multi-component nanoprobe-based “sandwich-type” DNA detection strategy.

Table 1. Sequences of oligonucleotides employed in this work [a].

Name	Sequence
Capture probe	5' GAAACCTATGTATGCTCTTTTTTTTT - (Biotin) 3'
Detection probe	5' SH - (CH ₂) ₆ - TTTTTTTTTTTTTTTGTATGAATTATAATCAAA 3'
Target DNA	5' GAGCATACATAGGGTTTCTCTGGTTTCTTTGATTATAATTCATAC 3'
1-mismatched DNA	5' GAGCATACATAGGGTTTCTCTGGTTTCTTTGATTATNATTCATAC 3' ("N" stands for T, C, or G)
Non-cognate DNA	5' ACACGCTTGGTAGACTTTTTTTTTTAGCATCGATAACGTT 3'

[a] The sequence of the target DNA is a segment from the wild-type BRCA-1 gene.

contrast, in the absence of target DNA, the “sandwich” complex could not be formed, thus the detection probe was not attached to the MMPs during the magnetic separation step. As a result, only in the presence of target DNA, HRP confined at the surface of AuNPs could catalytically oxidize the substrate and generate optical signals that reflected the quantity of target DNA. In addition, it is worthwhile to point out that the use of BSA as a non-specific blocker was critical for the suppression of non-specific signals. Indeed, we found that BSA-free nanoprobe showed strong adsorption toward MMPs, resulting in high background (data not shown).

In a typical colorimetric detection of DNA hybridization, we first mixed capture probe-loaded MMPs with the target DNA sample in the hybridization buffer. The sequence of the target DNA is a segment from the breast cancer-associated BRCA-1 gene.^[21] After this hybridization step, MMPs were magnetically collected, extensively rinsed with the washing buffer, and further hybridized with the signaling nanoprobe. Subsequently, the resulting complex was again magnetically collected and reacted with the substrate solution including a chromogenic reagent (2, 2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (ABTS) that showed blue color in the presence of HRP catalysis. As expected, we observed the appearance of the characteristic green color only in the presence of target DNA, and the color intensity was a sensi-

tive function of the target DNA concentration (Fig. 1-S, inset). Importantly, we could easily distinguish the color contrast between the 100 pM target DNA sample and the blank control even with the naked eye.

Instrument-based analysis offered increased sensitivity for nanoprobe-based DNA detection. For example, we could detect as few as 25 pM target DNA by using a microplate reader (Fig. 1-S, inset). The extinction intensities at 405 nm also provided a way to quantitatively measure target DNA concentrations. A series of diluted DNA targets were analyzed as described above, and then we plotted the absorbance difference with the DNA concentration, resulting in a sigmoid working curve in the concentration range of 25 pM–1.0 nM (Fig. 2). Also of note, the sensitivity of the nanoprobe-based assay could be further improved by using fluorescent detection. In our preliminary experiments employing a fluorescent substrate (3-(4-hydroxy) phenyl propionic acid, HPPA) for HRP, we obtained a detection limit of around 1 pM, exhibiting an improvement of over order of magnitude compared to the corresponding colorimetric assay.

We then evaluated the specificity of this nanoprobe-based assay and found that a large excess of non-cognate DNA (1 nM) did not produce significant absorption variation (Fig. 1-S, inset). More importantly, the nanoprobe could effectively discriminate even a single-base mismatch. As shown in Figure 3a, the one-base mismatched targets produced much smaller signals as compared to the fully complementary target. These assays clearly reflected the high specificity of our nanoprobe-based assay.

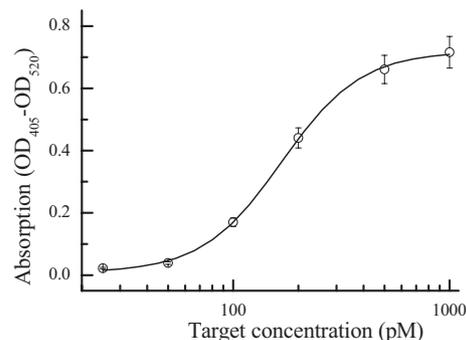


Figure 2. A dose-response curve for colorimetric detection of target DNA (BRCA-1 gene segment). Data were collected from at least three independent sets of experiments.

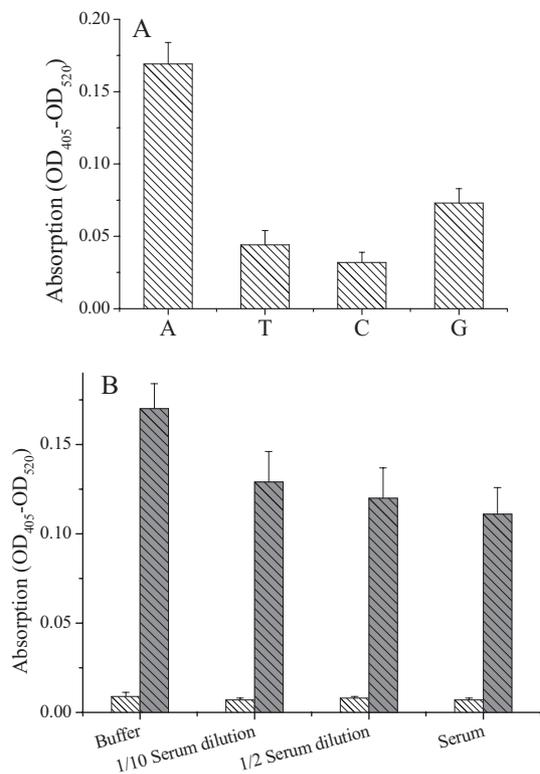


Figure 3. a) Nanoprobe-based colorimetric detection for single-base mismatches. The nanoprobe was challenged with 100 pM DNA target (A) mutated to either T, C, or G. b) Detection of 100 pM DNA target (gray bars) and 100 nM non-cognate DNA (white bars) in buffer, diluted (1/10 and 1/2) and undiluted sera. Data were collected from at least three independent sets of experiments.

In order to evaluate the applicability of using nanoprobe in real-sample detection, we tested nanoprobes for their ability to detect DNA targets in the presence of biological fluids. As demonstrated in Figure 3, while the signals obtained in sera (both diluted and undiluted) were slightly suppressed compared to those obtained in pure buffer, we could still easily differentiate the target (100 pM) from a large excess of non-cognate DNA (10 nM). This clearly showed that our nanoprobe could selectively identify DNA targets even in complexes such as undiluted serum.

The nanoprobe-based DNA assay demonstrated several advantages. First, the nanoprobe integrated both DNA detection probe and HRP at the surface of AuNPs, thus obviating the use of additional biotin-avidin bridges involved in conventional enzymatic bioassays. Second, one AuNP could accumulate 7 HRP molecules owing to its large surface, thus one hybridization event could bring multiple HRP molecules, leading to large signal amplification. Moreover, it has been well-documented that highly curved surfaces of nanoparticles improve the stability or enhance the activity of enzymes,^[22–24] which might also contribute to the observed high sensitivity of the nanoprobe-based DNA assay. Third, this assay protocol is simple, convenient and cost-effective. We demonstrated that even visual detection with naked eyes could detect as few as

100 pM target DNA, which was comparable to or even better than many previously reported instrument-based DNA analysis.^[25–28] Moreover, a detection limit in the low pM range was obtained by using a fluorescent approach. Also of note, there are several straightforward approaches to further improve this sensitivity in order to meet high-end applications, which include the use of large-size AuNPs that should carry more HRP molecules and bring about larger amplification; automated operation may lead to better reproducibility; and the use of electrochemical detection for HRP-catalyzed reactions may lead to higher sensitivity.^[22,29] Fourth, the described nanoprobe-based detection strategy could be easily generalized to detection of various analytes such as proteins, small molecules, or even heavy metal ions by replacing the signaling DNA with DNA or RNA aptamers.^[10,30,31]

In summary, we have demonstrated a novel multi-component nanoprobe-based DNA detection strategy. This nanoprobe was prepared by assembling AuNPs with DNA detection probe, HRP, and BSA, where the DNA detection probe was used to construct the sandwich complex, HRP was for enzymatic catalysis, and BSA was a non-specific blocker. By using this novel method, we could conveniently detect as few as 100 pM target DNA with the naked eye, and this sensitivity could be significantly improved by instrument-based assays (25 pM with absorption and 1 pM with fluorescence). Given the simplicity and high sensitivity of this method, we propose that it might be a promising approach to perform DNA-based diagnostics where resources are limited, such as small clinics in developing countries or field detection.

Experimental

Materials: Oligonucleotides were synthesized by Shanghai Sangon Biotechnology Co. HRP was purchased from Sino-American Biotechnology Co. 2,2'-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (ABTS), 3-(4-hydroxy) phenyl propionic acid (HPPA), BSA and poly (ethylene glycol) (PEG, Avg. M_w 3350) were purchased from Sigma Chemical Co. Streptavidin-coated MagnetSphere paramagnetic particles (ca. 1.0 μ m diameter, 1 mg mL⁻¹) were obtained from Promega Corporation. All other reagents were of analytical grade.

Solutions: Washing buffer (10 mM phosphate sodium buffer solution, pH 7.4, 100 mM NaCl), blocking solution (10 mM phosphate sodium buffer solution, pH 7.4, 100 mM NaCl, 5 % PEG, 2 % BSA), stock solution (10 mM phosphate sodium buffer solution at pH 7.4, 0.1 M NaCl, 1 % BSA, 0.01 % thiomersal), hybridization buffer (750 mM NaCl, 150 mM sodium citrate, pH 7.4), substrate solution for HRP catalyzed reactions (0.05 M phosphate-citrate buffer, pH 5.0, 0.01 % ABTS, 0.0075 % H₂O₂, freshly prepared before use), substrate solution for fluorescent assays (0.1 M Tris-HCl buffer, pH 8.5, 0.05 % HPPA, 0.0075 % H₂O₂, freshly prepared before use). All solutions were prepared using Milli-Q water.

Preparation of Multifunctional Nanoprobe: AuNPs of 15 nm were prepared as follows [32]: 3.5 mL of 1 % trisodium citrate solution was added to a boiling, rapidly stirred solution of HAuCl₄. The solution was kept boiling and stirred for 20 min. After being cooled to room temperature, the prepared Au NPs were stored at 4 °C.

The protocol for preparation of multi-component nanoprobe was as follows. 100 μ L of HRP solution (1 %, w/v) was added to 5 mL of Au NPs, the pH value of which was adjusted to 9.0 by adding diluted

NaOH solution. The mixed solution was incubated for 30 min at 37 °C with gentle shaking, and was concentrated to 1 mL (about 10 nM) by centrifuging (13000 rpm, 30 min, 4 °C). Subsequently, thiolated DNA detection probe was added to the solution to a final concentration of 1.5 μM. After 16-h incubation at room temperature, the concentration of NaCl in the solution was brought to 0.1 M during 24 h by stepwise addition of 1 M NaCl/10 mM phosphate sodium buffer solution (pH 7.4). Next, 100 μL of 10 % BSA solution was added to passivate the Au NPs for 30 min. The mixture was centrifuged (13000 rpm, 30 min, 4 °C, supernatant decanted) and rinsed with 1 mL of 10 mM PBS buffer (pH 7.4). The centrifuging/rinsing procedure was repeated three times to remove the unbound HRP and DNA. The final deposition was suspended in 500 μL of stock solution and stored at 4 °C for further use.

Preparation of Capture Probe Coated Magnetic Microparticles (MMPs): The MMPs modified with streptavidin were first washed with hybridization buffer twice. Then, a solution of biotinylated capture probe DNA was added to the collected MMPs (at a ratio of 1.25 nmol probe 1 to 1 mg MMPs). The mixture was incubated for 20 min at room temperature with gentle shaking, washed twice with washing buffer and suspended in blocking solution. After a 30 min incubation, the mixture was washed again and stored in stock solution at 4 °C for further use.

Detection of DNA Hybridization: In a typical experiment, 50 μL capture probe coated MMPs were added to a 1.5 mL microcentrifuge tube. The MMPs were washed with hybridization buffer, and magnetically collected. Then 1 mL of hybridization buffer containing target DNA of various concentrations was added and incubated with the MMPs for 1 h at 37 °C, with gentle shaking. Next, the complexes were magnetically collected and rinsed with washing buffer. Then 50 μL of nanoprobe working solution (37 μL of hybridization buffer, 2.5 μL of 10 % BSA, 0.5 μL of 100 μM non-cognate DNA, 10 μL of nanoprobe) was added and the mixture was incubated for another 1 h at 37 °C with gentle mixing. Subsequently, the complexes were magnetically collected again and rinsed with washing buffer. This washing procedure was repeated for 5 times to remove unbound nanoprobe.

Absorbance Measurements: Absorbance values were obtained with a Tecan GENios microplate reader at room temperature. The measurement wavelength was 405 nm and the reference wavelength 520 nm. 100 μL of substrate solution was added to react with the resulting complexes for 30 min at 25 °C with gentle shaking. After the magnetic separation, the absorbance (OD₄₀₅ – OD₅₂₀) of the supernatant was measured with the microplate reader.

Fluorescence Measurements: Fluorescence intensity was obtained with a fluorescence spectrophotometer at room temperature. 100 μL of substrate solution was added to react with the resulting complexes for 30 min at 25 °C with gentle shaking. After the magnetic separation, the intensity of fluorescence of the supernatant was measured at an excitation wavelength of 320 nm and emission wavelength of 330–600 nm.

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