Hairpin DNA Switch for Ultrasensitive Spectrophotometric Detection of DNA Hybridization Based on Gold Nanoparticles and Enzyme Signal Amplification

ARTICLE *in* ANALYTICAL CHEMISTRY · AUGUST 2010

Impact Factor: 5.64 · DOI: 10.1021/ac1006238 · Source: PubMed

6 AUTHORS, INCLUDING:

Youyu Zhang
Hunan University
118 PUBLICATIONS **1,924** CITATIONS
SEE PROFILE

Jun Wang
Nanjing University of Posts and Telecomm…
355 PUBLICATIONS **11,624** CITATIONS
SEE PROFILE

Hong-Xing Wu
Health Canada
218 PUBLICATIONS **15,998** CITATIONS
SEE PROFILE

Yuehe Lin
Washington State University
363 PUBLICATIONS **21,408** CITATIONS
SEE PROFILE

Anal. Chem. **2010,** *82,* 6440–6446

Hairpin DNA Switch for Ultrasensitive Spectrophotometric Detection of DNA Hybridization Based on Gold Nanoparticles and Enzyme Signal Amplification

Youyu Zhang,[†,‡] Zhiwen Tang,[‡] Jun Wang,[‡] Hong Wu,[‡] Aihui Maham,[‡] and Yuehe Lin*[,‡]

Key Laboratory of Chemical Biology and Traditional Chinese Medicine Research (Ministry of Education), College of Chemistry and Chemical Engineering, Hunan Normal University, Changsha 410081, People's Republic of China, and Pacific Northwest National Laboratory, Richland, Washington 99352

A novel DNA detection platform based on a hairpin DNA switch, nanoparticles, and enzyme signal amplification for ultrasensitive detection of DNA hybridization has been developed in this work. In this DNA assay, a "stem−loop" DNA probe dually labeled with a thiol at its 5′ end and a biotin at its 3′ end, respectively, was used. This probe was immobilized on the gold nanoparticles (AuNPs) anchored by a protein, γ-globulin, on a 96-well microplate. In the absence of target DNA, the immobilized probe with the stem−loop structure shields the biotin from being approached by a bulky horseradish peroxidase linked streptavidin (streptavidin−HRP) conjugate due to the steric hindrance. However, in the presence of target DNA, the hybridization between the hairpin DNA probe and the target DNA causes significant conformational change of the probe, which forces biotin away from the surface of AuNPs. As a result, the biotin becomes accessible by the streptavidin−HRP, and the target hybridization event can be sensitively detected via the HRP catalyzed substrate 3,3′,5,5′-tetramethylbenzidine using a spectrophometric method. Some experimental parameters governing the performance of the assay have been optimized. At optimal conditions, this DNA assay can detect DNA at the concentration of femtomolar level by means of a signal amplification strategy based on the combination of enzymes and nanoparticles. This approach also has shown excellent specificity to distinguish single-base mismatches of DNA targets because of the intrinsic high selectivity of the hairpin DNA probe.

It is important to detect a specific DNA sequence because of its broad applications in clinical diagnosis, genetics therapy, and a variety of biomedical studies.[1−4] In particular, some diseases or cancers, e.g., hepatitis C virus genotypes[1] and leukemia,[5,6] result from genetic disorders, and some specific genes are important biomarkers for diagnosing these diseases. Thus, selective, sensitive, and rapid methods for detecting specific DNA sequences are highly desirable.

Generally, detecting a DNA sequence is based on DNA hybridization.[7−9] To read out the DNA hybridization events, the common method is to label the target DNA or the detection DNA probe with radioactive species,[10] fluorescent dyes,[11,12] or electroactive markers.[13] Because the concentration of DNA is often low in biological samples, an ultrasensitive detection method for specific DNA sequencing is essential. Therefore, some approaches for ultrasensitive detection of a DNA sequence based on signal amplification have been developed.[14] For example, quantitative real-time polymerase chain reaction (qPCR)[15] and branched DNA[9] have been developed for ultrasensitive detection of DNA in biological samples. The qPCR method takes advantage of multiple-step amplification with DNA polymerase to increase the target amounts.[16] However, the PCR method, which is relatively sophisticated and time-consuming, could introduce bias during the cycle

* To whom correspondence should be addressed. E-mail: yuehe.lin@pnl.gov. Phone: 01-509-371-6241.

† Hunan Normal University.

‡ Pacific Northwest National Laboratory.

(1) Riccardi, C. d. S.; Kranz, C.; Kowalik, J.; Yamanaka, H.; Mizaikoff, B.; Josowicz, M. *Anal. Chem.* **2007**, *80*, 237–245.

(2) (a) Kleparnik, K.; BocÌŒek, P. *Chem. Rev.* **2007**, *107*, 5279–5317. (b) Liu, G.; Lin, Y. *J. Am. Chem. Soc.* **2007**, *129*, 10394–10401.

(3) Graziewicz, M. A.; Longley, M. J.; Copeland, W. C. *Chem. Rev.* **2005**, *106*, 383–405.

(4) Wang, Y.; Vaidya, B.; Farquar, H. D.; Stryjewski, W.; Hammer, R. P.; McCarley, R. L.; Soper, S. A.; Cheng, Y. W.; Barany, F. *Anal. Chem.* **2003**, *75*, 1130–1140.

(5) Balcome, S.; Park, S.; Quirk Dorr, D. R.; Hafner, L.; Phillips, L.; Tretyakova, N. *Chem. Res. Toxicol.* **2004**, *17*, 950–962.

(6) Bao, P.; Frutos, A. G.; Greef, C.; Lahiri, J.; Muller, U.; Peterson, T. C.; Warden, L.; Xie, X. *Anal. Chem.* **2002**, *74*, 1792–1797.

(7) Flechsig, G. U.; Peter, J.; Hartwich, G.; Wang, J.; Grundler, P. *Langmuir* **2005**, *21*, 7848–7853.

(8) Liu, G.; Wan, Y.; Gau, V.; Zhang, J.; Wang, L. H.; Song, S. P.; Fan, C. H. *J. Am. Chem. Soc.* **2008**, *130*, 6820–6825.

(9) Lee, A. C.; Dai, Z. Y.; Chen, B. W.; Wu, H.; Wang, J.; Zhang, A. G.; Zhang, L. R.; Lim, T. M.; Lin, Y. H. *Anal. Chem.* **2008**, *80*, 9402–9410.

(10) Carvajal, M. In *Food Contaminants*; Siantar, D. P., Trucksess, M. W., Scott, P. M., Herman, E. M., Eds.; ACS Symposium Series 1001; American Chemical Society: Washington, DC, 2008; pp 13−55.

(11) Rant, U.; Arinaga, K.; Fujita, S.; Yokoyama, N.; Abstreiter, G.; Tornow, M. *Nano Lett.* **2004**, *4*, 2441–2445.

(12) Sanborn, M. E.; Connolly, B. K.; Gurunathan, K.; Levitus, M. *J. Phys. Chem. B* **2007**, *111*, 11064–11074.

(13) Fojta, M.; Kostecka, P.; Trefulka, M. R.; Havran, L.; Palecek, E. *Anal. Chem.* **2007**, *79*, 1022–1029.

(14) Wang, J.; Liu, G. D.; Jan, M. R. *J. Am. Chem. Soc.* **2004**, *126*, 3010–3011.

(15) Bortolin, S.; Christopoulos, T. K.; Verhaegen, M. *Anal. Chem.* **1996**, *68*, 834–840.

(16) Zhou, L. B.; Otulakowski, G.; Lau, C. Y. *Vitam. Coenzymes, Part L* **1997**, *282*, 64–76.

10.1021/ac1006238
Published on Web 07/07/2010

of amplification. The branched DNA method is based on a large amount of enzymes attached onto the branched detection DNA probe for signal amplification.[9] This method is sensitive but involves multisteps for signal amplification; thus, it is also time-consuming. Other methods for ultrasensitive detection of DNA based on nanomaterial amplifications have also been developed recently.[17,18] For example, the detection of a DNA probe modified with a carbon nanotube carrying many enzymes was prepared for sensitive DNA detection.[14] Gold nanoparticles (AuNPs) loaded with a large amount of ferrocene have been developed for sensitive DNA sequencing.[19] However, these methods generally suffer from low specificity. For example, it is difficult for them to differentiate single-nucleotide polymorphism (SNP).

Molecular beacon, which is composed of a hairpin-like DNA stem−loop structure, was first developed by Tyagi and Kramer in 1996[20] and has been widely used for specific DNA detection and other biomedical applications.[21] The biggest advantage of the molecular beacon-based DNA assay is that it has excellent specificity for detecting nucleic acid targets, which is ascribed to the conformational change properties of the recognition event.[22,23] In addition, this method is label-free and straightforward for DNA detection, thus avoiding tedious labeling of target DNA. Generally, fluorescence has been used in the molecular beacon-based assay,[24] which is based on the stem-and-loop structure where several bases at its 5′ and 3′ ends are self-complementary. This brings the fluorophore and the quencher labeled at two ends into close proximity and efficiently quenches fluorescence. In the presence of target DNA, the complementary sequence of the loop part of the molecular beacon binds to the target DNA, leading to the conformation change of the beacon; therefore, the fluorescence of the labeled fluorophores is restored. The intensity of the restored fluorescence can be used as signal to quantify target DNA. Molecular beacon-based bioassays have also been used to monitor the interactions between DNA and proteins, such as DNA cleavage,[25] ligation,[26] and phosphorylation.[27] It has been proven that molecular beacon-based assays give excellent specificity and high stability as compared with linear single-stranded DNA (ssDNA) probes. Situma and co-workers reported that the molecular beacons immobilized on the solid surface presented better specificity than those in bulk solution.[28,29] Recently, electrochemical methods, e.g., using electroactive species such as ferrocene to replace fluorophore, have been developed for molecular beacon-based DNA assay.[30−35] An enzyme-based E-DNA sensor for the sequence-specific electrochemical detection of nucleic acids was reported by Liu et al.[8] This DNA sensor employs a "stem−loop" DNA probe dually labeled with biotin and digoxigenin (DIG) and exhibited the ability to detect DNA targets with excellent differentiation. However, relatively complicated multiple immobilization steps are involved in the sensor fabrication procedure. In general, the molecular beacon-based DNA assay can achieve excellent selectivity but is often limited by the sensitivity for real applications, such as detecting a low concentration of DNA in biological samples.

Recently, nanomaterials have been successfully used to improve the sensitivity of DNA bioassays.[36,37] Among these nanomaterials, AuNPs are particularly attractive for bioassays by virtue of their facile synthesis, large specific surface area, high chemical stability, favorable biocompatibility, good conductivity, and high affinity of binding to amine/thiol-containing molecules.[38] AuNP is an excellent material for immobilizing a molecular beacon probe by taking advantage of its large surface aspect and unique chemical properties. In parallel, enzymes are very efficient catalysts for biochemical reactions and are highly selective for catalyzing specific reactions with high sensitivity.[39] Therefore, enzymes are usually used in a signal amplification-based bioassay. For example, the enzyme-linked immunosorbent assay (ELSIA) is a popular method for bioassays.[40] However, the combination of a molecular beacon with a AuNP and an enzyme for ultrasensitive detection of DNA sequence has never been reported.

In this paper, we developed a selective and ultrasensitive DNA detection platform by the combination of a hairpin DNA switch probe, enzyme, and AuNP. In this novel approach, we designed a hairpin structure DNA probe to improve selectivity and use the enzyme and AuNPs for signal amplification. The results demonstrated that this method can be used for ultrasensitive detection of DNA with the detection limit at the femtomolar level. Therefore, this sensing platform may open up a new avenue for selective and ultrasensitive detection of DNA hybridization.

EXPERIMENTAL SECTION

Reagents and Apparatus. All oligonucleotide probes were obtained from Integrated DNA Technologies (Coralville, IA), and their sequences are shown in Table 1. The stem−loop oligonucleotide (probe) is modified with the −HS group at the 5′ end and

(17) Zheng, G. F.; Daniel, W. L.; Mirkin, C. A. *J. Am. Chem. Soc.* **2008**, *130*, 9644–9645.
(18) (a) Liu, G.; Wang, J.; Wu, H.; Lin, Y. Y.; Lin, Y. H. *Electroanalysis* **2007**, *19*, 777–785. (b) Liu, G.; Wang, J.; Lea, S. A.; Lin, Y. *ChemBioChem* **2006**, *7*, 1315–1319.
(19) Wang, J.; Li, J. H.; Baca, A. J.; Hu, J. B.; Zhou, F. M.; Yan, W.; Pang, D. W. *Anal. Chem.* **2003**, *75*, 3941–3945.
(20) Tyagi, S.; Kramer, F. R. *Nat. Biotechnol.* **1996**, *14*, 303–308.
(21) Tang, Z. W.; Wang, K. M.; Tan, W. H.; Li, J.; Liu, L. F.; Guo, Q. P.; Meng, X. X.; Ma, C. B.; Huang, S. S. *Nucleic Acids Res.* **2003**, *31*, e184.
(22) Tsourkas, A.; Behlke, M. A.; Rose, S. D.; Bao, G. *Nucleic Acids Res.* **2003**, *31*, 1319–1330.
(23) Bonnet, G.; Tyagi, S.; Libchaber, A.; Kramer, F. R. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 6171–6176.
(24) Wang, K. M.; Tang, Z. W.; Yang, C. Y. J.; Kim, Y. M.; Fang, X. H.; Li, W.; Wu, Y. R.; Medley, C. D.; Cao, Z. H.; Li, J.; Colon, P.; Lin, H.; Tan, W. H. *Angew. Chem., Int. Ed.* **2009**, *48*, 856–870.
(25) Li, J. J.; Geyer, R.; Tan, W. *Nucleic Acids Res.* **2000**, *28*, e52.
(26) Huang, Y.; Zhang, Y. L.; Xu, X. M.; Jiang, J. H.; Shen, G. L.; Yu, R. Q. *J. Am. Chem. Soc.* **2009**, *131*, 2478–2480.
(27) Tang, Z. W.; Wang, K. M.; Tan, W. H.; Ma, C. B.; Li, J.; Liu, L. F.; Guo, Q. P.; Meng, X. X. *Nucleic Acids Res.* **2005**, *33*, e97.
(28) Situma, C.; Moehring, A. J.; Noor, M. A. F.; Soper, S. A. *Anal. Biochem.* **2007**, *363*, 35–45.
(29) Situma, C.; Hashimoto, M.; Soper, S. A. *Biomol. Eng.* **2006**, *23*, 213–231.
(30) Wang, X.; Yun, W.; Dong, P.; Zhou, J. M.; He, P. G.; Fang, Y. Z. *Langmuir* **2008**, *24*, 2200–2205.
(31) Radi, A. E.; Sánchez, J. L. A.; Baldrich, E.; O'Sullivan, C. K. *J. Am. Chem. Soc.* **2006**, *128*, 117–124.
(32) Du, H.; Strohsahl, C. M.; Camera, J.; Miller, B. L.; Krauss, T. D. *J. Am. Chem. Soc.* **2005**, *127*, 7932–7940.
(33) Strohsahl, C. M.; Du, H.; Miller, B. L.; Krauss, T. D. *Talanta* **2005**, *67*, 479–485.
(34) Miranda-Castro, R.; de-los-Santos-Alvarez, P.; Lobo-Castanon, M. J.; Miranda-Ordieres, A. J. *Anal. Chem.* **2007**, *79*, 4050–4055.
(35) Zhang, J.; Qi, H.; Li, Y.; Yang, J.; Gao, Q.; Zhang, C. *Anal. Chem.* **2008**, *80*, 2888–2894.
(36) Wang, J. *Small* **2005**, *1*, 1036–1043.
(37) Hill, H. D.; Vega, R. A.; Mirkin, C. A. *Anal. Chem.* **2007**, *79*, 9218–9223.
(38) Song, Y.; Cui, K.; Wang, L.; Chen, S. *Nanotechnology* **2009**, *20*, 105501.
(39) Rasor, J. P.; Voss, E. *Appl. Catal., A* **2001**, *221*, 145–158.
(40) Sung, W.-C.; Chang, C.-C.; Makamba, H.; Chen, S. H. *Anal. Chem.* **2008**, *80*, 1529–1535.

Table 1. DNA Probe and Oligonucleotides Used in This Work[a]

probe	5′-/5ThioMC6-D/CCG GGT TGT TTG CCT CCC TGC TGC GGA CCC GG/3Bio/-3′
T1	5′-GTA CTT TCA CCG CAG CAG GGA GGC AAA CAG TAC TTG CA-3′
T2	5′-GTA CTT TCA CCG CAG CAG GGA GGC AAA CCG TAC TTG CA-3′
T3	5′-GTA CTT TCA CCG CAG CAG GGA GGC TAT ACG TAC TTG CA-3′
T4	5′-CAG GAT CAT GGT GAT GCT CTA CGT GCC GTA GCC-3′

[a] Note: T1, T2, T3, and T4 represent the perfectly matched target DNA, one-base matched DNA, four-bases mismatched DNA, and nonrelevant DNA, respectively.

a biotin at the 3′ end, respectively. It has six complementary bases at its 5′ and 3′ ends (five of them are G−C pairs), which results in the formation of the stem−loop structure at an appropriate ionic strength. Target 1 is complementary to the loop sequence of the probe; target 2 contains a one-base mismatch, whereas target 3 has four base mismatches. Target 4 is a random sequence that is noncomplementary to the probe sequence. Proteins such as bovine serum albumin (BSA) and γ-globulin were purchased from Sigma-Aldrich or Fluka. AuNPs (5, 10, and 20 nm, 0.01% $HAuCl_4$) were also purchased from Sigma-Aldrich Corporation.

The phosphate buffer solution (PBS) consisted of 0.01 M phosphate-buffered saline, 0.137 M NaCl, and 3 mM KCl (pH 7.4), and these were purchased from Sigma. The probe immobilization buffer solution consisted of a 0.01 M phosphate-buffered saline, 1 M NaCl, and 0.003 M KCl (pH 7.4). The blocking buffer solution was made up of a 3% (w/v) γ-globulin (pH 7.4) PBS. The hybridization and washing (H&W) buffer was a PBS solution containing 5 mM $MgCl_2$ (pH 7.4). The 3,3′,5,5′-tetramethylbenzidine (TMB)−H_2O_2 solution for spectrophotometric measurement was purchased from Sigma.

All other reagents were used as received, and all the solutions were prepared with deionized water.

A Safire 2 microplate reader (TECAN, Switzerland) was used to record the absorption signals of samples. A 96-well microplate was used as a platform for the probe immobilization, DNA hybridization, and spectrophotometric measurements.

Coating the Microplate with Protein. In order to select a good anchoring agent for both microplate and AuNPs, three proteins, BSA, casein, and globulin, were chosen to modify the inner surface of the wells in a 96-well microplate. Briefly, 50 μL of 3% BSA, 1% casein, and 3% γ-globulin PBS solution was added into each well of the 96-well plate, respectively, and then incubated at 4 °C overnight followed by washing with 250 μL of PBS for three times.

Immobilization of AuNPs. To increase the effective surface area for immobilization of the probes, AuNPs were used to modify the inner surface of the wells in a 96-well microplate. Two strategies were used to immobilize the AuNPs. One strategy was the self-assembling of AuNPs on the inner surface of a mercapto-modified 96-well microplate: 50 μL of 5, 10, or 20 nm AuNPs solutions was added into each well of the 96-well plate and then incubated at 4 °C for about 24 h followed by washing with PBS. The other strategy was immobilizing AuNPs on a γ-globulin-modified 96-well microplate. After modifying the microplate with γ-globulin, AuNPs solution was added and incubated for 3 h at 37 °C.

Immobilization of the Hairpin Probe. Stem−loop structure probes were immobilized on AuNPs by self-assembling. Briefly, 50 μL of 0.5 μM probe in PBS buffer (10 mM phosphate, pH 7.4, and 1 M NaCl) was added to AuNP-coated wells and incubated overnight at 4 °C. Subsequently, it was washed three times under shaking at 255 rpm for 5 min, and then 250 μL of 50 mM 6-mercaptohexan-1-ol was added into the 96-well plate and incubated for 3 h at room temperature. Here, 6-mercaptohexan-1-ol was used to block the surface of AuNPs through self-assembly for reducing nonspecific binding.

DNA Target Detection Strategies. After each well was washed with washing buffer (0.1 M PBS buffer containing 5 mM $MgCl_2$) three times, 50 μL of a different concentration of complementary DNA samples in a hybridization buffer was added and incubated at 37 °C, shaking at 100 rpm for 30 min. Then, the microplate was washed three times with PBS and added with the blocking buffer solution to prevent nonspecific binding. The microplate was washed one time to remove unbound γ-globulin. Finally, 50 μL of 1 μg/mL horseradish peroxidase labeled streptavidin (streptavidin-HRP), which was prepared in 5% γ-globulin in PBS buffer, was added and incubated for 5 min at room temperature. After the reaction, the solution was discarded, and the plate was washed again with PBS buffer three times to remove the unbound streptavidin-HRP. Then 50 μL of (TMB)−H_2O_2 solution was added in each well and incubated at room temperature with shaking at 100 rpm for 10 min to allow a sufficient color intensity to develop, which was read on a microplate reader.

Spectrophotometric Analysis. Spectrophotometric measurements were performed on a Safire 2 microplate reader with the advantages of its speed and high throughout. The ultraviolet (UV) absorption spectrum was collected by wavelength scan in the range of 450−750 nm. And the UV adsorption intensity was recorded at 650 nm.

RESULTS AND DISCUSSION

We first designed a stem−loop probe labeled with −SH and biotin at the 5′ and the 3′ end, respectively, for DNA assay. Figure 1 shows the strategy of DNA hybridization detection. To enhance the sensitivity of such an assay, an enzyme and a Au nanoparticle were used for amplified detection of DNA hybridization. In this strategy, the AuNPs were immobilized on 96-well microplate to increase the surface area for loading more probes labeled with −SH via the Au−S bond between gold and the thiol-labeled DNA probe. HRP was employed to amplify the detection signal.

To obtain the best detection sensitivity and selectivity for the DNA assay, some factors, such as the anchoring proteins, the AuNPs' size and concentration, the DNA probe concentration, and some testing conditions have been systematically investigated and optimized.

In this study, a good anchoring agent should have strong adsorption affinity to both the microplate's surface and the AuNPs.

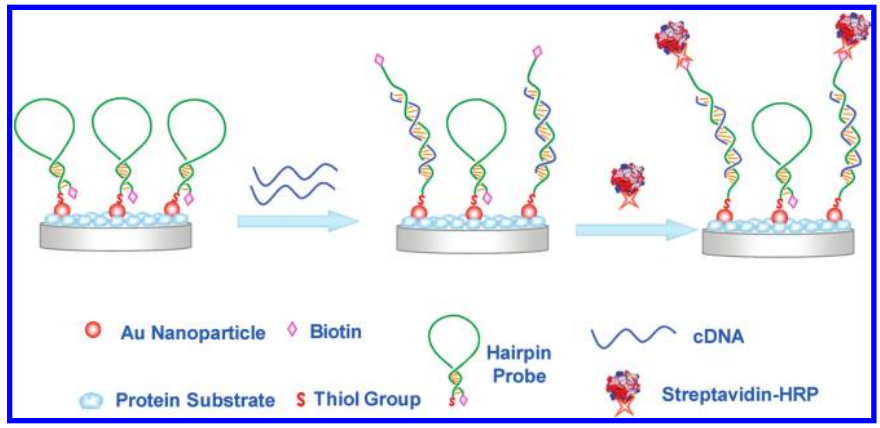

Figure 1. Scheme for DNA hybridization detection with the hairpin DNA switch. In the closed conformation, the biotin label is sterically shielded and thus inaccessible to the reporter enzyme. Upon target binding, the disruption of the stem−loop and the formation of the duplex make the streptavidin label HRP accessible for binding, which catalyzes the reduction of hydrogen peroxide.

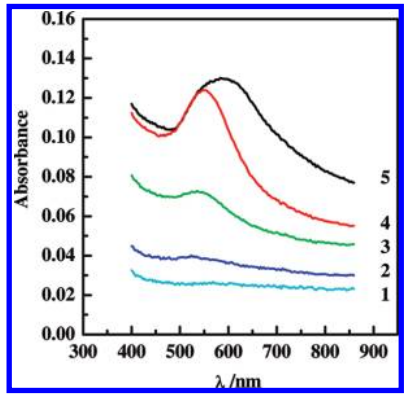

Figure 2. Absorption spectra of AuNPs immobilized on a 96-well plate. Curve 1 represents the inner surface modified with γ-globulin. Curve 2 represents a 5 nm gold nanoparticle coating on a thiol-functionalized microplate. Curves 3−5 represent 5, 10, and 20 nm AuNPs immobilized on a γ-globulin protein layer.

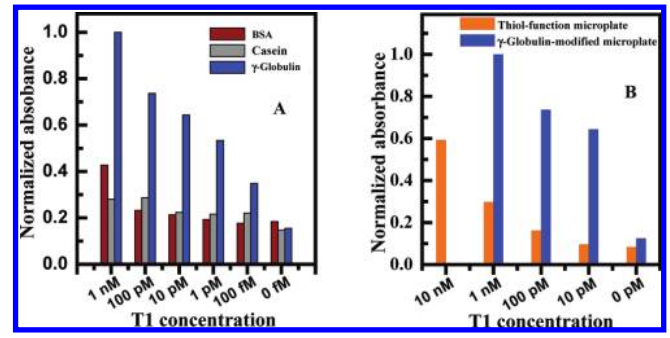

Figure 3. (A) Detection of hybridization of probe with complementary target using different proteins as anchoring proteins. (B) Detection of hybridization of probe with complementary target using a thiol-functionalized microplate and γ-globulin-modified microplate.

Here we examined several proteins, such as BSA, casein, and γ-globulin. As a comparison, we also investigated the performance of the microplate modified with thiol functional groups for AuNP immobilization. AuNPs were immobilized on the protein layer and the thiol-functionalized surface by using three different sizes of AuNPs with diameters of 5, 10, and 20 nm. After adding AuNPs into a protein-coated well for 3 h at 37 °C, a layer of pink-colored gold particles was visible on γ-globulin-modified wells. However, AuNPs coated on both BSA- and casein-anchored wells seemed to have very low density and appeared as a very light color. In comparison, the AuNPs on the thiol-functionalized microplate appeared nearly colorless. To quantify the AuNP's immobilization, we detected a UV absorption spectrum of AuNPs at different conditions, and the results are shown in Figure 2. The UV detection results were consistent with visual observation by the naked eye. In the case of the γ-globulin-anchored surface, the absorbance of AuNPs was found for the immobilization of all three sizes of nanoparticles. But for the BSA- and casein-modified surface, no AuNPs absorbance was observed (data not shown). A very weak absorption peak of AuNPs can be observed on a thiol-functionalized surface. These experimental results suggest that γ-globulin protein is the best-anchored agent for AuNP immobilization.

We also performed DNA hybridization detection on the AuNPs-modified surface anchored by the different proteins, and the thiol-functionalized microplate was used for comparison (Figure 3). As shown in Figure 3A, an increase of responses with increasing target concentrations was observed using γ-globulin as the anchoring agent. However, the use of BSA and casein as anchor agents did not show good responses for the same samples. For the thiol-functionalized microplate, much lower responses shown in Figure 3B were observed as compared to those obtained at the γ-globulin-modified microplate, indicating that the γ-globulin-anchored platform has better performance than the thiol-functionalized one. These results are consistent with those obtained from the investigation of the loading of AuNP with these anchoring agents, which indicated that the amount of AuNP immobilized greatly affected the sensitivity of the DNA assay. The γ-globulin-coated surface shows the largest loading of AuNPs due to its strongest adsorption affinity, thus giving the highest signal. Therefore, γ-globulin protein has been selected as the anchoring agent to fabricate the hairpin DNA switch for DNA target detection.

Selection of Size and Concentration of AuNPs. The amount of DNA probe immobilized on a microplate can directly affect the sensitivity of DNA detection. To increase the efficiency of the immobilization of the DNA probe, increasing the effective surface area is an effective, direct, and feasible route. It is well-known that nanoparticles have a very large surface-to-volume ratio. Therefore, nanoparticles that are immobilized on a solid surface can remarkably increase the effective surface area. We chose

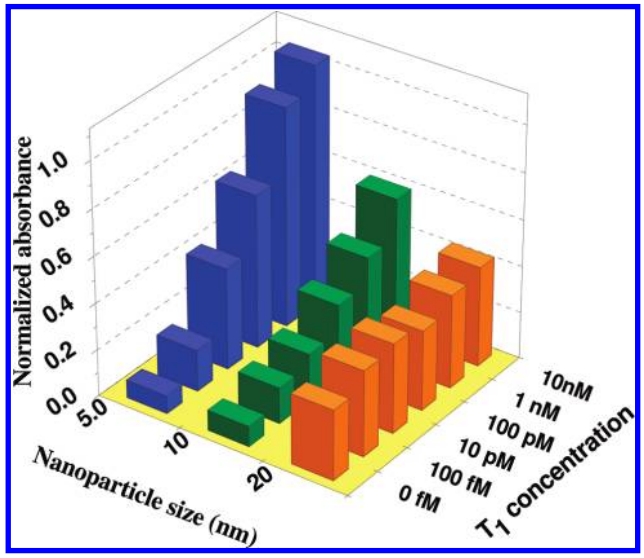

Figure 4. Detection of hybridization of probe with complementary target using different sizes of AuNPs modifying the microplate.

Table 2. Ratio of Signal to Noise Obtained at Different Dilution Times of Stock Solution for AuNPs with the Diameter of 5 nm

	dilution times					
target concentration	4	5	6	7	8	9
1 nM	1.41	2.74	3.52	6.90	24.21	7.98
10 pM	1.02	1.87	2.15	4.25	9.03	5.22

AuNPs of three different sizes for coating the microplate and tried to find the best NP size for the assay since the size of nanoparticles can directly affect their surface area. Diluted AuNPs (5, 10, and 20 nm, 0.0015% $HAuCl_4$) were used to modify the microplate inner surface to immobilize the thiolated DNA probe for DNA hybridization detection. The results are shown in Figure 4. It is evident that the response signals from 5 nm gold were about twice as high as those from 10 nm AuNPs. From 20 nm AuNPs, there is not much difference in the responses with the increase of the target concentration. These results suggested that higher sensitivity can be achieved when the inner surface of the microplate is coated with smaller AuNPs, which may be due to two reasons: (1) the smaller size of AuNPs means that they have a larger surface-to-volume ratio, therefore providing a larger surface area to immobilize more DNA probes, and (2) the small size of AuNPs makes it easy to form a uniform monolayer on the surface modified with γ-globulin than the large size. Therefore, 5 nm AuNPs were chosen for this study.

The AuNPs' concentration used to modify the microplate can significantly affect the DNA detection sensitivity. The DNA assay suffered from high background or low sensitivity when the concentration of the AuNPs was too high or too low. To achieve a high signal-to-noise ratio, we optimized the concentration of AuNPs by investigating the signal-to-noise ratio of the DNA assay with different dilutions of stock AuNPs. The results are listed in Table 2. High signal-to-noise ratio could be obtained when a stock AuNPs' solution is diluted 8 times, which is 24.21 for a high target DNA concentration of 1 nM and 9.03 for a low target concentration

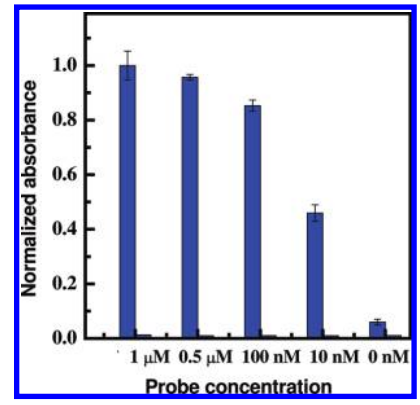

Figure 5. Optimization of probe concentration: plot for concentration of probe vs normalized absorbance. The perfectly matched DNA concentration was 1 pM.

of 10 pM, respectively. Therefore, 8 times dilution of stock AuNPs (5 nm) was used to modify the 96-well microplate in this study.

Selection of Probe Concentration. To achieve the assay's best performance, the amount of the probe immobilized on the 96-well was optimized using different concentrations of the probe. The 5′−HS-labeled stem−loop probe was immobilized on the AuNPs through self-assembling. We found that the response increases as the amount of probe immobilized on the surface increases,. However, it should be verified that the high loading of the probe on the surface does not affect the stem−loop structure of the probe on the surface and the access of target DNAs for hybridization. Therefore, the effect of the hairpin probe concentration on the performance of the assay was studied by hybridizing with 1 pM complementary target DNA after the immobilization. The results are presented in Figure 5. It can be seen from this figure that the signal response tends to level off at 0.5 μM, indicating that the probe concentration at 0.5 μM starts to be saturated. Therefore, the 0.5 μM probe solution was used for the probe immobilization.

Optimization of Testing Conditions. Many experimental parameters, such as hybridization temperature, hybridization time, and Mg^{2+} ion strength, can affect the detection sensitivity of the assay. A series of optimizations of these parameters were carried out to improve the sensitivity and selectivity of the DNA assay.

Supporting Information Figure S1 shows the effect of the hybridization temperature on the efficiency of DNA hybridization. In this study, a 100 pM complementary DNA target was incubated with the immobilized stem−loop probe for 30 min at a series of temperatures ranging from 25 to 45 °C. As shown in Supporting Information Figure S1, the highest signal is obtained at 37 °C. Therefore, a hybridization temperature of 37 °C was selected in the following experiments.

The effect of the hybridization time has also been investigated. The target DNA was incubated with an immobilized probe at 37 °C for different times from 10 to 50 min. The signal responses of the assay to the different hybridization times are shown in Supporting Information Figure S2. It can be seen from this figure that the signal increases rapidly when the hybridization time increases from 10 to 30 min and then decreases slightly from 30 to 50 min, indicating that the hybridization reaches equilibrium

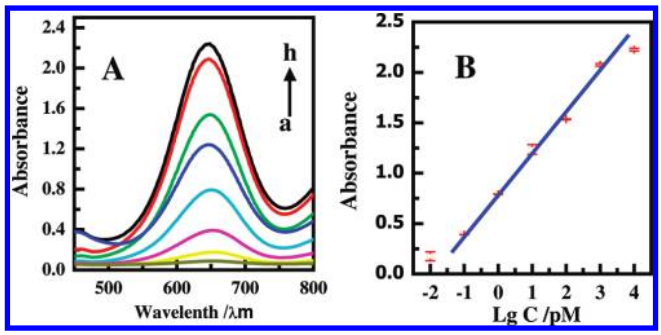

Figure 6. (A) Absorption spectrum of hybridization of the probe with complementary ssDNA. The a−h complementary concentrations are 0, 0.001, 0.01, 0.1, 1, 10, 100, 1000, and 10 000 pM, respectively. (B) Plot of absorbance as a function of the complementary target DNA concentration. The absorbances are averages for three repeats.

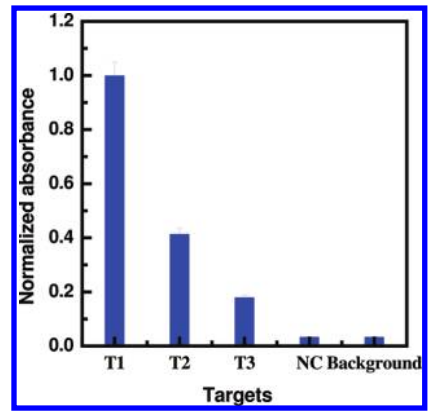

Figure 7. Specificity of the DNA assay detecting different targets: T1, perfectly matched DNA; T2, one-base matched DNA; T3, four-bases mismatched DNA; NC, nonrelevant DNA. The target concentrations of T1, T2, T3, and NC are 100 pM. All data were normalized to the perfect matched target sample.

at 30 min. As a result, a reaction time of 30 min was selected for the DNA hybridization.

The cation Mg^{2+} plays an important role in DNA hybridization. For a hairpin DNA probe-based DNA assay, there is hybridization competition between intramolecular stem part hybridization and intermolecular target DNA−probe hybridization. Therefore, Mg^{2+} concentration in the hybridization buffer should be balanced. Although a high concentration of Mg^{2+} can enhance target DNA hybridized to the loop of the structure, it may also strengthen the binding of the stem part of the probe, which may adversely affect the performance of the assay. Supporting Information Figure S3 shows the effect of Mg^{2+} concentration in the hybridization buffer on the response signal of the assay. As shown in this figure, the assay produces the highest signal when the Mg^{2+} concentration is 5 mM. Accordingly, a 5 mM Mg^{2+} concentration was used throughout the experiments to obtain the highest signal.

Performance of the DNA Assay. At optimal conditions, the DNA assay was challenged with a series of target DNA concentrations (Figure 6). As shown in Figure 6A, the signals of the assay increase as the concentration of the target DNA increases. In particular, we found that the assay was still responsive with 10 fM target DNA (as shown in Figure 6A). This high signal to background is an inherent merit of the hairpin structure DNA probe used in this approach. It was found that the response signal was logarithmically proportional to the target concentration in the range from 10 fM to 1.0 nM (Figure 6B). The detection limit was experimentally found to be less than 10 fM, which is superior to those obtained from other methods, such as the electrochemical impedance measurement,[41] and was comparable to those obtained from an enzyme-based E-DNA sensor.[8] This ultrahigh sensitivity reflected the high signal amplification of the enzyme combining with the immobilization of AuNPs.

Specificity of DNA Hybridization. The specificity of the hairpin DNA-based assay method was also investigated by using a single-base mismatched oligonucleotide (T2) and four bases of mismatched oligonucleotide (T3) as well as a complete noncomplementary oligonucleotide (NC). All results are displayed in Figure 7 (the signal was normalized with the signal of perfect complementary DNA). As shown in Figure 7, the signal from the one-base mismatched (T2) is approximately 40% of the signal from the fully complementary sequence (T1), suggesting that this novel DNA detection method is highly specific and may be used to differentiate even a single-base mismatch. The four-base mismatched oligonucleotide (T3) gave about one-fifth the signal of a perfect matched sequence. The response from the complete noncomplementary oligonucleotide is negligible. These results indicate that this novel DNA assay has excellent specificity to distinguish the single-base mismatched DNA target, which benefits from using the stem−loop DNA probe as the sensing component in this approach. The high specificity is important for many genetic assays in clinical and forensic applications, such as detecting SNPs.

CONCLUSION

We have demonstrated a highly specific and sensitive DNA detection platform based on a combination of hairpin DNA switch, AuNPs, and enzyme amplification. In this novel approach, the hairpin DNA probe was used to selectively detect the presence of target DNA while the AuNPs were used to facilitate the efficient immobilization of the DNA probe. The enzyme was employed to transduce the conformation change of the hairpin DNA probe induced by target DNA recognition to an optical signal with remarkable amplification. This novel DNA assay demonstrates high sensitivity and excellent specificity toward the target DNA due to the hairpin structure DNA probe and a signal amplification strategy using AuNPs and HRP. The results demonstrated that this novel method can detect a femtomolar-level DNA target with excellent selectivity to differentiate single-base mismatches of mutant DNA, which makes it promising for biomedical and bioanalytical applications requiring high-performance genetic target analysis.

ACKNOWLEDGMENT

The work was done at Pacific Northwest National Laboratory (PNNL) and was supported by the PNNL Laboratory Directed Research and Development program and by Grant U54 ES16015 from the National Institute of Environmental Health Sciences (NIEHS), NIH. Its contents are solely the responsibility of the

(41) Wang, Y.; Li, C. J.; Li, X. H.; Li, Y. F.; Kraatz, H. B. *Anal. Chem.* **2008**, *80*, 2255–2260.

authors and do not necessarily represent the official views of the federal government. PNNL is operated for the U.S. Department of Energy (DOE) by Battelle under contract DE-AC05-76RL01830. The characterization was performed using EMSL, a national scientific user facility sponsored by the Department of Energy's Office of Biological and Environmental Research and located at PNNL. Y. Zhang acknowledges the support from the National Natural Science Foundation of China (20975037).

SUPPORTING INFORMATION AVAILABLE

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

Received for review March 8, 2010. Accepted June 18, 2010.

AC1006238