A Simple, Universal Colorimetric Assay for Endonuclease/Methyltransferase Activity and Inhibition Based on an Enzyme-Responsive Nanoparticle System

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ABSTRACT An enzyme responsive nanoparticle system that uses a DNA—gold nanoparticle (AuNP) assembly as the substrate has been developed for the simple, sensitive, and universal monitoring of restriction endonucleases in real time. This new assay takes advantage of the palindromic recognition sequence of the restriction nucleases and the unique optical properties of AuNPs and is simpler than the procedure previously described by by Xu *et al*. (*Angew. Chem. Int. Ed. Engl.* 2007, *46*, 3468—3470). Because it involves only one type of ssDNA modified AuNPs, this assay can be directed toward most of the endonucleases by simply changing the recognition sequence found within the linker DNA. In addition, the endonuclease activity could be quantitatively analyzed by the value of the reciprocal of hydrolysis half time ($t_{1/2}^{-1}$). Furthermore, our new design could also be applied to the assay of methyltransferase activity since the methylation of DNA inhibits its cleavage by the corresponding restriction endonuclease, and thus, this new methodology can be easily adapted to high-throughput screening of methyltransferase inhibitors.

KEYWORDS: endonucleases · nanoparticles · palindromic DNA · methyltransferase · inhibitors

Endonucleases, a family of nucleases that can hydrolyze the internal phosphodiester bonds in DNA or RNA, are among the most important enzymes in molecular biology.[1−3] The specific actions of these enzymes are essential in a variety of fields ranging from biotechnology to pharmacology, and catalyze processes involving replication, recombination, DNA repair, molecular cloning, genotyping, and mapping.[4−8] Recent attempts to attach restriction endonucleases to nanostructures have been undertaken due to the well-established ability of the endonucleases to cleave DNA at specific recognition sites.[9−12] Therefore, assays of enzyme activities and evaluations of the kinetic parameters are of great importance in the fields of clinical diagnostics, drug discovery, and nanoscience. Historically, the detection methods have included gel electrophoresis, high-performance liquid chromatography (HPLC), filter binding, and enzyme-linked immunosorbent assay (ELISA), *etc*.[13−16] However, these protocols are discontinuous, time-intensive, DNA-consuming, laborious, and usually require isotope labeling. Many of these limitations are now being addressed by the development of fluorescence assays based on fluorescence quenching or fluorescence resonance energy transfer (FRET).[17−21] For example, a "molecular break lights" application based on an intramolecular fluorescence-quenching technique has been developed for the highly sensitive and convenient assay of restriction endonucleases using a single-stranded oligonucleotide as the substrate.[17,18] An alternative approach has been described recently for real-time monitoring of endonuclease activity using the hairpin fluorescence molecular beacon (MB)-based DNA probes.[20,21] Although each technique has its own advantage, they are compromised by the requirement for double-labeled DNA probes, limited chemical stability, and interferences by external nonspecific events. Thus, the search for a sensitive, simple, and economical assay for nucleases is ongoing.

The photostability, ease of synthesis, and ability to conjugate to biological molecules make nanoparticle-based materials attractive alternatives to the fluorescent or radiolabeled enzyme substrates of traditional enzymatic assays. Recently, gold nanoparticles (AuNPs) have been successfully employed as a colorimetric probe for the detection of nucleic acids, [22−24] enzymes,[25−30] proteins,[31,32] metal ions,[33−36] small molecules,[37−39] and for screening of DNA binders[40−42] based on their unique size- and distance-dependent optical

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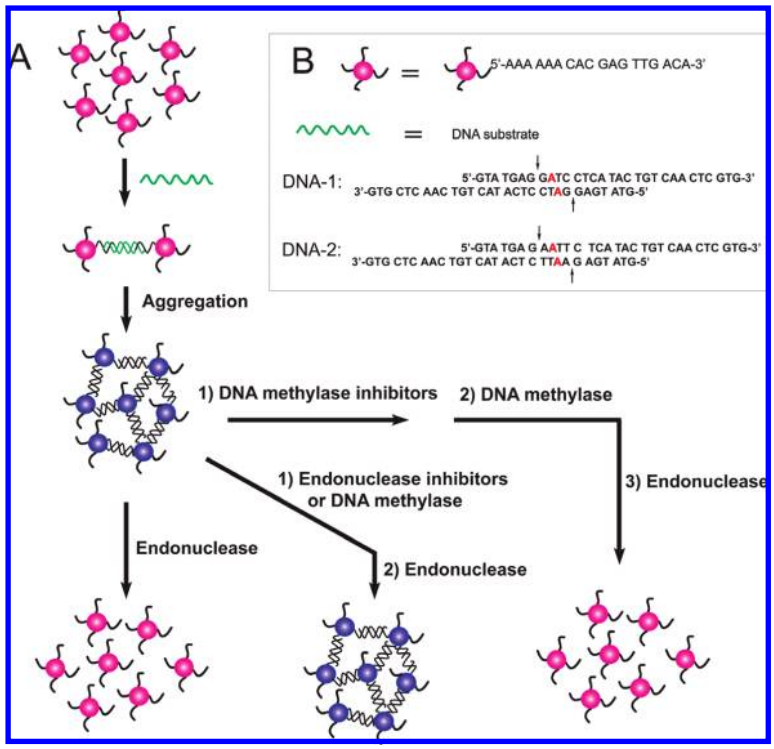

Scheme 1. (A) Schematic representation of the assay for endonuclease and methyltransferase activity and inhibition. (B) Sequences of DNA probe, DNA-1, and DNA-2. DNA-1,2 contain the recognition sites of Dpn II/Dam MTase and EcoR I/EcoR I MTase, respectively. The arrows show the cleavage sites, and the letters in red indicate the points of methylation.

properties. The major advantage of AuNP-based assays is that the molecular recognition events can be transformed into color changes which can be observed by the naked eye. Moreover, these assays provide much higher sensitivity because of the extremely high extinction coefficients of AuNPs. Xu *et al.* recently reported a colorimetric assay for the detection of DNase I endonuclease activity and inhibition using DNA-functionalized AuNPs.[30] In this previously published method, the dispersion of the aggregates of AuNPs interconnected by a DNA duplex leads to significant color changes when the endonuclease degrades the DNA duplex. Although highly selective and more sensitive than conventional methods, this visual inspection assay is limited by the need to prepare probes by functionalizing two separate batches of AuNPs with two different thiol-modified oligonucleotide strands. And for each of the enzymes, two specific DNA-functionalized AuNPs must be redesigned and synthesized, which makes the assay complex, expensive, and time-consuming. Herein, we demonstrated a new concept that overcomes this limitation by achieving enzyme-responsive nanoparticle systems that are not only sensitive and reliable, but also universal, simple, and economical in operation.

Our strategy was inspired by the phenomenon that the majority of restriction endonucleases are type II restriction enzymes (there are currently 3805 biochemically or genetically characterized restriction enzymes in REBASE, of which 3698 are type II restriction enzymes).[43] The recognition sites of type II endonucleases are typically short, palindromic sequences of double-stranded DNA. As shown in Scheme 1, our system is composed of two elements: only a single type of DNA-functional AuNP probe and an appropriate oligonucleotide linker that can hybridize with the probe DNA. The linker was designed to contain a self-complementary region, which can form a duplex structure with a base-pair overlap containing the recognition sites and overhanging 3′-ends. The overhanging portion was complementary to the oligonucleotide probe, and DNA−AuNPs assembly formed based on the hybridization of oligonucleotide linkers with the probe DNA immobilized on surface of the AuNPs. The thus designed AuNPs assembly could be used as the substrate for the endonuclease detection, as disassembly of AuNPs can be achieved by incubation with an endonuclease that cleaves double-stranded DNA in a site-specific manner. Significantly, it is expected that this approach can be directed toward most endonucleases by simply changing the recognition sequence found within the linker DNA. Furthermore, this system enables us to assay DNA methyltransferase activity and screen its inhibitors since the methylation of DNA inhibits its cleavage by its corresponding restriction endonucleases.

RESULTS AND DISCUSSION

Preparation of DNA−AuNPs Assembly Substrate. Dpn II restriction endonuclease was used as a model system in the current studies. As shown in Scheme 1B, the linker DNA-1 was designed to contain an 18-mer base-pair overlap which could form a stable duplex with 12-mer dangling ends in the enzymatic buffer solution. The single restriction site 5′-GATC-3′ was incorporated into the duplex region and the dangling ends at the 3′-termini were complementary to the 12-mer probe sequences. AuNPs (13 nm) were functionalized with 5′-thiol-(A_6)-CACGAGTTGACA-3′ (probe DNA), and the thus prepared nanoparticles had an average of 80∼100 oligonucleotides per particle. In a typical procedure, the solution of DNA-modified AuNPs was mixed with the linker DNA so that both sticky ends of the linker DNA duplex could bind to the complementary strands attached to the AuNPs in a tail-to-tail hybridization. The thus synthesized AuNP assembly was then used as the substrate to assay endonuclease and DNA methyltransferase activity.

Assay for Restriction Endonuclease Activity. In the UV−vis spectrum of an initial suspension of DNA−AuNPs assembly, a broad absorption spectrum (540−750 nm) re-

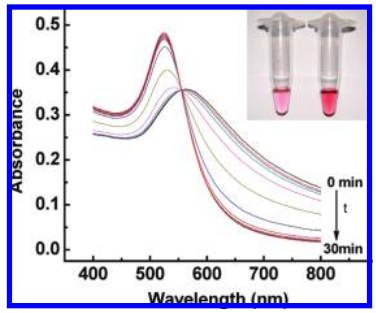

Figure 1. UV−vis spectra of DNA−AuNP assembly (AuNPs, 2 nM) taken at 2 min intervals for 30 min after the addition of Dpn II endonuclease (10 units). Inset, the color change of the nanoassembly in the absence (left) and presence (right) of Dpn II endonuclease (10 units, 30 min).

sults from the cross-linked network of nanoparticles, which is visualized as purple/blue (Figure 1). Upon addition of Dpn II restriction endonuclease, a decreased absorbance intensity at 700 nm and an increase of the plasmon band at 520 nm were both observed. As the endonuclease degraded the DNA−duplex interconnections, nanoparticles were released, regenerating a red color due to the dispersed nanoparticles (Figure 1, inset). On the other hand, the DNA−AuNPs assembly was not degraded by the enzyme when a linker DNA (DNA-2) that lacks the recognition site for Dpn II was introduced (Figure S1), thus demonstrating the selectivity of the DNA−AuNPs assembly for its specific endonuclease.

To demonstrate the application of the DNA−AuNPs assembly for monitoring the nuclease activity in real-time, the ratios of absorbance of 524 and 700 nm (A_{524}/A_{700}) were studied as a function of nuclease concentration. With a high ratio associated with red-colored dispersed particles and a low ratio associated with blue-colored aggregates, the ratiometric method allowed the determination of aggregation states independently of sample conditions, and increased the sensitivity of the assay. Figure 2A shows the time curves of the digestion reactions of the DNA−AuNPs assembly with various concentrations of Dpn II. The hydrolysis half time ($t_{1/2}$, the time at which 50% of the aggregates are hydrolyzed) decreased as the amount of the Dpn II restriction endonuclease increased, since a higher concentration of enzyme cleaves substrate more rapidly at the same initial concentration of DNA.

To address the universal approach for restriction endonucleases, detection of enzyme activity of the restriction endonuclease *Eco*R I was also studied. The specific substrate DNA-2 for *Eco*R I was obtained by simply replacing the recognition sequence with 5′-GAATTC-3′ within the linker DNA (Scheme 1B). Upon addition of the *Eco*R I enzyme, the red color was regenerated and an increased absorbance of the plasmon band at 520 nm was observed (data not shown). Similar hydrolysis curves were also obtained with varied amounts of *Eco*R I (Figure 2C). Note that it took relatively longer for the DNA−AuNPs assembly to start to be dispersed by the *Eco*R I enzyme than by Dpn II, and the hydrolysis curves based on the modified DNA−AuNPs assemblies were different from those obtained when the directly used oligonucleotides served as the substrates.[17] These differences in enzymatic activity could simply reflect the distinctions in the enzymes and/or the chemical assay buffers.

As the presence of AuNPs could distinctly affect the enzymatic reactions, we hypothesized that the enzyme-responsive AuNP system mainly consisted of two steps: (a) the binding of the enzyme to its recognition sites and (b) the enzymatic hydrolysis to release the nanoparticles. Recent work by Seferos *et al.*[44] demonstrated that the enzyme-nanoparticle binding is not inhibited when DNA−AuNPs are used, and the kinetics of the system might be limited by the release of nanoparticle. Therefore, traditional methods for obtaining the velocity of the enzymatic reaction could not be applied in our AuNPs systems. Interestingly, as shown in Figure 2B,D, the plots of the reciprocal of hydrolysis half time ($t_{1/2}^{-1}$) *versus* nuclease concentration showed good linear relationships ($R^2 = 0.995$, 0.991, respectively) for both enzymes. Furthermore, a good linear correlation ($R^2 = 0.989$) could also be obtained with another endonuclease, DNase I, by analyzing previous hydrolysis data.[30] Thus, we demonstrated that the nucleases activities could be quantitatively analyzed by using the plot of

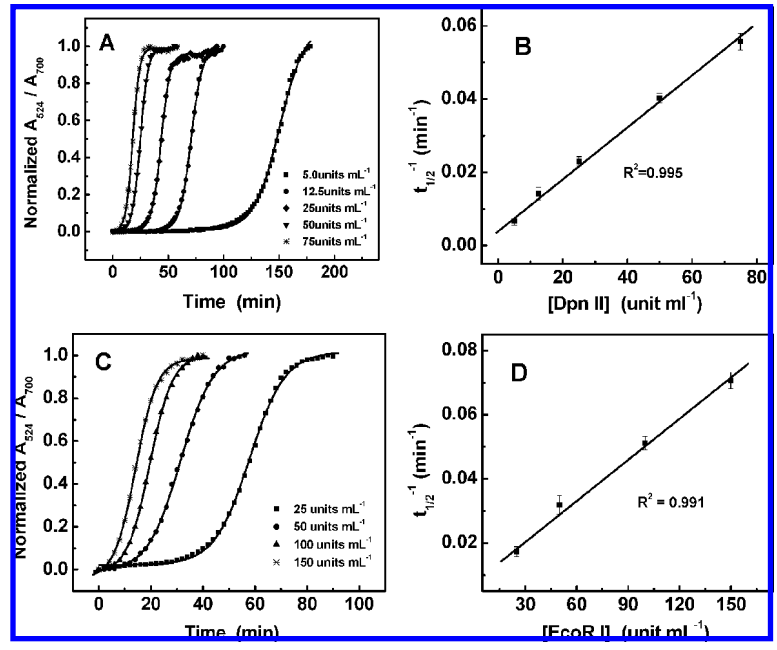

Figure 2. Normalized dissociation curves for the DNA−AuNP aggregates (5 nM) at different concentrations of Dpn II (A) and EcoR I (C). The data were recorded at 2 min intervals; the reciprocal of hydrolysis half time ($t_{1/2}^{-1}$) *versus* concentrations of Dpn II (B) and EcoR I (D).

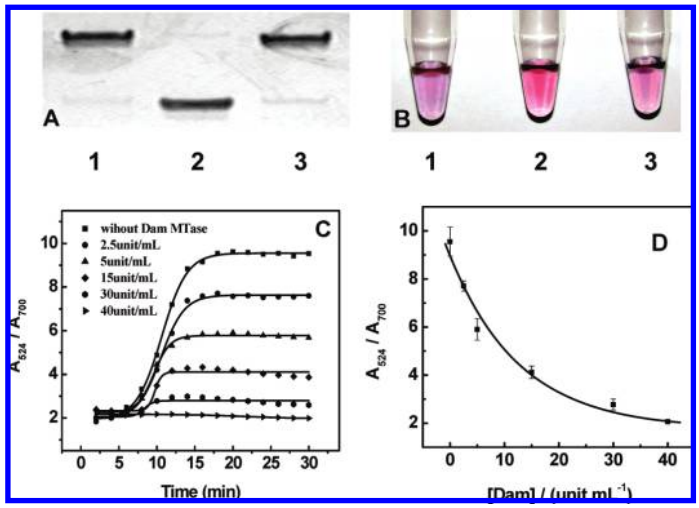

Figure 3. (A) The gel electrophoresis images of DNA-1 with and without Dam MTase in the presence of Dpn II: (1) DNA-1 alone; (2) DNA-1 with Dpn II; (3) DNA-1, Dam MTase, and Dpn II. (B) Color changes of the DNA−AuNPs assembly with and without DNA methylation in the presence of Dpn II: (1) DNA−AuNP only; (2) DNA−AuNP and Dpn II; (3) DNA−AuNP, Dam MTase, and Dpn II. (C) The hydrolysis curves for the DNA−AuNP assembly with various Dam MTase concentrations at fixed Dpn II concentration (10 units). (D) The ratio of absorbance at 524/700 nm *versus* Dam MTase concentrations under the same concentration condition as in panel C.

$t_{1/2}^{-1}$ *versus* enzyme concentrations in this kind of enzyme-responsive nanoparticle system.

Assay for DNA Methyltransferase Activity. DNA methylation is an epigenetic process which is carried out by DNA methyltranferases (MTases) that catalyze the transfer of a methyl group from *S*-adenosyl-L-methionine (SAM) to adenine or cytosine residues in DNA. It occurs in both prokaryotes and eukaryotes, and plays a critical role in the control of gene expression, maintenance of genomic integrity, virulence, and cancer origin.[45−47] As such, methyltransferases represent an exciting and novel family of targets, and the pharmacological inhibition of DNA MTases provides a broad spectrum of therapeutic applications, including but not limited to antibiotics, antiviral drugs, and anticancer therapeutics.[48−50] Here we show that our simple DNA−AuNPs assembly can also be utilized to assay DNA MTase activity and to screen MTase inhibitors, based on the general principle that the cleavage of methylated DNA by restriction endonuclease is blocked by the methylation at the specific palindromic sequence in duplex DNA. DNA-1 and Dam methylase were chosen as the model DNA and methyltransferase, respectively (Scheme 1). The suspension of DNA-1−AuNPs assembly was first treated with Dam MTase, which catalyzed the methylation reaction at the recognition sequence to yield the methylation duplex DNA 5′-GAmTC-3′. The suspension was subsequently centrifuged and treated with Dpn II endonuclease, which recognizes the same sites and can specifically cleave DNA-1, but not methylated DNA-1. In this case, the methylated DNA was not cleaved (as observed by electrophoreses shown in Figure 3A), and the AuNPs assembly could not be dispersed (Figure 3B). A series of standard Dam MTase solutions were prepared at a fixed DNA−AuNPs and Dpn II nuclease concentrations, and the ratios of absorbance of 524 and 700 nm (A_{524}/A_{700}) was used for the quantitative detection of the Dam MTase activity. With a high ratio associated with dispersed particles and a low ratio associated with aggregates, the results indicate that the methylation of DNA-1 inhibited its cleavage by Dpn II nuclease (Figure 3C). The value of A_{524}/A_{700} gradually decreased with the increasing concentrations of methylase (Figure 3D), which shows that the higher concentration of methylase is beneficial to the methylation of DNA substrate. Furthermore, the concept of universal assay can also be applied because the methylation of DNA inhibits its cleavage by its corresponding restriction endonucleases. For example, EcoR I methylase has a specific recognition site 5′-GAATTC-3′ and its substrate DNA-2 can be obtained by simply replacing the recognition sequence within the linker DNA. As observed in the case of Dam MTase, the DNA-2−AuNPs assembly could be used to assay the EcoR I methylase activity through the inhibition of its cognate EcoR I nucleases (Figure S2).

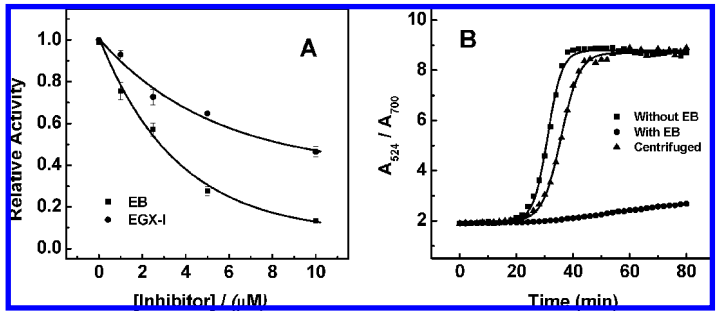

Figure 4. (A) Inhibition efficiency of Dam MTase by EB and EGX^{-I}. The IC_{50} values were obtained from the fitting curve. (B) Dissociation curves for the DNA−AuNPs aggregates (2 nM) in the absence and presence of EB (2 μM), subjected to washing with enzyme buffer at a Dpn II concentration of 25 unit mL^{-1}.

Assay of the Inhibition of DNA MTase Activity. This simple and sensitive assay was also applied to screen for inhibitors of DNA MTase. Transferring of the methyl group to the DNA residue was blocked in the presence of inhibitor, and consequently, the DNA−duplex interconnections were degraded and particles were dispersed (Scheme 1A). The inhibition effect can be quantitatively analyzed by using the ratios of absorbance A_{524}/A_{700}. Increasing the inhibitor concentration lowers the content of methylation, and therefore, the ratios of absorbance A_{524}/A_{700} increased. Dam MTase was used as a model system and two small molecules, cyclopentaquinoline carboxylic acid (EGX^{-I}) and ethidium bromide (EB) (Figure S3 for the structures), were selected for our studies. EGX^{-I} is a promising small molecule recently discovered by Mashhoon *et al.*[50] which could efficiently inhibit Dam MTase activity, and EB is a typical DNA intercalator which has never been reported as a DNA MTase inhibitor. The IC_{50} value, the inhibi-

tor concentration required to reduce enzyme activity by 50%, was obtained from the plot of relative activity (RA) *versus* inhibitor concentration (Figure 4A). EGX^{-I} and EB were found to inhibit Dam MTase with IC_{50} values of 8.58 ± 1.13 and 2.79 ± 0.61 μM, respectively. The IC_{50} for EGX^{-I} is in good agreement with the reported literature value of 9.7 μM determined by other methods.[50]

Since there were two enzymes involved in the system, it was necessary to eliminate the effect of small molecules on the endonuclease before studying the effect of small molecules on the activity of DNA MTase. In the traditional restriction-protection based assays, extra control experiments must be carried out. Our assay has a technological advantage in terms of simplicity: by using the DNA−AuNPs assembly as the substrate, the interferences can easily be eliminated through repeated centrifuging and washing steps. For example, as shown in Figure 4B, upon addition of EB to the DNA−AuNPs suspension, the gold nanoparticle assembly was dispersed very slowly by Dpn II endonuclease; with washing, centrifugation, and resuspension, the aggregate could be dispersed at a rate similar to a preparation not treated with EB. We believe that this unique property of the AuNPs makes it an ideal probe for assaying substances which require multiple separation steps.

CONCLUSION

In summary, we have demonstrated a unique design of an enzyme-responsive nanoparticle system for monitoring restriction endonuclease/methyltransferase activities and inhibitions using DNA−AuNPs assemblies as the substrates. The unique optical properties of AuNPs and the palindromic recognition sequence of the nucleases make this novel assay not only sensitive and reliable, but also universally applicable in its operation and easily adaptable to high-throughput screening. In addition, the assay presented here is simpler in format than that presented by Xu *et al.*[30] It involves only a single type of ssDNA-modified AuNPs, which offers the advantages of simplicity and cost efficiency. Furthermore, for the first time, the endonuclease activity has been quantitatively analyzed by the value of the reciprocal of hydrolysis half time ($t_{1/2}^{-1}$). These findings may contribute to the future application of nanoparticle-based technologies to therapeutic agents and to the development of chemical tools for the controlled assembly and manipulation of nanostructures.

METHODS

Materials. Tetrachloroauric acid ($HAuCl4 \cdot 3H2O$) and sodium citrate ($Na_3C_6H_5O_7 \cdot 2H_2O$) were purchased from Alfa Aesar Co. Ltd. Cyclopentaquinoline carboxylic acid was obtained from ChemBridge Co. (San Diego, CA). All other chemicals were purchased from Sigma-Aldrich Co. Ltd. and used without further purification. Dam and EcoR I methylase, Dpn II and EcoR I endonuclease, and SAM were purchased from New England Biolabs Inc. DNA oligonucleotides were purchased from Sangon (Shanghai, China) and used without further purification, and their concentration were determined by measuring the absorbance at 260 nm after melting. Extinction coefficients were estimated by the nearest-neighbor method.

The sequences are as follows.

Probe: HS-(CH_2)6−5′-AAA AAA CAC GAG TTG ACA-3′
DNA-1: 5′-GTA TGA GGA TCC TCA TAC TGT CAA CTC GTG-3′
DNA-2: 5′-GTA TGA GAA TTC TCA TAC TGT CAA CTC GTG-3′

Preparation and Functionalization of AuNPs. The 13-nm AuNPs were prepared using a standard citrate method.[51] The DNA−AuNPs were prepared according to the previously reported method.[52,53] Before DNA loading, the thiol functionality on the probe oligonucleotides was deprotected by treatment with 1.5 equiv of TCEP for 1 h at pH 5.2 at room temperature. AuNPs were functionalized by derivatizing aqueous Au colloid with deprotected thiol-oligonucleotides (final concentration, 3.6 μM for oligonucleotides and 10 nM for AuNPs, respectively). The resulting solution was then incubated at room temperature for 24 h. The solution was incubated for an additional 24 h at room temperature after the NaCl concentration of the solution was increased to 100 mM. These DNA−AuNPs were purified three times by centrifugation (30000*g* for 45 min) in 10 mM phosphate buffer (pH 7.0), and the nanoparticles were stored in the same buffer at 4 °C.

Preparation of DNA−AuNP Assembly. After combining of DNA-1 (or DNA-2) and the probe DNA−AuNPs in hybridization buffer (phosphate buffer (10 mM, pH 7.5) containing 300 mM NaCl), the mixture was heated to 90 °C and held at this temperature for 10 min. The solution was cooled slowly to room temperature, which resulted in aggregation of the particles and the concomitant red-to-purple color change. The aggregates were centrifuged briefly with a benchtop microcentrifuge and precipitated to the bottom of the microcentrifuge tube. The aggregates were washed with nuclease buffer and then resuspended in 1 mL nuclease buffer. This solution can be used to assay the enzymatic activity.

Assay of Endonuclease Activity. Since the standard enzymatic buffers contain dithiothreitol (DTT), which was found to precipitate gold nanoparticles,[54] for the present work we prepared buffers without DTT. The DNA−AuNPs assembly was first suspended in the enzymatic buffer at a final concentration of 2 nM (50 mM MES, 50 mM NaCl, 10 mM $MgCl_2$, pH 6.0 for Dpn II endonuclease; 50 mM tris-HCl, 100 mM NaCl, 10 mM $MgCl_2$, pH 7.5 for EcoR I endonuclease). Different amounts of endonuclease were then added to the suspension of the DNA−AuNPs assembly, and the UV−vis spectra of the AuNPs were monitored every 2 min at 37 °C using a UV−vis spectrometer (Cary 300, Varian Inc., Palo Alto, CA). The hydrolysis curves were obtained from the spectra and the hydrolysis half time was calculated from the corresponding hydrolysis curves.

Assay of Methylation Activity by Gel Electrophoresis. The reaction mixture consisted of 5.0 μM DNA-1, 1× methylase buffer (50 mM HEPES, 50 mM NaCl, 1 mM EDTA, pH 7.5), 80 μM SAM, and 10 units of Dam MTase. In the gel electrophoresis assays, the 10-μL sample was incubated at 37 °C for 3 h and then a 40 μL buffer (50 mM MES, 50 mM NaCl, 10 mM $MgCl_2$, pH 6.0) and 10 units of Dpn II endonuclease were added and incubated at 37 °C for another 3 h. Subsequently, the samples were applied to a polyacrylamide gel (20%) to separate the cleaved products from the substrate. The electrophoresis was carried in 1× TBE (pH 8.0) at 100 V constant voltage for 3 h. The gels were silver-stained.

Assay of DNA Methyltransferase Activity. The methylation experiment was performed in 50 μL methylase buffer (50 mM HEPES, 50 mM NaCl, 1 mM EDTA, pH 7.5 for Dam MTase; 50 mM Tris · HCl, 50 mM NaCl, 10 mM EDTA, pH 8.0 for EcoR I methylase) containing 80 μM SAM, 5 nM DNA−AuNP assembly and a varying amount of DNA MTase at 37 °C for 90 min. The suspension was then centrifuged and the precipitates were resuspended with 200 μL endonuclease buffer. Then 10 units of corresponding en-

donuclease was added and incubated at 37 °C, and the hydrolysis was monitored by UV−vis spectroscopy.

Inhibition of Dam MTase Activity. Inhibitors (0−10 μM) were preincubated at varying concentrations with DNA-1-AuNP assembly (5 nM) in the methylase buffer (50 mM HEPES, 50 mM NaCl, 1 mM EDTA, pH 7.5) at 37 °C for 15 min. Then Dam MTase (8 units) and SAM (80 μM) were added, and the resulting suspension were incubated at 37 °C for 90 min. The suspension was then centrifuged and the precipitate was resuspended with 200 μL of endonuclease buffer. Dpn II endonuclease (10 units) was added and the mixture was incubated for 30 min at 37 °C. The value of A_{524}/A_{700} was recorded using the UV−vis spectrometer. The relative activity ($R_{activity}$) of Dam MTase in the presence of inhibitors could be determined by monitoring concentration changes of methylated DNA by A_{524}/A_{700} using the following equation:

$$R_{activity} = \frac{R_0 - R_i}{R_0 - R_i} 100\%$$

in which R_0, R_t and R_i are the value of A_{524}/A_{700} of the AuNPs without Dam, with Dam, and with Dam in the presence of inhibitors, respectively. The inhibition efficiency was calculated from the plot of relative Dam activity *versus* inhibitor concentrations.

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Supporting Information Available: Supporting figures and structures. This material is available free of charge *via* the Internet at http://pubs.acs.org.

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