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RESEARCH PAPER

A colorimetric assay of DNA methyltransferase activity based on peroxidase mimicking of DNA template Ag/Pt bimetallic nanoclusters

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Abstract
DNA methylation catalyzed by DNA methyl transferase (MTase) is a significant epigenetic process for modulating gene expression. Abnormal levels of DNA MTase enzyme have been regarded as a cancer biomarker or a sign of bacterial diseases. We developed a novel colorimetric method to assay M.SssI MTase activity employing peroxidase-like activity of DNA template Ag/Pt NCs without using restriction enzymes. Based on inhibiting the peroxidase reaction that occurred in the TMB-H_2O_2 system, in the presence of MTase, a highly sensitive and selective colorimetric biosensor was fabricated with a detection limit (LOD) of 0.05 U/mL and a linear range from 0.5 to 10 U/mL. The changes in absorption intensity were monitored to quantify the M.SssI activity. This strategy had a high selectivity over other proteins. Furthermore, it is also demonstrated that this method can be used for the evaluation and screening of inhibitors for DNA MTase.

Keywords Ag/Pt nanoclusters · DNA methyltransferase · Colorimetric detection · Enzyme mimic

Introduction

DNA methylation, a common epigenetic modification of the genome, plays a critical role in both prokaryotes and eukaryotes [1]. It has received great attention because of its close relationship with various biological events, such as transcription, gene imprinting, chromatin structure, embryonic development, X chromosome inactivation in females, and tumors [2, 3]. DNA methylation refers to methyl transfer from the S-adenosyl methionine (SAM) donor to cytosine or adenine residues, in particular short palindromic sequences, which are achieved by the catalysis effect of DNA methyltransferases (MTase) [4, 5]. It has been demonstrated that alterations of MTases activity leads to aberrant DNA methylation patterns which are regarded as biomarkers of cancer in various types of cancer such as gastric [6], lung [7], prostate [8], colon [9], and is associated with several genetic diseases [10, 11]. The detection and quantification of MTase activity has drawn much attention because of its significant role in both fundamental research and clinical applications. Up to now, some conventional methods for the detection of DNA methylation and assay of MTase activity have been developed, which include

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radioisotope labeling of [methyl-3H]–SAM [12], capillary electrophoresis [13], high performance liquid chromatography (HPLC) and mass spectroscopy [14], polymerase chain reaction (PCR) [15], SERS (surface enhanced Raman spectroscopy) [16], Western blotting [17], bisulfite treatment [18], and immune reaction [19]. However, all of them carry obvious drawbacks such as radioisotope labeled substrate, expensive antibodies, fluorescently labeled substrates, large-scale detection equipment, high operation cost, time-consuming sample preparation, insufficient sensitivity and so on. Thus, further efforts are needed in the development of rapid, sensitive, simple, and economical methods for accurate DNA methylation detection and MTase activity assays. In order to improve the detection sensitivity and specificity, many new strategies for the detection of DNA MTase activity have been developed, including electrochemical [20–22], chemiluminescent [23, 24], fluorescence [25–28] methods, photoelectrochemistry [29], and colorimetric assays [30–33]. Among them, the colorimetric strategy has received widespread attention owing to the advantages of rapidity, simplicity, cost-effectiveness, and especially, it may be easily read out by the naked eye without requiring expensive analytical instruments. Several colorimetric biosensors have been successfully developed for DNA MTase activity analysis. For example, Song et al. [34] and Liu et al. [35] proposed DNA MTase assay employing DNA-modified gold nanoparticles (AuNPs) dispersion and aggregation, Wu et al. [36] have reported a colorimetric biosensor for DNA MTase detection based on MTase protection of the DNA-gold nanoparticles (AuNPs). Li et al. [37] developed a colorimetric method for detection of DNA MTase activity with the LOD of 6 U/mL based on methylation responsive DNAzyme. A label-free colorimetric method was established based on methylation-blocked cascade amplification [38]. However, these methods were challenged with the limitation of use because of complex reaction conditions of enzymes, the instability of the AuNPs, and signal amplification system requirement. Thus, an assay with more stability and simplicity is still needed. It is well known that DNA has the capacity to be an efficient template for in situ fabrication of some nanomaterials such as metal NPs/NCs. These DNA-templated nanomaterials have shown unique properties such as facile synthesis, controllable size, and biocompatibility. Although DNA-templated Ag NCs and DNA-templated Au NCs are reported as fluorescent probes in many bioassays [39–45], they show poor catalytic properties in enzyme-mimicking activity [46]. Recently, Pt nanomaterials were found to be unique peroxidase enzyme mimetic [47, 48]. Higuchi et al. established a colorimetric bioassay of thrombin based on a peroxidase-mimicking DNA–Pt complex [49]. Zheng et al. improved the previous work by developing a facile approach to produce DNA-Ag/Pt bimetallic nanoclusters with peroxidase-like catalytic activity for colorimetric detection of thrombin [46]. Then DNA-Ag/Pt NCs was applied in the colorimetric analysis of some analytes such as Hg^{2+} [50], L-Cys [51], the vascular endothelial growth factor (VEGF) [52]. In this article, the activity and inhibition of M.SssI MTase was explored in terms of the peroxidase-like activity of the DNA-Ag/Pt NCs because of its easy operation on unmodified oligonucleotides. M.SssI, a prokaryotic DNA MTase, was employed in this work since it is an important model and experimental tool in DNA methylation study that shares the specificity of eukaryotic DNA MTase [53]. We designed a C-rich ds-DNA as a synthesis template with internal CCGG palindromic site it is recognizable by M.SssI. It was found that DNA methylation by M.SssI can effectively inhibit the catalytic activity of DNA-Ag/Pt NCs selectively. Based on this finding, a colorimetric assay for DNA MTase activity detection was developed. The changes in blue color intensity were monitored to quantify the M.SssI activity. A LOD of 0.05 U/mL was obtained with no amplification. Further study of the inhibition of atypical drug on the activity of M.SssI MTase was also investigated. This method was designed without employing methylation-specific restriction endonuclease beside MTase enzyme. Also, to the best of the authors' knowledge, it is the first DNA MTase colorimetric assay that does not use gold nanoparticles or any amplification strategy, which were employed by previous reported assays [36, 38]. This method has significant advantages, including reproducibility, high sensitivity, good selectivity, rapidity, and cost effectiveness.

Experimental

Reagents and equipment

CpG methyltransferase (M.SssI) and SAM were purchased from Thermo Scientific. According to the supplier, M.SssI is stored at –20 ^{0}C in a buffer containing 10 mM potassium phosphate (pH 7.0), 400 mM KCl, 1 mM DTT, 1 mM EDTA, 0.2 mg/mL BSA, and 50% (v/v) glycerol. $AgNO_3$ and sodium borohydride ($NaBH_4$) were purchased from Merck. 3, 3′, 5, 5′-tetramethylbenzidine (TMB), and K_2PtCl_4 was purchased from Sigma Aldrich.

The base sequences of the oligonucleotides use in this work are listed in the Electronic Supplementary Material (ESM), Table S1. The oligonucleotides were synthesized and purified with PAGE by Shanghai Generay Biotech Co. The oligonucleotides were prepared with TE buffer (containing 1 MTris-HCl and 0.5 mL EDTA, pH 7.5). All chemicals were of analytical grade and used without further purification.

Equipment

Ultraviolet-Visible (UV-Vis) spectra were measured using Specord 250 spectrophotometer (Analytik Jena, Germany).

Characterization of DNA-Ag/Pt NCs was determined by Transmission Electron Microscope (TEM) (Zeiss, EM10C, 80 KV, Germany) and Atomic Force Microscopy (Multimode 8, AFM, Bruker, USA). Energy dispersive X-ray (EDX) was recorded on MIRA III field emission scanning electron microscope (TESCAN, Czech Republic). The annealing process was carried out with a Sprint thermal cycler (Themo Scientific Hybaid, USA).

Preparation of DNA-Ag/Pt NCs

The DNA-Ag/Pt NCs have been prepared according to the method described by Zheng et al. [46] with a minor modification. Annealing reaction of two oligonucleotides was carried out by heating to 90 ^{0}C followed by slow cooling for 1 h in a thermocycler. Briefly, $AgNO_3$ solution (50 μL, 150 μM), K_2PtCl_4 solution (120 μL, 125 μM), and the templatesS1 and S2 DNA solution (300 μL, 2 μM each) in phosphate buffer (10.0 mM, pH 7.4) were added together. After incubation for 30 min in the dark, freshly prepared $NaBH_4$solution (40 μL, 5 mM) was added immediately under vigorous shaking for 5 min in order to initiate the reduction reaction. Finally, the mixture was allowed to react at 37 °C for 3 h. The obtained DNA-Ag/Pt NCs can be stored at 4 °C for 1 mo.

Gel electrophoresis analysis

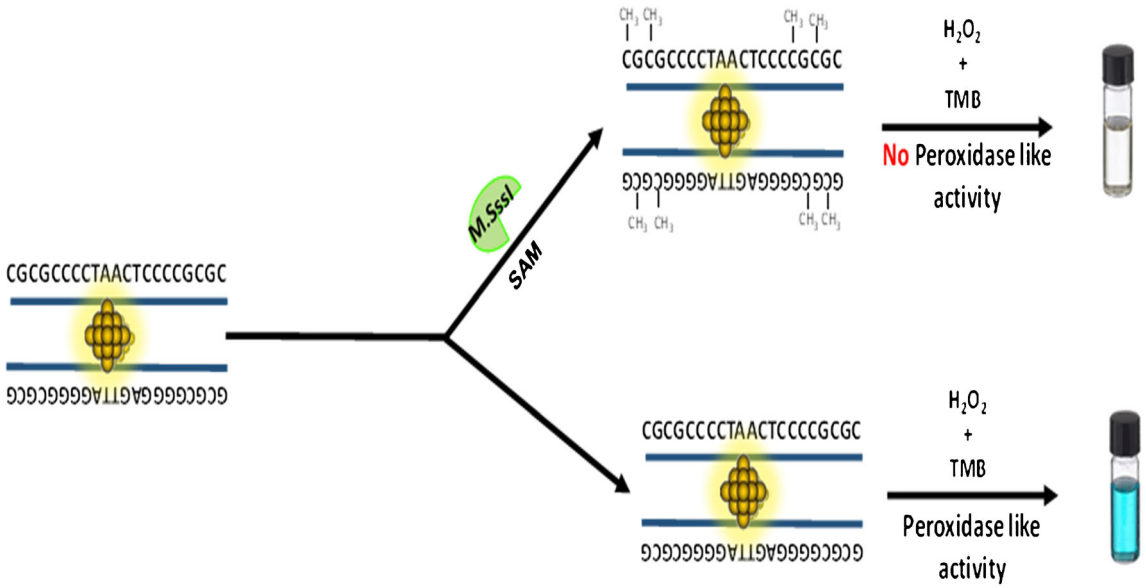
Gel electrophoresis was carried out to verify the hybridization of Cytosine-rich DNA strand and the complementary strand. The experiment was performed in phosphate buffer 10.0 mM, (pH 7.4), same buffer used for the formation of the nanoclusters. All oligonucleotides were diluted to 4 μM solution in buffer, and 15 μL of unmethylated (S1) or methylated (S3) sequence were mixed to 15 μL of complementary strand (S2) to obtain 2 μM final concentration. The mixtures were heated for 5 min at 95 °C and cooled for 1 h at 20 °C in a thermocycler. The samples were loaded on a polyacrylamide gel (10 % polyacrylamide in 1×TBE- 89 mM Tris–boric acid, pH 8.0, 0.002 M EDTA pH 8.0) to identify double-stranded nanocluster templates. Electrophoresis was carried in 1×TBE) at 40 V constant voltage for 2 h at room temperature. After staining with SYBR Gold nucleic acid gel stain solution (Invitrogen), the gel was imaged using a Gel Doc 1000 (Biorad). Gel images analysis was performed using ImageJ software (imagej.nih.gov). For the analysis of the intensity of the bands, the profiles of gel lanes were plotted after background subtraction. Peaks where selected and integrated to get the area. The ratio between the peak areas was then calculated.

Catalytic activity evaluation of DNA-Ag/Pt NCs

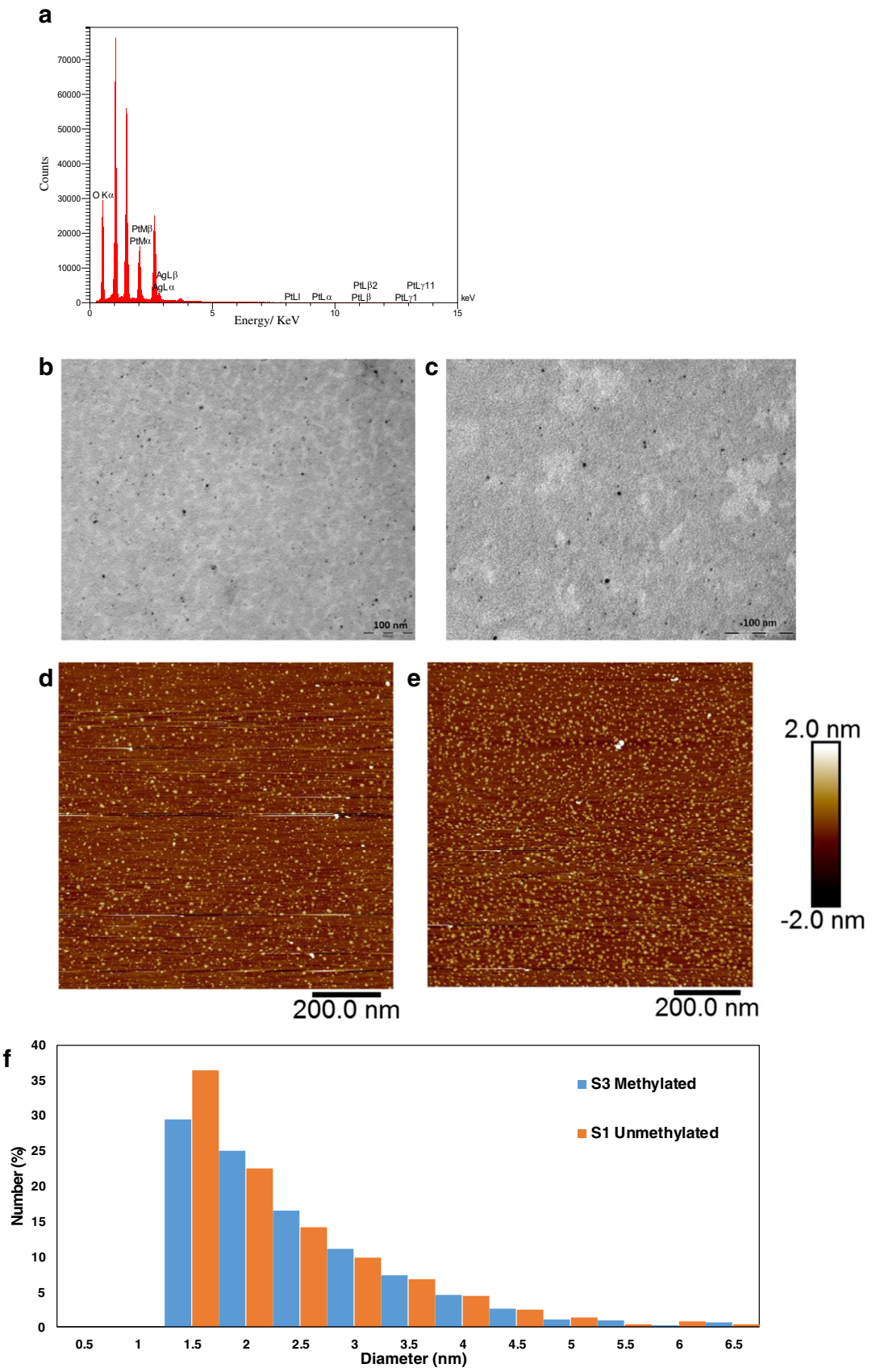
Time-dependent absorbance change was monitored at 652 nm at 25 °C (see ESM Fig. S1). In order to detect the peroxidase-like activity of DNA-Ag/Pt NCs in a typical colorimetric reaction, 10 μL of DNA-Ag/Pt NCs solution was added into 200 mM acetate buffer at pH 4.0; then 20 μL H_2O_2 0.5 M and 40 μL TMB 4 mM was added into above solution to initiate color reactions, which were monitored with the absorption at 652 nm. All the experiments were repeated three times for reproducibility.

M.SssI activity detection

To assay the M.SssI MTase activity, different concentrations of M.SssI with SAM substrate and buffer solution (10 mM potassium phosphate pH 7.0, 400 mM KCl, 1 mM DTT, 1 mM



Scheme 1 Schematic illustration of the proposed colorimetric assay for the detection of M.SssI MTase activity


a
Counts
Energy/ KeV
O Kα
PtMβ
PtMα
AgLβ
AgLα
PtLl
PtLα
PtLβ2
PtLβ
PtLγ11
PtLγ1
keV
b
100 nm
c
100 nm
d
200.0 nm
e
200.0 nm
2.0 nm
-2.0 nm
f
S3 Methylated
S1 Unmethylated
Number (%)
Diameter (nm)

◀ **Fig. 1** **(A)** EDX spectrum of DNA-Ag/Pt NCs. TEM images of synthesized Ag/Pt NCs by unmethylated **(B)** and methylated **(C)**. AFM images of synthesized Ag/Pt NCs by unmethylated **(D)** and methylated **(E)** DNA template. **(F)** DNA and size distribution histogram of Ag/Pt NCs of unmethylated and methylated DNA

EDTA, 0.2 mg/mL BSA, and 50% v/v glycerol) were added to a constant amount of DNA/Ag NCs solution. Then the mixtures was incubated at 37 °C for 30 min, Afterwards, 20 μL of H_2O_2 (0.5 M) and 40 μL of TMB (4 mM) were added. Finally, the mixture was incubated at 37 °C for 15 min, and absorbance values were recorded by UV-Vis spectrophotometer at 652 nm. Further color production of Ag/Pt NCs with methylated oligonucleotide and unmethylated oligonucleotide DNA templates were compared, which proved the methyl group effect on peroxidase-like activity of nanoclusters.

Selectivity and inhibition of M.SssI activity

BSA and Dpn I were selected as the potential interfering proteins. The selectivity experiments were conducted in the same way as the M.SssI activity detection procedure with 5 U mL^{-1} Dpn I and 1 μM BSA. Gentamicin inhibition effect on M.SssI MTase activity was evaluated by incubation of different concentration of gentamicin with DNA-Ag/Pt NCs and M.SssI MTase (10U/mL), SAM and buffer solution for 15 min at 37 °C. After the addition of H_2O_2 and TMB, the absorption at 652 nm was recorded.

Results and discussion

Principle of colorimetric M.SssI MTase activity detection

In this work, we present a facile strategy for the assay of M.SssI activity and the detection of DNA methylation based on DNA-Ag/Pt peroxidase-like activity of DNA-Ag/Pt NCs. The assay strategy is shown in Scheme 1. A cytosine-rich single stranded DNA and its complement were designed for this assay, based on previous works [46] with a slight modification. The subsequence 5′-CCGG-3′ was inserted inside, which is specifically recognized by M.SssI. After hybridization, the DNA duplex can serve as a template for Ag/Pt NCs formation by following the mechanism. Ag(I) has strong

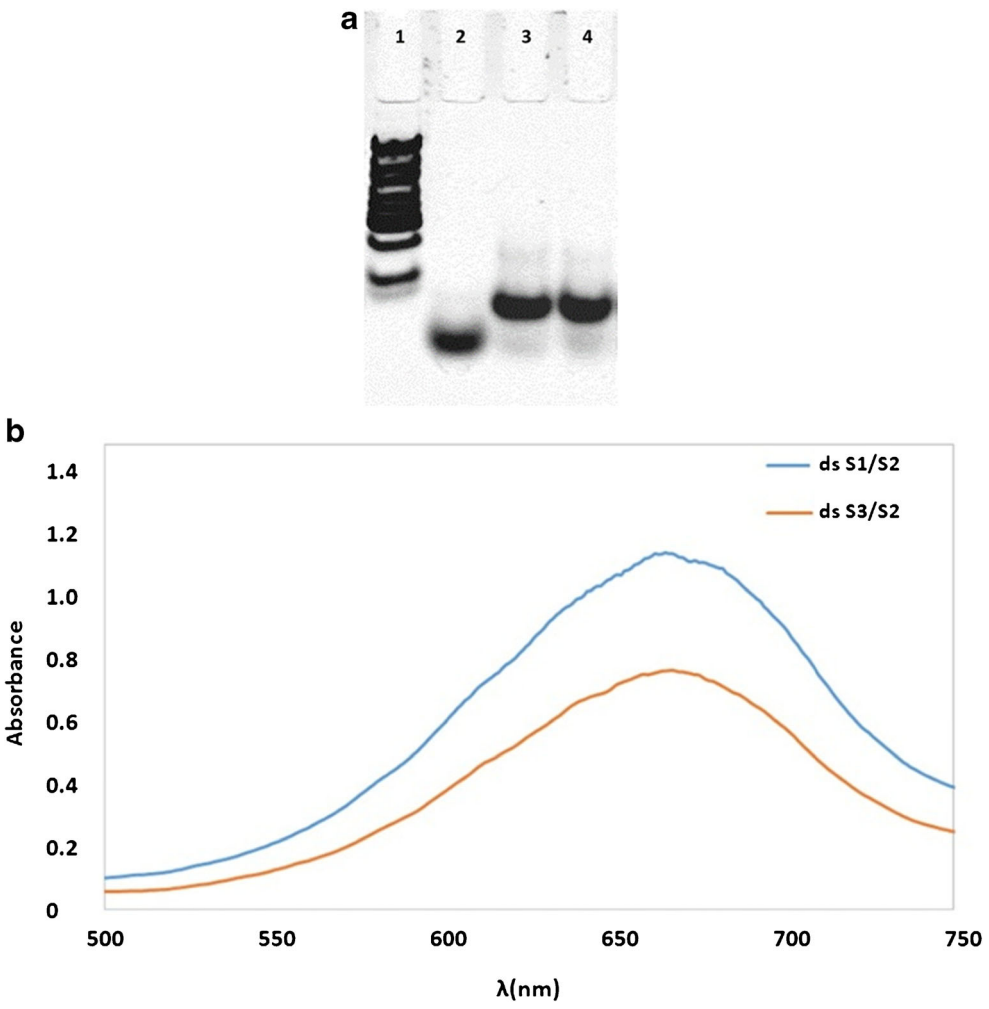
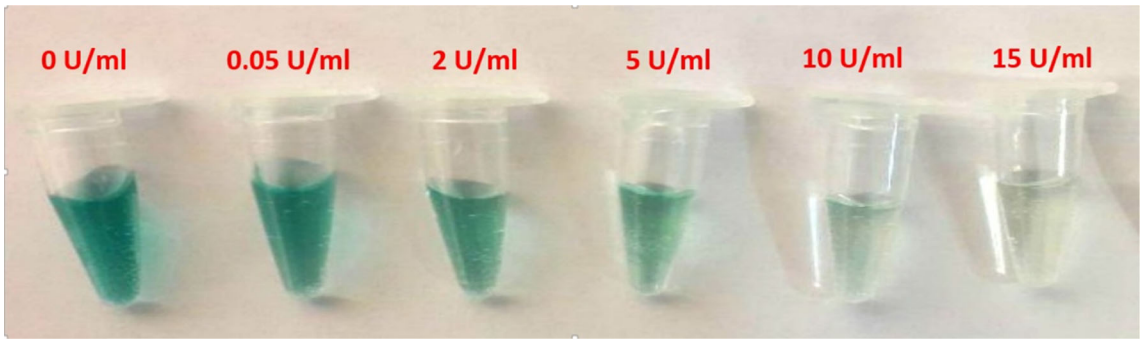
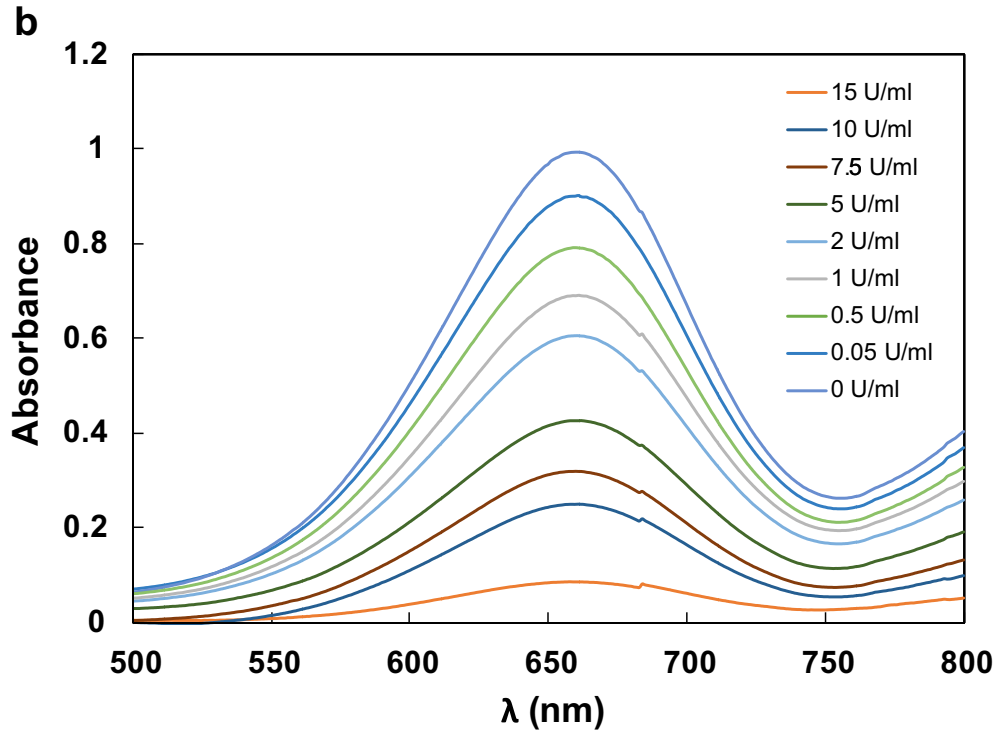
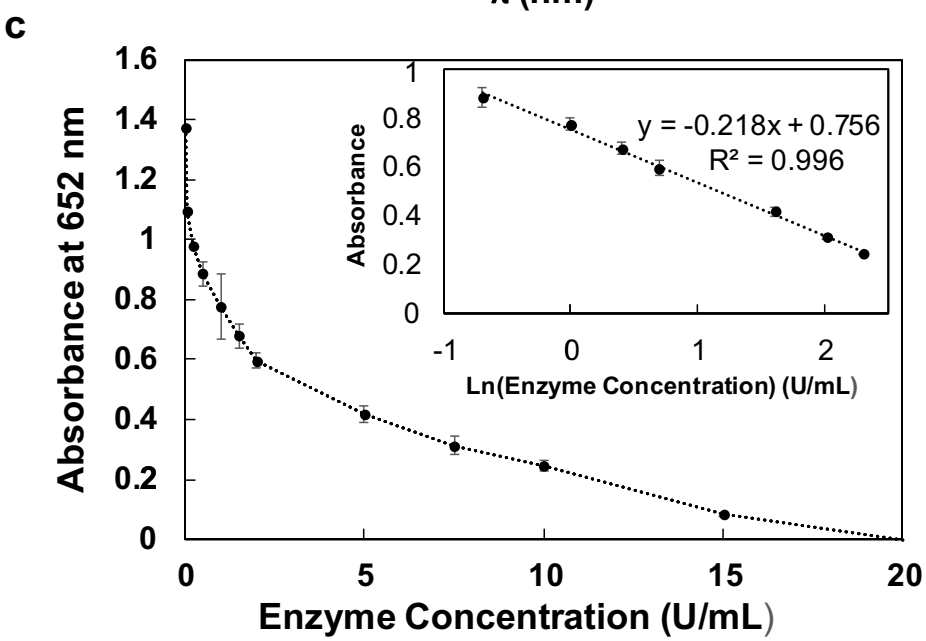


Fig. 2 **(A)** Gel electrophoresis analysis of double stranded template for Ag/Pt NCs formation. Lane 1: S1; Lane 2: S1/S2; Lane 3: S3/S2. **(B)** Spectrum of absorbance measured at 652 nm showing the catalytic activity of double stranded


a
0 U/ml
0.05 U/ml
2 U/ml
5 U/ml
10 U/ml
15 U/ml


b
Absorbance
λ (nm)
15 U/ml
10 U/ml
7.5 U/ml
5 U/ml
2 U/ml
1 U/ml
0.5 U/ml
0.05 U/ml
0 U/ml


c
Absorbance at 652 nm
Enzyme Concentration (U/mL)
y = -0.218x + 0.756
R² = 0.996
Absorbance
Ln(Enzyme Concentration) (U/mL)

◀ **Fig. 3** Changes in blue color intensity **(A)** photograph, and **(B)** absorption spectra in the presence of different concentration of M.SssI. The curves from bottom to top were obtained with different concentration 15, 10, 7.5, 5, 2, 1.5, 1, 0.5, 0.25, 0.05, and 0 U/mL M.SssI. **(C)** The relationship between the absorption intensity at 652 nm and the concentration of M.SssI MTase. The inset shows linear correlation between absorption intensity at 652 nm and the logarithm of M.SssI concentration in the range of 0.5 to 10 U/mL. Error bars show the standard deviation of three experiments

affinity to cytosine base and interacts with C-rich DNA and forms Ag NCs after being reduced with $NaBH_4$. Then, owing to galvanic replacement reaction between Ag(0) and Pt(II), Pt(0) growth would be initiated on the surface of Ag NCs and continue because of reduction with $NaBH_4$. Since the peroxidase-like activity of DNA-Ag/PtNCs is attributed to deposition of PtNCs onto AgNCs, AgNCs are considered to support for PtNCs deposition. Indeed, PtNCs could not be formed without presence of AgNCs [46]. It has been known for many years that silver has high affinity for binding to cytosine bases compared with the other bases. Methyl cytosine plays an intermediate role between cytosine and thymine, which has a low affinity for silver [54, 55]. As reported in the previous studies, addition of methylated cytosines in the DNA sequence resulted in the reduction of the fluorescence emission of DNA AgNCs in a linear range [39, 40]. This could be originating from the possible changes in orientation, intercalation, or interaction of Ag NCs with cytosine bases, which lead to formation of new silver atom bond lengths or angles. Figure S2 (see ESM) exhibits the fluorescence emission of the DNA/Ag NCs with methylated and unmethylated DNA templates. The fluorescence peak of Ag NCs decreased when methylated DNA was used as the nanocluster template and Ag/Pt NCs did not show any fluorescence. Thus, it can be concluded that formation of AgNCs is affected by the methylation of cytosines and consequently the deposition of PtNCs on AgNCs may be changed.

DNA-Ag/Pt NCs could catalyze the oxidation of TMB by H_2O_2, which produces a bright blue product and results in an intense absorption peak at 652 nm, which is the characteristic peak of oxidized TMB. Subsequently, in the presence of M.SssI MTase and SAM, the DNA hybrid was methylated resulting in decrease in DNA/Ag-Pt NCs catalytic activity. Therefore, MTase activity could be quantified by monitoring the change in produced blue color intensity of solutions. Since methylation of DNA can be restrained in the presence of inhibitors, this assay could also be used to screen M.SssI MTase inhibitors.

Characterization of synthesized Ag/Pt NCs

A typical energy-dispersive X-ray (EDX) spectrum of DNA-Ag/Pt NCs is shown in Fig. 1A, where elemental Ag and Pt are clearly detected, indicating the Ag/Pt bimetallic NCs have been formed successfully. The size and morphology of the obtained nanoclusters were characterized by using TEM (Fig. 1B and C). The DNA-Ag/Pt NCs were about 2 nm in size, roughly spherical, and well distributed. The results found in AFM imaging (Fig. 1D and F) matched those from TEM. TEM and AFM imaging do not show significantly different results on the nanoclusters obtained from methylated and unmethylated oligonucleotides, although it could be noted that for methylated DNA template a slightly higher percentage of nanoclusters are smaller than 1.5 nm, obviously paired to a lower percentage of nanoclusters in bigger size ranges compared with unmethylated DNA-Ag/Pt NCs (Fig. 1E). Even if this could be weakly related to the differences in catalytic activity, we conclude that nanoclusters are equally formed on both the methylated and unmethylated template DNAs and in general no strong differences in shape and dimensions can be appreciated.

The feasibility of the designed system

To demonstrate how practical the proposed strategy is to detect DNA methylation, some experiments were conducted. The specific sequence S1 and the methylated sequence S3 have been annealed to complementary sequence S2 to form double-stranded DNA (as verified by gel electrophoresis (Fig. 2A). Nanocluster formation was performed on double-stranded DNA S1/S2 and S3/S2, and the DNA-Ag/Pt NCs were tested for their catalytic activity (Fig. 2B). These results showed that both methylated and unmethylated DNA scaffold provide suitable condition for nanocluster formation, but as observed in the case of single-stranded DNA, the presence of methyl groups on cytosine residues reduced the catalytic activity of Ag/Pt NCs. This experiment is a further proof that the described strategy is suitable for a colorimetric assay of MTase activity.

Methyltransferase activity assay

To further investigate the analytical performance of the designed method for M.SssI activity analysis, after choosing the optimal conditions, DNA-Ag/Pt NCs were first incubated with M.SssI at various concentrations, then the colorimetric reaction described in section "M.SssI activity detection" was carried out. Subsequently the absorption spectrums was recorded with increase of M.SssI from 0.05 to 15 U/mL. As seen in Fig. 3A (left to right), the color changed from blue to colorless with the increase of the M.SssI concentrations. The corresponding UV vis absorption spectra are represented in Fig. 3B. As the concentration of M.SssI MTase increased, the absorption intensity decreased. Designed strategy showed a linear correlation with the logarithm of M.SssI enzyme concentration in the range of 0.5–10 unit/mL

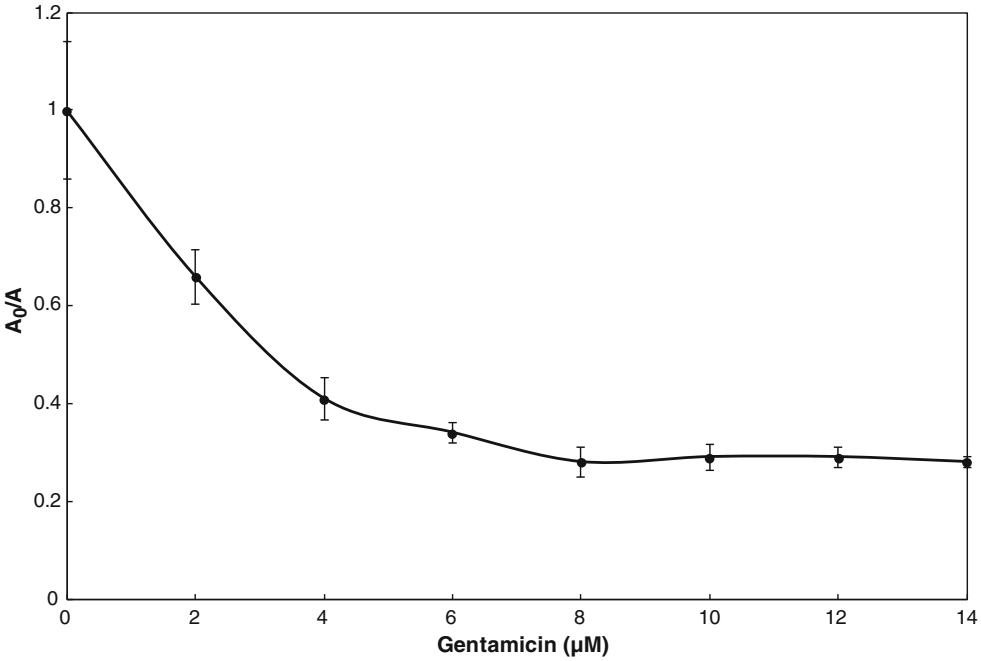


Fig. 4 Inhibitory effect of gentamicin on the activity of M.SssI. The concentration of M.SssI is 5 U/mL. (A_0 is absorbance intensity of Ag/Pt NCs incubated with 5 U/mL M.SssI, and A is the absorbance intensity of AgNCs incubated with 5 U/mL M.SssI with different concentration of gentamicin: 0, 2, 4, 6, 8, 10 μM). Error bars show the standard deviation of three experiments

M.SssI with a correlation coefficient of 0.996 and the LOD was 0.05 unit/mL, which is comparable to recent reports [31] (Fig. 3C).

M.SssI methyltransferase inhibition assay

To check the validity of our method in screening the inhibitors of M.SssI, we employed gentamicin as an inhibitor model [56]. We evaluated the inhibitor effects by incubating DNA-Ag/Pt NCs and M.SssI MTase and SAM with different doses of gentamicin (Fig. 4). The activity of the M.SssI decreases with the increase in concentration of gentamicin, showing a dose-dependent inhibition. The IC_{50} value is the inhibitor concentration required to reduce enzyme activity by 50%. In this study IC_{50} was found to be 3.1 μM, which indicates that this is a promising method to screen the inhibitor effects of drugs on MTase [56, 57].

Selectivity study

To evaluate the selectivity of the proposed assay, we investigated the effect of BSA and Dpn I as nonspecific enzymes. BSA is an irrelevant protein and DpnI is an enzyme that can only recognize the sequence (5′-GATC-3′) in ds DNA. As shown in Fig. 5, 5 U/mL M.SssI could induce a remarkable decrease of absorbance of DNA-Ag/Pt NCs solution. On the other hand, other proteins at concentration of 1 μM for BSA and 5 U/mL for DpnI had no obvious effects on absorbance,

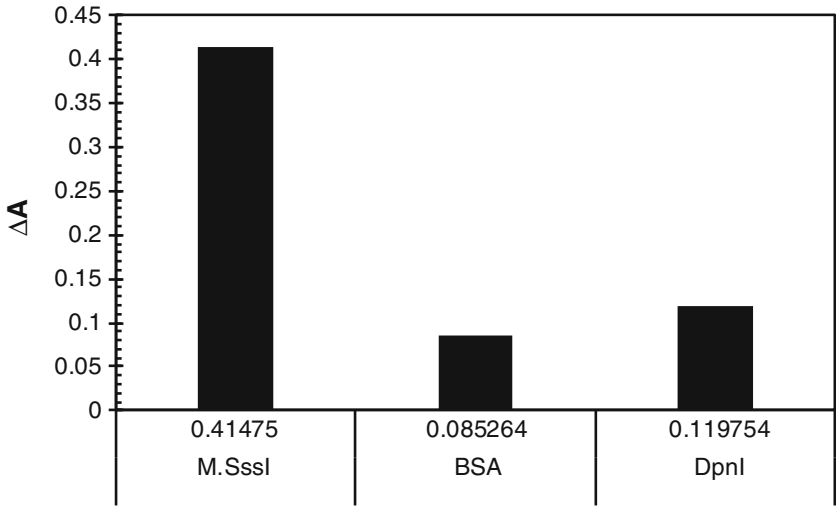


Fig. 5 Absorption response of the assay for different proteins: M.SssI, BSA, Dpn I. Error bars show the standard deviation of three experiments

indicating that our system could be a potential colorimetric tool for sensitive detection of M.SssI DNA methyltransferase activity.

Conclusion

We have successfully developed a novel Ag/Pt NCs-based colorimetric strategy for detecting MTase activity with high sensitivity and selectivity. To the best of our knowledge, it is the first DNA MTase colorimetric assay apart from the using of gold nanoparticles or amplification strategies. We attempted to characterize the DNA-Ag/Pt NCs and presented novel approach for better understanding of mechanism and the possible effects of methylation. The assay was designed without employing the endonuclease enzyme and any amplification step. The preparation of the DNA Ag/ Pt NCs probe including hybridization and reduction steps is facile, low-cost, and accessible to numerous laboratories. Moreover, there is no requirement of additional functionalization of DNA-templated nanoclusters with biomolecules and exploiting complex operations such as separation, precipitation, or washing. We have evaluated further inhibitor screening capability of the proposed method, which can be extended to screen other suitable inhibitors as anticancer and antimicrobial agent.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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