Gold nanorods-based FRET assay for ultrasensitive detection of DNA methylation and DNA methyltransferase activity

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A fluorescence method for the detection of DNA methylation and the assay of methyltransferase activity is proposed using gold nanorods as a fluorescence quencher on the basis of fluorescence resonance energy transfer. It is demonstrated that this method is capable of detecting methyltransferase with a detection limit of 0.25 U mL\(^{-1}\), which might make this method a good candidate for monitoring DNA methylation in the future.

1. Introduction

DNA methylation, a well-known epigenetic event, plays an essential role in regulating gene transcription, cell growth and proliferation.\(^1\) It has been well-confirmed that DNA methylation is carried out with the aid of methyltransferase (MTase) using S-adenosyl-L-methionine (SAMe) as the methyl donor.\(^2\) Aberrant gene-specific methylation can significantly alter gene expression and lead to numerous diseases.\(^3\) Thus, the development of simple, sensitive, and effective methods for DNA methylation detection and DNA MTase activity assay would be valuable for the diagnosis of genetic disease.

Currently, several new methods have been reported for the detection of DNA methylation, including polymerase chain reaction-based techniques,\(^4,5\) high-performance liquid chromatography,\(^6\) capillary electrophoresis,\(^7\) gel electrophoresis,\(^8\) electrochemistry,\(^9-14\) DNA microarray,\(^15\) light scattering,\(^16\) colorimetry,\(^17\) and fluorescence methods.\(^18-20\) Interestingly, with the development of materials science and nanotechnology, several nanomaterials, such as gold nanoparticles,\(^21,22\) magnetic nanoparticles,\(^23\) graphene,\(^24\) and carbon nanoparticles,\(^25\) are exploited for the quantification of methylation. Most of these methods, however, have the limitation of being time-intensive, DNA-consuming, requiring laborious treatment, and usually having a radiolabeling substrate requirement. Therefore, it is highly necessary to develop sensitive, simple, and effective methods for the detection of DNA methylation and the assay of MTase activity.

Recently, gold nanorods (AuNRs) received widespread interest because of their anisotropically configuration and unique optical properties. The AuNRs have been applied for chemical and biochemical sensing with applications, including sensing metal ions,\(^26,27\) antibodies,\(^28,29\) DNA,\(^30,31\) and in photo-thermal therapy.\(^32,33\) More recently, the interaction between DNA and AuNRs has attracted significant attention and has undergone substantial growth due to their collective behaviour.

Herein, we proposed a simple, sensitive and cost-effective homogeneous assay for methyltransferase activity and inhibition study that utilizes fluorescence resonance energy transfer (FRET) between AuNRs and fluorescein (FAM)-tagged single-stranded DNA. As illustrated in Scheme 1, the assay is based on the fact that positively charged AuNRs have a higher affinity for double-stranded DNA (dsDNA) than for single-stranded DNA because the surface charge density of dsDNA is significantly larger than that of ssDNA. Dam MTase and Dpn I were chosen as the model MTase and restriction endonuclease, and a FAM-labelled DNA (FDNA1) and complementary DNA (cDNA1) were chosen as the substrate DNA. In the absence of methyltransferase, the fluorescence of FDNA1 was quenched...
because of the electrostatic attraction-based FRET between AuNRs and FDNA1. The formation of FDNA1/cDNA1 hybrid enhanced the electrostatic interaction between AuNRs and DNA, leading to an increase in FRET efficiency and a significant decrease in fluorescence intensity. However, when FDNA1/cDNA1 was treated with Dam MTase, methylate was generated. Fluorescence enhancement was observed on further treatment with Dpn I, which cleaved FDNA1/cDNA1 hybrid substrate into small fragments. Because the electrostatic interaction between AuNRs and small DNA fragments is weaker than that between FDNA1 and cDNA1, the fluorescence of the system was restored.

2. Experimental section

2.1 Reagents and materials

Tetrachloroauric acid trihydrate (HAuCl₄·3H₂O), sodium borohydride (NaBH₄), cetyltrimethylammonium bromide (CTAB), and ascorbic acid were purchased from Chengdu Chemical Reagent Co., Chengdu, China. All the chemicals were of analytical reagent grade and were used without further purification. The DNA adenine methylation methyltransferase (Escherichia coli), Dpn I endonuclease, S-adenosyl-L-methionine (SAMe), and the corresponding buffer solutions were purchased from New England Biolabs (NEB, UK). All these standard solutions were prepared under 4 °C. DNA oligonucleotides were synthesized by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd., Shanghai, China. DNA stock solutions (1 mM) were prepared using 10 mM HCl buffer solution (pH 7.4) containing 0.5 M NaCl and maintained at −20 °C. DNA oligonucleotides were synthesized by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd., Shanghai, China. DNA stock solutions (1 mM) were prepared using 10 mM HCl buffer solution (pH 7.4) containing 0.5 M NaCl and maintained at −20 °C. The sequences of oligonucleotides used in this work are listed as follows:

FAM-DNA1 (FDNA1): 5′-CCCTTTGATCATTTT-FAM-3′; cDNA1: 3′-GGAAAACCTAGTTAAA-5′; cDNA2: 3′-GGAAACTCGTAAA-5′.

2.2 Apparatus

Fluorescence spectra were recorded using a Hitachi F-2700 fluorescence spectrophotometer (Hitachi Ltd., Japan) equipped with a xenon lamp excitation source. The samples were excited at 480 nm with an emission range from 510 to 620 nm. pH values were measured using a pH-3C precision pH meter (Leici Device Factory of Shanghai, China). Ultraviolet-visible absorption spectra were measured using a Shimadzu UV-2450 spectrophotometer (Suzhou Shimadzu Instrument Co., Ltd., China). Transmission electron microscopy (TEM) image was obtained with a JEM-2000 instrument at 200 kV.

2.3 Preparation of gold nanorods

AuNRs were synthesized by a chemical reduction process using a seeding growth method, as previously reported by Murphy. Briefly, the seed solution was prepared by mixing HAuCl₄ (250 μL, 10 mM) and CTAB (7.5 mL, 0.1 mM) in a beaker. Then, freshly prepared ice cold NaBH₄ (600 μL, 10 mM) solution was added all at once under stirring for 2 min. The solution immediately turned brown after adding NaBH₄, indicating the formation of gold seeds. The seed solution was maintained at room temperature for 2 h and then used for the subsequent growth of AuNRs.

In the growth solution, CTAB (40 mL, 0.1 M) was mixed with HAuCl₄ (1.7 mL, 10 mM) and AgNO₃ (250 μL, 10 mM). After gentle mixing, 270 μL of ascorbic acid was slowly added to the solution. The solution immediately became colorless immediately after the addition of the ascorbic acid. Finally, 0.42 mL of gold seed solution was added to the growth solution mentioned above to initiate the growth of AuNRs. The solution immediately turned blue purple immediately after adding the gold seed solution, indicating the generation of AuNRs. The AuNRs solution was kept undisturbed at 30 °C for at least 16 h. Afterwards, excess CTAB was removed from the solution by repetitive centrifugations at a rate of 10 000 rpm for 10 min, and AuNRs were re-dispersed in distilled water. The AuNRs were stored in a refrigerator at 4 °C before use. The resulting AuNRs were characterized by transmission electron microscopy (TEM) and ultraviolet-visible absorption spectroscopy (UV). The TEM image shows that the obtained AuNRs were nearly mono-dispersed with an average length of 30.24 nm and width of 9.73 nm (Fig. 1A). The UV-Vis absorption spectrum of the initial AuNRs exhibits two peaks at 513 nm and 763 nm (Fig. 1B). The concentration of AuNRs (0.36 nm) was estimated by UV/vis spectroscopy based on an extinction coefficient of 4.2 × 10³ M⁻¹ cm⁻¹ at λ = 763 nm for AuNRs.

2.4 Assay of Dam methyltransferase activity

A series of standard Dam MTase solutions were prepared from 0.5 to 200 U mL⁻¹. To protect the activity of Dam MTase, all the standard solutions were prepared at 4 °C and then stored at −20 °C. The methylation experiment was conducted at 37 °C for 2 h, and incubated in 20 μL of Dam MTase buffer (50 mM NaCl, 10 mM Tris–HCl, 10 mM MgCl₂, pH 7.5) containing 1 μM FDNA1, 1 μM cDNA2, 80 μM SAMe, 4 U of Dpn I endonuclease and different amounts of Dam MTase. When the dsDNA molecules were methylated by DNA MTase and cleaved by Dpn I, 10 μL of 0.36 mM as-prepared CTAB coated AuNRs suspension was added to the system. The mixture was sequentially added to 470 μL of 10 mM Tris–HCl buffer solution (pH 7.4) and incubated for 5 min. Fluorescence measurements were performed using a Hitachi F-2700 fluorescence spectrophotometer at excitation and emission wavelengths of 480 and 528 nm, respectively.

![Fig. 1](image-url) (A) TEM image of AuNRs. Inset shows that the AuNRs have a length of 30.24 nm and a width of 9.73 nm. (B) The corresponding absorption spectrum of AuNRs.
2.5 Evaluation of DNA methylation inhibitor

To further extend the potential application of this assay in the inhibition assay, the influence of drugs on Dam MTase activity was investigated by using 5-fluorouracil (anti-cancer drug) as a model inhibitor of Dam MTase. The inhibition experiments were similar to that described above, except that the drug was added along with Dam MTase.

3. Result and discussion

3.1 Sensing strategy

To the best of our knowledge, FRET will take place occurs when there is an appreciable overlap between the emission spectrum of the donor and the absorption spectrum of the acceptor. As illustrated in Fig. 2, the absorption spectrum of as-prepared AuNRs shows two absorption peaks at 513 and 763 nm, respectively. At the same time, the FAM-DNA possesses an emission maximum at 528 nm. Thus, AuNRs can act as electron acceptors to quench fluorophores in the photoinduced electron transfer process because the AuNRs have an absorption peak, which overlaps with the emission peak of FAM.

Fig. 3 shows the fluorescence spectra under different conditions. If the solution only contained FDNA1, a strong fluorescence signal was detected (curve a). In the absence of methyltransferase, the fluorescence of FDNA1 was quenched because of electrostatic attraction-based FRET between AuNRs and FDNA1 (curve b). Upon the addition of complementary cDNA1 to FDNA1, the formation of FDNA1/cDNA1 hybrid enhanced the electrostatic interaction between AuNRs and the DNA, leading to an increase in FRET efficiency and a significant decrease in fluorescence intensity (curve c). However, if FDNA1/cDNA1 was treated with Dam MTase, DNA hybrid was methylated by methyltransferase. Fluorescence enhancement was observed when the DNA hybrid was further treated with Dpn I, which cleaved FDNA1/cDNA1 hybrid substrate into small fragments, leading to the weakening of electrostatic interaction between AuNRs and small DNA fragments. Thus, the fluorescence of the system was restored (curve d). By monitoring the change in fluorescence, the activity of Dam MTase and its inhibition were systematically investigated, thus providing a practical tool for studying DNA methylation and the assay of activity of methyltransferase and its inhibition.

The sensing mechanism has been was further verified by TEM images and fluorescence anisotropy. It can be seen from images a and b in Fig. 4 that AuNRs are well-dispersed in the aqueous medium in the absence and presence of single-stranded DNA1, illustrating that the electrostatic interaction between single-stranded DNA1 and AuNRs is not strong enough to change the suspension state of AuNRs. However, AuNRs aggregated when DNA1/cDNA1 duplex formed in the presence of cDNA1 (see image c of Fig. 4). It was found that CTAB-coated AuNRs without any modification were stable for 4 months at 4°C. Therefore, the aggregation of AuNRs is largely because of the strengthened electrostatic interaction between dsDNA and AuNRs because dsDNA has a higher density of surface charge than ssDNA. However, the aggregation of AuNRs is significantly reduced in the presence of Dam MTase and Dpn I endonuclease that can cleave the DNA1/cDNA1 hybrid at a specific recognition site, which is mainly attributed to the weakened electrostatic attraction between the short DNA fragment and AuNRs. This is in accordance with the fluorescence measurements. Therefore,
this FRET system was again demonstrated to be reasonable and reliable for assaying the activity of DNA MTase.

3.2 Optimization of experimental conditions

To the best of our knowledge, DNA methylation was affected by several molecular species. To achieve the best conditions for the performance of DNA methylation assay, several variables of the monitoring system should be optimized. First, the influence of the amount of AuNRs on DNA methylation assay was investigated by comparing the FRET efficiency. The quenching rate ($Q$) and recovery rate ($R$) were calculated using the following equations:

$$Q = \frac{F_0 - F}{F_0} \times 100\%$$

$$R = \frac{F_a - F}{F_0 - F} \times 100\%$$

where $F_0$ and $F$ are the fluorescence of FDNA/cDNA1 in the absence and presence of AuNRs, respectively. $F_a$ is the fluorescence of the FDNA/cDNA1 duplex and AuNRs system after the addition of MTase and Dpn I. As shown in Fig. 5A, when 10 µL of 0.36 mM AuNRs was added to the measuring system, both the fluorescence quenching and recovering efficiency attained a good response. Thus, the optimal amount of AuNRs is 10 µL.

Second, the methylation time was studied. As illustrated in Fig. 5B, the fluorescence intensities reached a maximum value at 120 min and almost remained constant after that. Therefore, 120 min was chosen as the methylation time in the following studies. It is reported that Mg$^{2+}$ is an essential coenzyme for restriction endonuclease (including Dpn I endonuclease) and hinders the process of methylation. Fig. 5C shows the influence of Mg$^{2+}$ concentration on DNA methylation. The velocity of DNA methylation and cleavage reached the maximum when the Mg$^{2+}$ concentration was 10 mM. Thus, the optimum concentration of Mg$^{2+}$ for the system was 10 mM. Another important factor for DNA methylation is the concentration of SAMe. As can be seen in Fig. 5D, the methylation/cleavage velocities increased on varying the concentration of SAMe from 0 to 160 µM. The initial methylation/cleavage velocities increased slowly when the concentration of SAMe exceeded 80 µM. In addition, SAMe was not very stable during in vitro experiments. Therefore, the concentration of SAMe was optimized to 80 µM.

3.3 DNA adenine methylation methyltransferase detection

Under optimized conditions, the fluorescence spectra of the FDNA1/DNA1 probe at different concentrations of Dam MTase solutions were recorded, and are shown in Fig. 6A. It can be seen from Fig. 6A and B that fluorescence intensity gradually increased with the increasing concentration of Dam MTase. This is consistent with the fact that in the presence of higher concentrations of Dam MTase, more dsDNA probes were methylated and cleaved into small ssDNA fragments by Dpn I, resulting in a decrease in electrostatic interaction with AuNRs, which led to the recovery of fluorescence. As illustrated in Fig. 6B, the calibration curve (Fig. 6B, inset) shows that the fluorescence intensity is proportional to the logarithmic value of Dam MTase concentration ranging from 0.5 to 20 U mL$^{-1}$. 

Fig. 5 Effect of species on methylation. (A) Effect of the amount of AuNRs, (B) effect of methylation time, (C) effect of the concentration of Mg$^{2+}$, and (D) effect of the concentration of SAMe. The concentrations of FDNA1 and cDNA1 were 40 nM. The methylation experiment was conducted at 37 °C for 2 h. Relative initial velocity = $F_n/F_0$, where $F_n$ is the fluorescence of the methylation/cleavage reaction with different concentrations of Mg$^{2+}$ and SAMe, and $F_0$ is the fluorescence of methylation/cleavage reaction without Mg$^{2+}$ and SAMe.

Fig. 6 (A) Fluorescence emission spectra of the FDNA1/DNA1 probe with increasing concentration of Dam MTase. The concentrations of Dam (from bottom to top): 0, 0.5, 1.0, 2.5, 5, 10, 20, 30, 50, 75, 100, 150, 200 U mL$^{-1}$. (B) The response of fluorescence intensity versus the concentration of Dam MTase.
with a detection limit of 0.25 U mL$^{-1}$ at a signal/noise ratio of 3, which is low compared to some previous reports, suggesting that this method is efficient to detect DNA methylation and the assay activity of Dam MTase.

3.4 Investigation of the selectivity of the proposed method

To investigate the selectivity of the proposed method, a one-base mismatched single strand DNA (cDNA2) was chosen to evaluate the cleavage selectivity of Dpn I. As shown in Fig. 3 (curve e), unchanged fluorescence intensity was observed in the FDNA1/cDNA2 system, indicating that Dpn I could not cleave FDNA1/cDNA2 hybrid because Dpn I can only recognize the sequence 5′-G-A-T-C-3′. This result demonstrates that the present approach has high sequence selectivity for Dam MTase.

3.5 Estimation of the inhibition of DNA adenine methylation methyltransferase activity

In addition, we investigated whether our method can be applied for evaluating and screening Dam MTase inhibitors such as antibiotics and anticancer drugs. In the present work, 5-fluorouracil, an anticancer drug, was chosen as a model inhibitor. As shown in Fig. 7, the relative activity of Dam MTase decreased with the increasing concentration of 5-fluorouracil, indicating that the activity of Dam MTase was inhibited. This was in accordance with the dose-dependent inhibition of drugs. Therefore, the proposed method was available to screen the inhibitors to the DNA MTase.

4. Conclusions

In summary, a fluorescence method for the detection of DNA methylation and assay of methyltransferase activity is proposed with AuNRs as a fluorescence quencher on the basis of FRET. The detection limit of the method is as low as 0.25 U mL$^{-1}$, which might promise this method to be a good candidate for monitoring DNA methylation in the future. Furthermore, the protocol may offer new prospects for evaluating and screening the inhibitors of methyltransferase, which might be a great help for the discovery of anticancer drugs.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (nos 21273174 and 20975083) and the Municipal Science Foundation of Chongqing City (no. CSTC-2013jjB00002).

Notes and references