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Fluorescent copper nanoclusters as a nano-dye for DNA methyltransferase activity analysis and inhibitor screening

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ABSTRACT

Fluorescent copper nanoslusters (CuNCs) as a new class of fluorophores have attracted more and more attention due to their ease of synthesis, excellent optical properties, and low cost. In this study, a novel label-free fluorescent method was developed for the detection of DNA methyltransferases based on template length-dependent of dsDNA-CuNCs. In the absence of DNA adenine methylation methyltransferase (Dam MTase), the dsDNA containing the methylation-responsive sequence could effectively template the formation of fluorescent CuNCs with bright fluorescence. When the dsDNA substrate is methylated by Dam MTase, the methylation-sensitive restriction endonuclease Dpn I cleaves the methylated dsDNA and produces shorter dsDNA product, which fails to template fluorescent CuNCs. So, the Dam MTase activity could be identified by the changes of CuNCs' fluorescence. Based on this method, a linear range of 0.5–10 U/mL was achieved with high sensitivity and selectivity. Moreover, we also demonstrate the proposed method can be applied to evaluation and screening of inhibitors for Dam MTase.

1. Introduction

Due to their unique physical, electrical, and optical properties, ultrasmall fluorescent nanoparticles have obtained extensive applications, ranging from energy conversion and storage to biomedical imaging [1–4]. The ultrasmall fluorescent nanoparticles mainly included semiconductor nanocrystals (usually referred to as quantum dots (QDs)), fluorescent metal nanoclusters, carbon-based nanomaterials, up-conversion nanocrystals, and silicon nanoparticles. Among them, fluorescent metal nanoclusters (e.g., Au, Ag, and Cu) have attracted special research interest in the past decade due to their ease of synthesis, extreme brightness, low-toxicity, and good biocompatibility [5]. In general, fluorescent metal nanoclusters can be prepared by reduction of metal precursors or etching of large nanoparticles in the presence of strong stabilizers such as small thiol-molecules, polymers, proteins, peptides, and DNA [6]. In addition to binding to complementary nucleic acids and non-nucleic acid targets (aptamers) [7], the flexibility, nanosized structure, excellent programmable properties make the DNA molecules good templates for the synthesis of luminescent metal nanoclusters [8]. Since the first successful demonstration of DNA templated silver nanoclusters (AgNCs) by the group of Dickson in 2004 [9], DNA-AgNCs have been widely studied and successfully applied for bioimaging and biosensing [10–12]. In 2010, Mokhir et al. have reported that the random dsDNA could act as an efficient template for the formation of fluorescent copper nanoclusters (CuNCs) at a low concentration of $CuSO_4$, whereas the random ssDNA or triplex template can not [13]. Subsequently, Qing et al. found that specific ssDNA (poly T) could also template CuNCs with excellent fluorescence [14]. Those DNA-CuNCs could be facilely prepared within 5 min at room temperature by reducing Cu^{2+} ions with ascorbic acid in the presence of nucleic acid. Due to their ease of synthesis and excellent fluorescence properties, DNA-CuNCs have been used as fluorescent indicators in the field of biochemical analysis [15–17]. More recently, they also investigated the effect of sequence composition on fluorescence of dsDNA templated CuNCs, and found that the dsDNA templated CuNCs were poly (AT-TA) dependent [18]. In another word, the fluorescence intensity of dsDNA-CuNCs is highly-dependent on the polymerization degree and the length of poly (AT-TA). Only the relatively long poly (AT-TA) could template fluorescent CuNCs formation, while the fluorescence induced by poly (AT-TA) of less than 12 base pairs (bp) was negligible practically, which holds an immense potential for biochemical sensing based on length-change of nucleic acid.

DNA methylation is associated with transcriptional silencing of tumor suppressors or other genes important for normal cellular function

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and plays an important role in the development of cancer and other diseases (such as breast and colorectal cancers) [19]. DNA methyltransferases (MTases) are a family of enzymes that could recognize particular short palindromic sequences and transfer a methyl group from S-adenosyl- L-meth-ionine (SAM) to target adenine or cytosine residues in the palindromic sequences during the biological DNA methylation process [20]. An abnormal level of DNA MTase leads to the aberrant level of DNA methylation which has been regarded as biomarkers of early cancers [21]. Accordingly, DNA MTase is regarded as a potential target for anticancer therapy and drug screening [22,23]. Therefore, monitoring the DNA MTase activity and screening inhibitors (anti-methylation drugs) are of significance in fundamental biochemical research, clinical diagnostics, drug discovery, and disease therapy. Up to now, many analytical methods including high performance liquid chromatography, radioactive labeling, methylation-specific polymerase chain reaction and gel electrophoresis have been employed for detection of DNA MTase activity [24–28]. However, these methods are time-intensive, laborious, or require isotope labeling. In order to address these issues some new approaches have been introduced such as electrochemical, fluorescence, colorimetry, and bioluminescence based techniques [29–34]. For instance, Zhou et al. developed an electrochemical immunosensing platform for DNA mehtyltransferase activity analysis and inhibitor screening [35]. Zhang et al. develop a sensitive fluorescence method for DNA MTase activity based on RNA polymerase-mediated transcription amplification and duplex-specific nuclease assisted cyclic signal amplification [36]. Jiang and coworkers developed a novel biosensor platform for colorimetric detection of DNA methyltransferase based on terminal protection of the DNA-gold nanoparticle probes by mechanistically covalent trapping of target enzymes [37]. These methods have provided considerable advancements, but the design and modification of these strategies are still not facile enough. Moreover, most of the proposed fluorescence sensors require double-labeled oligonucleotide substrates, which will not only increase the cost of sensing system but also probably reduce the cleavage efficiency. Therefore, substantial efforts were still needed for the development of simple, label-free and efficient methods to meet the increasing demand for detecting DNA MTase activity.

To address these challenges, based on the excellent fluorescence performance of dsDNA-CuNCs, a label-free and sensitive fluorescent method was developed for detection of DNA MTase activity and inhibitor screening. In this method, a (AT/TA)-rich dsDNA containing the methylation-responsive sequence is ingeniously designed, and in the absence of Dam MTase, the relatively long (AT/TA)-rich dsDNA could template the formation of CuNCs with bright fluorescence. Once Dam MTase is added, the specific methylation-sensitive restriction endonuclease Dpn I cleaves the methylated dsDNA and produces shorter dsDNA, which fails to template fluorescent CuNCs. Thus, the Dam MTase activity could be identified by the changes of CuNCs' fluorescence.

2. Experimental

2.1. Materials and reagents

The DNA oligonucleotides used in this work were synthesized and HPLC-purified by Shanghai Sangon Biological Engineering Technology & Services Co. Ltd. (Shanghai, China). The sequences of the oligonucleotides used in this work were shown in Table S1. Dam MTase, Hha I MTase, Msp I MTase, Dpn I endonuclease, S-adenosylmethionine (SAM) and BSA were purchased from New England Biolabs (Beijing, China). 3-(*N*-Morpholino) propanesulfonic acid (MOPS), sodium chloride, copper sulfate, and sodium ascorbate were commercially obtained from Dingguo Biotechnology Company, Ltd. (Beijing, China), they were at least analytical grade and used without further treatment. 5-fluorouracil was purchased from New England Biolabs (Beijing, China). The reaction buffer (50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, pH 7.5) was used for Dam MTase dilution and enzymatic digestion reaction. 1 mg/mL BSA was mixed into the buffer to reduce the absorption of Dam MTase onto the plastic wall in the dilution and reaction process. The MOPS buffer (10 mM MOPS, 150 mM NaCl, pH 7.6) was used for the formation of fluorescent CuNCs. Deionized water, prepared through the Nanopure In finity ultrapure water system (Barnstead/Thermo-lyne Corp.) was used throughout the experiments. The serum samples were obtained from a health volunteer in Langfang People's Hospital (Langfang, China), and informed consent was obtained for the use of human serum. All experiments were performed in compliance with the relevant laws and institutional guidelines and approved by the Life-Science Ethics Review Committee of Langfang People's Hospital.

2.2. Apparatus

All fluorescence measurements were performed on an F-7000 fluorescence spectrophotometer (Hitachi, Japan). Both the excitation and emission slit were set at 5.0 nm, with a 700 V PMT voltage and a 0.2 s response time. The fluorescence emission spectra of CuNCs were collected from 500 nm to 660 nm at room temperature with a 340 nm excitation wavelength. All fluorescence measurements were carried out at room temperature unless stated otherwise.

2.3. Detection of dam MTase activity

To avoid intramolecular structures, all sequences used in this work are annealed by heating to 95 °C for 5 min and followed by slowly cooling down to room temperature. Typically, 20 μL reaction system containing 0.20 μM dsDNA template, 10 units (U) Dpn I endonuclease, and a varying amount of Dam MTase to carry out the methylation reaction and cleavage reaction. After incubation at 37 °C for a corresponding time, 170 μL MOPS buffer was mixed with the prepared solutions. Then, 4 μL sodium ascorbate of 100 mM, and 4 μL of copper sulfate of 10 mM were introduced and triggered the formation reaction of fluorescent CuNCs. The fluorescence spectra and emission images of formed CuNCs were recorded at room temperature 5 min later. The selectivity experiments were the same as the above procedure, except for the Dam MTase was replaced by Hha I or Msp I MTase.

2.4. Methylation assay by gel electrophoresis

In the gel electrophoresis assay, nucleic acid samples were prepared as above-mentioned methylation reaction and analyzed by 10% agarose gel. All the contents in the sample were the same as that of Dam MTase activity detection but without the MOPS, sodium ascorbate and copper sulfate. The electrophoresis was carried in 1 × TBE buffer (89 mM Tris Borate, 2.0 mM EDTA, pH 8.3) for 6 h at 60 V constant voltages. Final gel electrophoresis products were stained for 30 min by 1 × SYBR Gold and imaged on a Tanon-2500 R gel imaging system (Shanghai, China).

2.5. Influence of inhibitor on dam MTase activity

For Dam MTase inhibition assay, the inhibitors (5-fluorouracil) with different concentration were added into the methylation system together with Dam MTase (200 U/mL). Other procedures were the same as detection of Dam MTase activity.

3. Results and discussions

3.1. Principle of dam MTase-responsive sensing system

As the proof-of-concept of our approach, the Dam MTase and Dpn I endonuclease were chosen as the model MTase and endonuclease to be investigated. The Dam MTase catalyzes the transfer of a methyl group from SAM to the N6 position of the adenine residues in the symmetric tetranucleotide 5′-G-A-T-C-3′, and the Dpn I endonuclease can