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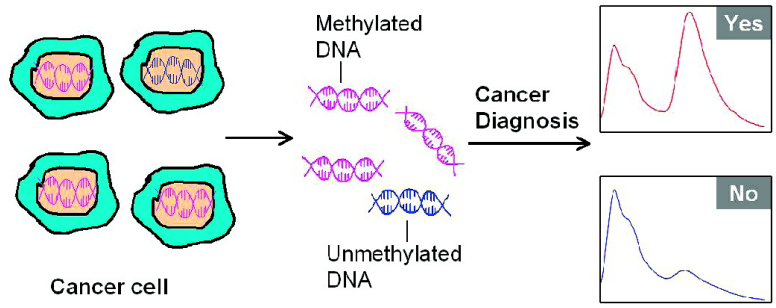

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Fluorescent Conjugated Polyelectrolyte as an Indicator for Convenient Detection of DNA Methylation

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Abstract: A convenient, sensitive, and label-free method to determine the DNA methylation status of CpG sites of plasmid and human colon cancer cell has been developed. The system relies on highly selective single base extension reaction and significant optical amplification of cationic conjugated polyelectrolytes (CCP-1). The higher fluorescence resonance energy transfer efficiency between CCP-1 and fluorescein-labeled dGTP (dGTP-Fl) is correlated to the incorporation of dGTP-Fl into the probe DNA by single base extension reaction when the target/probe pair is complementary at the methylation site. As low as 1% methylation status can be determined by this new assay method. Because of the optical amplification property of CCP-1, the method exhibited high sensitivity with a concentration of analyte DNA at the picomolar level. The CCP-1 can form a complex with negatively charged DNA through electrostatic interactions, avoiding labeling the DNA target and probe by covalent linking. The isolation steps employed in other typical assays were avoided to simplify operations and increase repeatability. These features make the system promising for future use for early cancer diagnosis.

Introduction

DNA methylation is an essential part of epigenetics, associated with DNA replication and repair, genomic imprinting, X chromosome inactivation, and regulation of gene expression.[1–3] DNA methylation in mammals occurs by addition of a methyl group to the 5 position of cytosine almost exclusively within a CpG dinucleotide.[4] Hypermethylation of the CpG islands in promoter regions of genes has been regarded as a hallmark of various diseases, cancer in particular.[5] Cancer-linked DNA hypermethylation often downregulates tumor suppressor gene expression and is responsible for tumor formation and progression.[6] Diagnosis based on DNA methylation alterations is advantageous and applicable for clinical purposes because of its robust performance arising from high DNA stability and very low methylation level at CpG islands in normal tissues. Thus, an approach for the rapid detection of DNA methylation could provide a powerful method for early cancer diagnosis. In particular, the detection of site-specific CpG methylation undoubtedly can be used to determine specific cancer types.[7,8]

Recently, new methods have been developed for investigation of global and regional hypermethylation in cancer based on the polymerase chain reaction (PCR) technique. Nearest-neighbor analysis can quantify the methylation level at CpG dinucleotides, with the one drawback being radioactive handling.[9] Chemical DNA sequencing combined with ligation-mediated PCR (LM-PCR) requires pretreatment with hydrazine and piperidine to modify nonmethylated cytosine and cleave DNA, followed by primer extension, ligation of linker, PCR, and gel sequencing, amounting to laborious operations.[10] A few methods, such as restriction landmark genome scanning (RLGS) and methylation-sensitive arbitrary primed PCR (MS-AP-PCR), employ restriction enzymes for methylation analysis with high reliability, but dependence upon restriction sites limits their applications, and radioactive labeling may be necessary.[11,12] Currently, techniques based on bisulfite-induced deamination are the most extensively applied for determination of methylation status. Methylation-specific PCR (MSP) facilitates the precise mapping of methylation patterns in CpG islands with extremely high sensitivity, while the false-positive possibility should be carefully considered.[13,14] Combined bisulfite restriction analysis (COBRA) employs restriction enzyme digestion to detect sequence variations in PCR products, dependent on recognition sites, although

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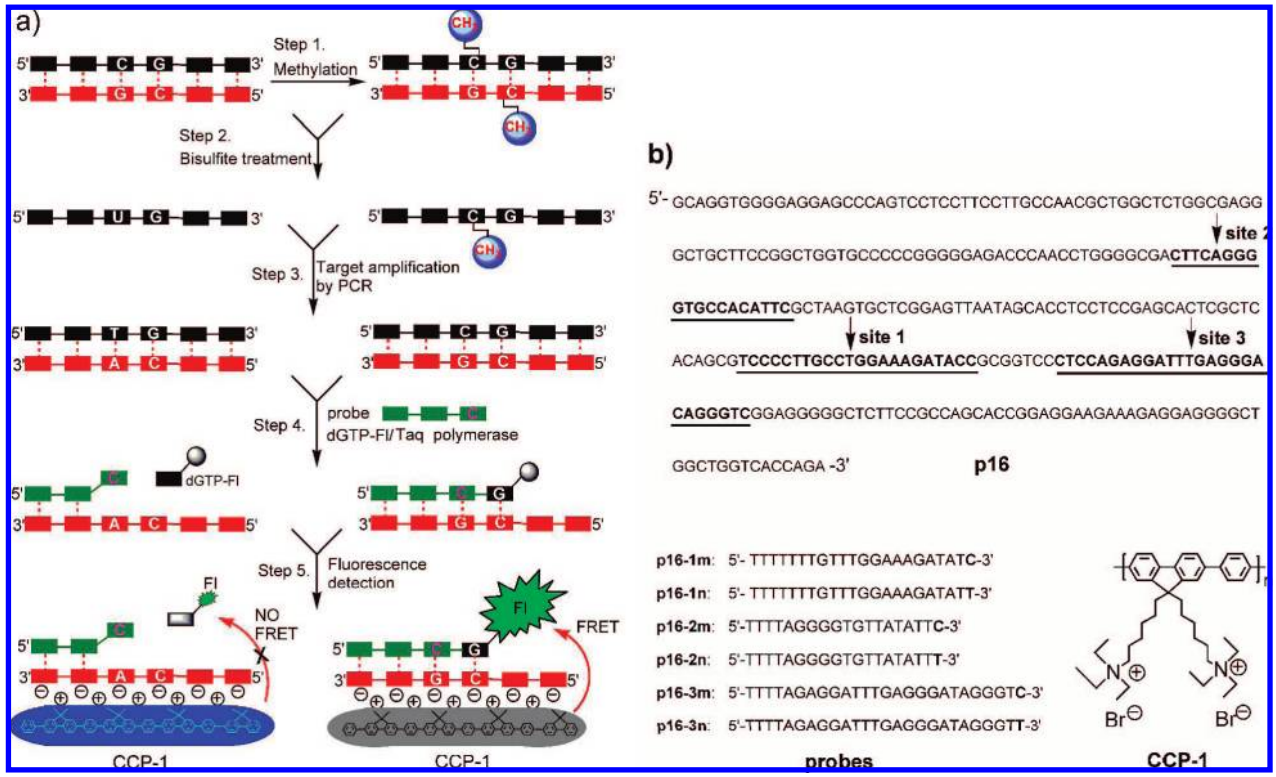
Scheme 1. (a) Schematic Representation of the Assay for Methylation Status of Specific CpG Sites and (b) Chemical Structure of CCP-1, the Sequences of DNA Probes for Single Base Extension, and the Target DNA Sequence and CpG Sites



it is a quantitative technique.[15] The MethyLight technique provides a quatitative, high-throughput, and fluorescent real-time resolution, but double-labeled probes as well as quantitative polymerase chain reaction (qPCR) apparatus make it too expensive and difficult to popularize.[16] Methylation-sensitive single nucleotide primer extension (Ms-SNuPE) assays analyze methylation status at specific CpG dinucleotides in a quantitative fashion, avoiding utilization of restriction enzymes.[17] To overcome the drawback of radioactive handling in Ms-SNuPE assays, combination of SNuPE assays with other techniques, such as ion pair reverse-phase high-performance liquid chromatography (IP-RP-HPLC) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-MS), is indispensable.[18,19]

Herein, we report a convenient, homogeneous, sensitive, and label-free cationic conjugated polyelectrolyte (CCP)-based approach to determine the methylation status of specific CpG sites. CCPs have received great interest due to their extraordinary light-harvesting and optical amplification properties, particularly in the application of nucleic acid biosensors.[20–25] A CCP has two advantages as a biosensor transducer: (i) in ratiometric assays, it behaves as an energy donor and transfers energy to an acceptor molecule, binding to its backbone through fluorescence resonance energy transfer (FRET),[26] leading to a significant optical amplification of the acceptor. This feature is favorable for increasing detection sensitivity. (ii) It can form a complex with oppositely charged DNA through electrostatic interactions to avoid labeling the DNA by covalent linking, in contrast to typical small-molecule fluorescent dyes.[27]

Results and Discussion

The CCP-based assay reported herein takes advantage of single nucleotide base extension reaction and significant optical amplification of CCP to determine the methylation status of a specific CpG site (Scheme 1a). One 283-bp sequence in the promoter region of the *p16* gene and three CpG sites in the sequence were of interest (Scheme 1b) since *p16* has been proved to be a tumor-suppressor gene.[28] Plasmid pUC57 that carried the sequence was fully methylated by methylase (M.SssI). Control experiments were performed in the same manner except lacking the methylation step. In brief, after DNA denaturation, bisulfite treatment was carried out using a modified

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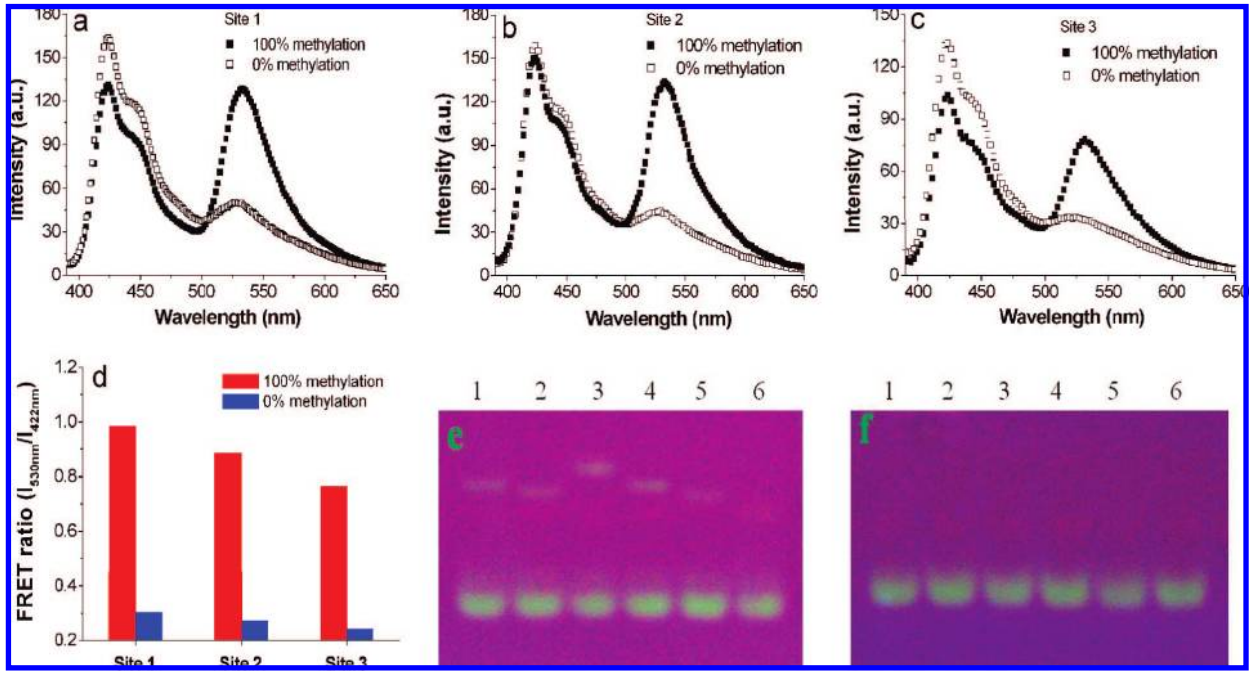

Figure 1. Fluorescence spectra (a−c) and FRET ratio (I_{530nm}/I_{422nm}) (d) from solutions containing CCP-1 and single nucleotide base extension products of methylated plasmid and nonmethylated plasmid using methylation-specific probes in 1 mL of HEPES buffer solution (25 mM, pH 8). The probes used in extension reactions were p16-1m for site 1, p16-2m for site 2, and p16-3m for site 3. The amount used were 0.67 pmol of probe, 1.67 pmol of dGTP-Fl, and [CCP-1] = 0.25 μM in RUs. The excitation wavelength is 380 nm. Also shown is electrophoresis analysis of single nucleotide base extension products involving a complementary target/probe pair (e) and a mismatched target/probe pair (f). In (e), lanes 1−3 respectively stand for p16-1m, p16-2m, and p16-3m in the presence of methylated DNA; lanes 4−6 respectively stand for p16-1n, p16-2n, and p16-3n, in the presence of nonmethylated DNA. In (f), lanes 1−3 respectively stand for p16-1m, p16-2m, and p16-3m in the presence of nonmethylated DNA; lanes 4-6 respectively stand for p16-1n, p16-2n, and p16-3n in the presence of methylated DNA.

agarose bead-based method similar to that described in the literature,[29] followed by PCR to amplify the target sequence. For nonmethylated plasmid pUC57, the cytosine of the CpG site was induced into uracil upon bisulfite treatment, and the uracil was substituted by thymine after PCR amplification. For methylated plasmid pUC57, the cytosine of the CpG site remained unchanged upon bisulfite treatment and PCR amplification. The single nucleotide base extension was performed using fluorescein-labeled dGTP (dGTP-Fl) and specific probe p16-1m for site 1 (p16-2m for site 2 or p16-3m for site 3; see their sequences in Scheme 1b) in the presence of Taq polymerase. The 3′-terminal base of the p16-1m probe is C, which is complementary to the target sequence from methylated plasmid pUC57, and the dGTP-Fl was incorporated into the probe by extension reaction. Upon addition of the cationic conjugated polyelectrolyte (CCP-1, see its chemical structure in Scheme 1b), strong electrostatic interactions between DNA and CCP-1 bring the fluorescein close to CCP-1, and efficient FRET from CCP-1 to fluorescein occurs. In contrast, FRET was sharply weakened when a mismatched probe was used (Scheme 1a; for instance, p16-1m for nonmethylated plasmid, p16-1n for methylated plasmid), which implied single base extension was not allowed due to the mismatch at the 3′-terminus of the probe. Thus, the methylation status of a specific CpG site can be monitored by fluorescence spectra in view of the observed CCP-1 or Fl emission changes in aqueous solutions. In other words, CCP-1 serves as an indicator for methylation status by mixing the treated DNA samples and CCP-1.

Figure 1a−c compares the emission spectra observed upon adding CCP-1 ([CCP-1] = 0.25×10^{-6} M in monomer repeat units (RUs)) to probe extension solutions in HEPES buffer (25 mM, pH 8). Before fluorescence measurement, enzyme SAP was added to degrade excess dGTP-Fl. After addition of CCP-1, comparison of the resulting fluorescence from fluorescein obtained by excitation at 380 nm shows ∼2−4 times higher signal for the complementary target/probe pair (p16-1m, p16-2m, and p16-3m for sites 1−3 of methylated plasmid) relative to that for the mismatched target/probe pair (p16-1n, p16-2n, and p16-3n for methylated plasmid). Considering there exist three enzymes (Taq polymerase, SAP, and *Exo*I) in the PCR product, the effect of the enzymes on the emission of CCP-1 was studied. As shown in Figure S1 (Supporting Information), almost no change was observed for the emission spectra of CCP-1 upon addition of these enzymes. This demonstrates that the enzymes in the PCR product do not interfere with the assays.

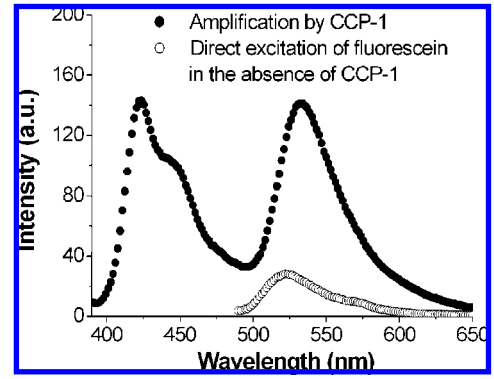

Figure 2. Emission spectra of dGTP-Fl with excitation wavelength at 480 nm and that of CCP/dGTP-Fl with excitation wavelength at 380 nm in HEPES buffer solution (2.0 mM, pH 8.0). The amounts used were 0.67 pmol of probe, 1.67 pmol of dGTP-Fl, and [CCP-1] = 0.25 μM in RUs.

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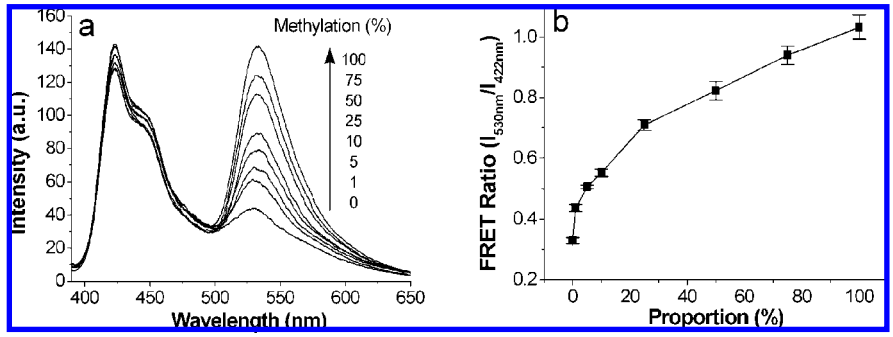
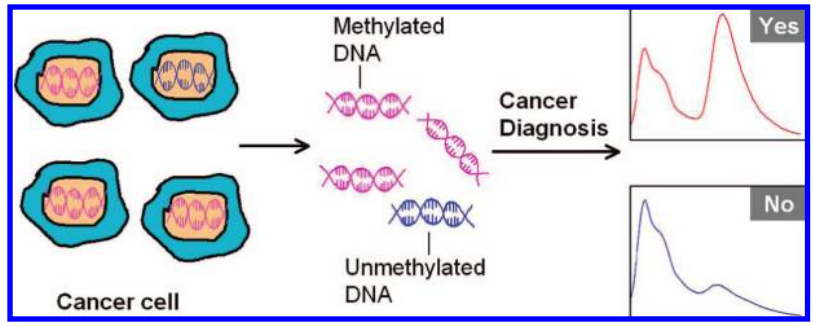


Figure 3. Fluorescence spectra (a) and FRET ratio (I_{530nm}/I_{422nm}) (b) of the extension production with varying proportions of methylated DNA in the presence of CCP-1. The amounts used were 0.67 pmol of probe, 1.67 pmol of dGTP-Fl, and [CCP-1] = 0.25 μM in RUs. The excitation wavelength is 380 nm.

Scheme 2. Schematic Representation of the Assay for DNA Methylation Status of Human Colon Cancer Cell Line HT29



The FRET ratios (I_{530nm}/I_{422nm}) for specific extension are ~3−5 times higher than that of the nonspecific extension (Figure 1d), which demonstrates the good selectivity of this assay for the detection of methylation status of specific CpG sites. To validate the results, non-denaturing polyacrylamide electrophoresis analyses were performed devoid of staining (Figure 1e,f), and the fluorescence of fluorescein was observed under UV light. The specific extension products yield one faster moving band and one slower moving band that emit green fluorescence. The faster moving bands are the unreacted dGTP-Fl, and the slower moving ones are the probe extension products where the dGTP-Fl is incorporated into the DNA probes. For the nonspecific extension products, no slower moving extension product band was observed. These results are well consistent with the FRET results shown in Figure 1a−c. To eliminate false-negative results, the control experiments were performed using a complementary nonmethylation-specific probes/nonmethylated plasmid pair at the same conditions as those of the methylation-specific probes/methylated plasmid pair (Figure S2, Supporting Information). The good FRET efficiencies show that our new technique is specific for methylation of CpG sites and eliminates false-negative results.

Note that the fluorescein emission intensity for the CCP1/specific extension product at 380 nm excitation is 5-fold larger than that obtained by direct excitation with its absorption maximum (480 nm, Figure 2). The improved fluorescein emission by FRET shows the optical amplification by the CCP-1 macromolecule. The background noise arising from nonspecific interactions between CCP-1 and dGMP-Fl was rather low. The detection method exhibited high sensitivity using analyte DNA at the picomolar level. Furthermore, isolation steps employed in other typical assays were avoided.

Quantitative determination of DNA methylation has important significance for study of the association between methylation and diseases. We demonstrate the potential of the proposed assay for determination of the DNA methylation by investigating the relationship between the methylation proportions and the FRET ratio (I_{530nm}/I_{422nm}) of extension production of test samples, where site 1 is used as the target CpG site. As shown in Figure 3, as the methylation proportion decreases, the FRET ratio also declines in a nonlinear fashion, owing to the decreasing amounts of extension products. Even for 1% methylation, FRET is still able to distinguish the signal from 0% methylation in light of fluorescence spectra, which reflects the amplification capability of CCP-1. As a result, CCP-1 could be used to quantitatively detect the methylation status of a specific CpG site.

On the basis of the detection model, we investigated the methylation status of the three CpG sites in the *p16* promoter region of human colon cancer cell line HT29 (Scheme 2). After bisulfite conversion of the genomic DNA, a nested-PCR amplification, which was necessary to diminish the false-positive possibility arising from the incomplete bisulfite conversion and to improve the specificity, was performed to obtain a 290-bp fragment containing the three target CpG sites (Scheme S1).[30] The sequence of interest was amplified with high specificity, as indicated in the electrophoresis analysis (Figure 4a). After the completion of the single base extension reaction, FRET analysis was performed in the presence of CCP-1 (Figure 4b−d). Significant FRET was present when DNA probes (p16-1m, p16-2m, or p16-3m) were effectively extended to the target DNA, while the FRET efficiency was rather low when using nonmethylation-specific probes (p16-1n, p16-2n, or p16-3n). These results suggest that hypermethylation occurs in the *p16* promoter region of HT29 cells, which is consistent with a

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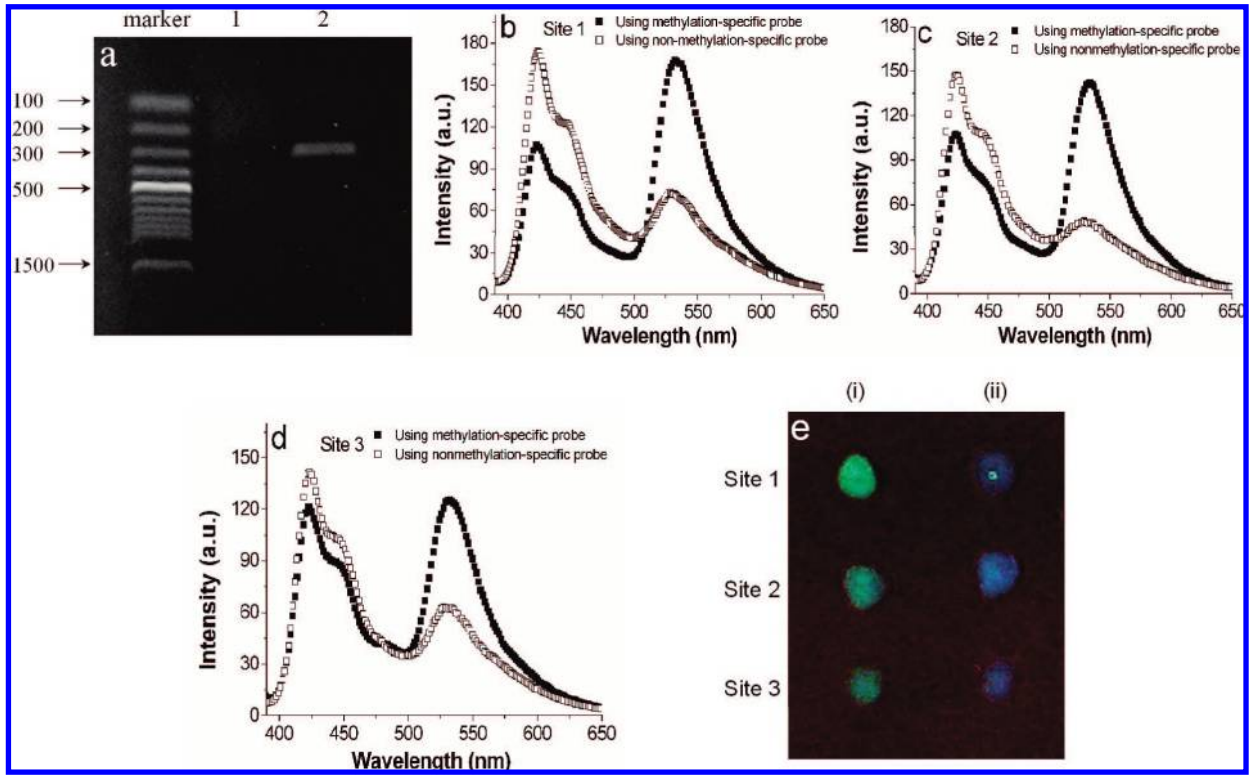

Figure 4. (a) Agarose electrophoresis analysis of PCR products. Lane 1, first round of PCR; lane 2, second round of PCR. (b−d) Fluorescence spectra from solutions containing CCP-1 and single nucleotide base extension products of human colon cancer cell line HT29 using methylation-specific probes (p16-1m, p16-2m, and p16-3m) and nonmethylation-specific probes (p16-1n, p16-2n, and p16-3n). (e) Images of extension products in the presence of CCP-1 under a 300 nm UV light. The probes used in extension reactions were (i) p16-1m for site 1, p16-2m for site 2, and p16-3m for site 3; (ii) p16-1n for site 1, p16-2n for site 2, and p16-3n for site 3.

previous report.[31] To make the method compatible with a high-throughput system (HTS), we studied the optical behavior of CCP-1 in the presence of extension products on glass slides. Each mixture of CCP-1 and single base extension products of HT29 were dotted in a volume of 1 μL, containing 0.8 μL of extension products and 0.2 μL of 0.5 mM CCP-1, on the surface of one glass slide. Under UV light with excitation of 300 nm, the array showed fluorescence emission from the six spots of the mixture (Figure 4e). Green emissions arising from efficient FRET correspond to the existence of methylation at the target CpG sites by using methylation-specific probes, and blue emissions due to the blocked FRET correspond to the existence of nonmethylation at these CpG sites. The results provide the possibility of potential application in chip-assisted analysis for methylation status of CpG islands in human genes and in determining specific cancer types.

Conclusion

In summary, we take advantage of the optical properties of CCP to develop an optical platform for assaying the methylation status of specific CpG sites. This new technique offers several advantages over current DNA methylation detection methods. First, the method is continuous and homogeneous and does not require handling radioactive materials. Therefore, isolation and washing workups are avoided, which simplifies operations and increases repeatability. Second, the method requires only microgram amounts of DNA or less, whether it is from human cells or plasmid, useful for analyzing trace amounts of DNA. Third, in contrast to the previously reported fluorescence technique, this method does not require designing dye-labeled DNA probes, which should significantly reduce the cost. In addition, the method could be expanded to fluorescence-based high-throughput screening assays. Finally, the turn-on response of fluorescence is rapid since it behaves in a "mix and detect" fashion, and CCP acts as an indicator of the methylation status of specific CpG sites. In principle, this sensor mechanism might be highly generalizable and provides a means for detecting the methylation status of specific CpG sites of other cancer genes. These advantages makes the system promising for future use for cancer diagnosis.

Experimental Section

Materials and Measurements. CCP-1 was synthesized according to the procedure in the literature.[32] PCR primers, DNA probes, and plasmid pUC57 that carries the 283-bp gene *p16* promoter sequence were purchased from Sino-American Biotechnology Co. HotStart Taq polymerase, SAP, and *Exo*I were obtained from Takara Co., M.SssI was from New England Biolabs (NEB), and dGTP-Fl was purchased from Jingmei Biotechnology Co. All other reagents were obtained from commercial sources. The water was purified using a Millipore filtration system. The concentrations of oligonucleotides were determined by measuring the absorbance at 260 nm in a 160 μL quartz cuvette. Fluorescence spectra were obtained in 3 mL polystyrene cuvettes containing 1 mL of samples using a Hitachi F-4500 fluorometer equipped with a Xeron lamp excitation source. The excitation wavelength was 380 nm.

Preparation of Genomic DNA. HT29 cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum in a humidified

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incubator containing 5% CO_2 at 37 °C. A single-cell suspension was obtained, and genomic DNA was extracted with removal of RNA by a TIANamp Genomic DNA kit (Tiangen Biotech Co., Ltd.) according to the manufacturer's instruction.

Preparation of Methylated Plasmid DNA. Aliquots of 5 μL of NEB buffer, 8 nmol of *S*-adenosylmethionine (SAM), and 5 U of M.SssI were added to 2 μg of plasmid in a final volume of 50 μL, followed by incubation at 37 °C. After 5 h, 8 nmol of SAM and 5 U of M.SssI were added again, and the resulting solution was incubated overnight. The enzyme was inactivated at 65 °C for 20 min, and the solution was directly used for bisulfite treatment.

Bisulfite Treatments of Methylated Plasmid and Genomic DNA. Bisulfite treatment was performed by a bead-based method as described by Olek et al. with minor modifications.[29] First, 21 μL of plasmid or genomic DNA (the total amount of DNA was not more than 1.0 μg) was denatured in a boiling water bath for 10 min, and then 4 μL of freshly prepared 2.0 M aqueous NaOH was added. The resulting solution was incubated at 50 °C for 15 min. Next, 50 μL of 2% LMP agarose solution was added to the DNA solution. Agarose beads were formed by gently pipetting 10 μL aliquots into 300 μL of cold heavy mineral oil. A 1.9 g amount of sodium metabisulfite was dissolved in a mixed solution of 2.5 mL of H_2O and 700 μL of 2.0 M NaOH, and 0.5 mL of 1.0 M hydroquinone was added to obtain a clear bisulfite solution. Aliquots of 500 μL of bisulfite solution were added into cold mineral oil, and then the beads were incubated with light exclusion for 4 h at 50 °C and and rinsed four times (each 15 min) with 1.0 mL of TE buffer solution (pH 8.0). Desulfonation was achieved in 500 μL of 0.2 M NaOH two times (each 15 min) at 37 °C, followed by washing steps with 1.0 mL of TE buffer solution (two times, each 15 min). The beads were stored at 4 °C overnight or longer and rinsed with water (two times, each 10 min) before PCR assay.

PCR Amplification. For plasmid DNA, amplification of the *p16* promoter sequence was performed in 50 μL aqueous solution containing one bead, 0.1 mM dNTPs, 5 μL of 10× Taq buffer, 0.5 μM of primers (5′-GTAGGTGGGGAGGAGTTTAGT-3′ and 5′-TCTAATAACCAACCAACCCCTCC-3′), and 2.5 U of HotStart Taq polymerase in a thermocycler under the following conditions: 94 °C for 2 min, followed by 40 cycles of 94 °C, 68 °C for 30 s, and 72 °C for 30 s. For genomic DNA, nested-PCR amplifications were performed in a total volume of 50 μL in the following conditions: 95 °C for 3 min, 35 cycles at 94 °C for 30 s, 56 °C for 45 s, 72 °C for 45 s (for the outer primer), and 95 °C for 3 min, or 35 cycles at 94 °C for 30 s, 58 °C for 45 s, 72 °C for 45 s (for the inner primer). Outer primer: 5′-GAG GGG GTA GGG GGA TAT-3′ (forward), 5′-ACC AAT CAA CCA AAA ACT CCA TAC TA-3′ (backward). Inner primer: 5′-GTA GGT GGG GAG GAG TTT AGT T-3′ (forward), 5′-CCC ACC CTC TAA TAA CCA ACC AA-3′ (backward). Agarose electrophoresis analysis was performed to check PCR products.

Detection of Site-Specific CpG Methylation. To 19 μL of the PCR mixture was added 6 μL of an enzymatic cocktail containing 2.5 μL of 10× SAP buffer, 1 U of SAP, and 10 U of *Exo*I. After incubation at 37 °C for 45 min, the enzymes were inactivated at 95 °C for 15 min to degrade excess PCR primers and dNTPs. Single base extension reactions were performed in a total volume of 10 μL of aqueous solution containing 5 μL of treated PCR product, 10 μM dGTP-Fl, 4 μM CpG-specific probe, and 1.25 U of Taq polymerase. The reaction mixture, covered with 30 μL of mineral oil, was incubated at 94 °C for 1 min, followed by 50 cycles of 94 °C for 20 s, 56 °C for 30 s, and 72 °C for 30 s. At the end of the reaction, 2 μL of an enzymatic cocktail containing 1 μL of 10× SAP buffer and 1 μL of 1 U/μL SAP was added, followed by incubation at 37 °C for 20 min and inactivation at 65 °C for 20 min to degrade excess dGTP-Fl. To a 3 mL polystyrene cuvette containing 1 mL of HEPES buffer (25 mM, pH 8.0) and 0.25 μM CCP-1 was added 2 μL of extension mixture. Fluorescence spectra were measured with excitation at 380 nm.

Image Experiment. The solutions containing 0.8 μL of extension products and 0.2 μL of 5.0×10^{-4} M CCP-1 were dotted on a glass slide with an Eppendorf pipet. The images were taken with a Canon IXUS 750 digital camera in a WD-9403F UV viewing cabinet (Beijing Liuyi instrument factory, Bejing) under 300 nm transmission light.

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Supporting Information Available: Fluorescence spectra from solutions containing CCP-1 and single nucleotide base extension products of methylated plasmid and nonnmethylated plasmid using nonnmethylation-specific probes in HEPES buffer solution; sequence of the *p16* promoter region of human colon cancer cell line HT29 containing three methylated CpG sites; complete ref 14. This material is available free of charge via the Internet at http://pubs.acs.org.

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