analytical chemistry

Technical Note

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High-Sensitivity and High-Efficiency Detection of DNA Hydroxymethylation in Genomic DNA by Multiplexing Electrochemical Biosensing

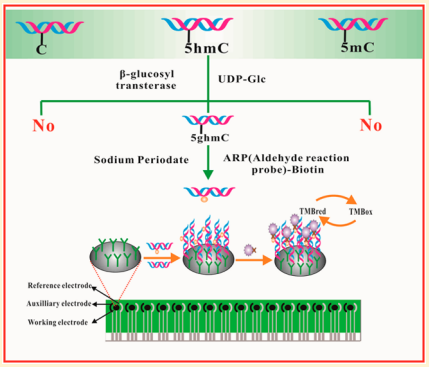
Shixing Chen,[†] Yanzhi Dou,[†] Zhihan Zhao,[†,‡] Fuwu Li,[†] Jing Su,[†] Chunhai Fan,[*,†] and Shiping Song[*,†]

[†]Division of Physical Biology and Bioimaging Centre, Shanghai Synchrotron Radiation Facility, CAS Key Laboratory of Interfacial Physics and Technology, Shanghai Institute of Applied Physics, Chinese Academy of Sciences, Shanghai 201800, China

[‡]College of Sciences, Shanghai University, Shanghai 200444, China

Supporting Information

ABSTRACT: DNA hydroxymethylation (5-hmC) is a kind of new epigenetic modification, which plays key roles in DNA demethylation, genomic reprogramming, and the gene expression in mammals. For further exploring the functions of 5-hmC, it is necessary to develop sensitive and selective methods for detecting 5-hmC. Herein, we developed a novel multiplexing electrochemical (MEC) biosensor for 5-hmC detection based on the glycosylation modification of 5-hmC and enzymatic signal amplification. The 5-hmC was first glycosylated by T4 β-glucosyltransferase and then oxidated by sodium periodate. The resulting glucosyl-modified 5-hmC (5-ghmC) was incubated with ARP-biotin and was bound to avidin-HRP. The 5-hmC can be detected at the subnanogram level. Finally, we performed 5-hmC detection for mouse tissue samples and cancer cell lines. The limit of detection of the MEC biosensor is 20 times lower than that of commercial kits based on optical meaurement. Also, the biosensor presented high detection specificity because the chemical reaction for 5-hmC modification can not happen at any other unhydroxymethylated nucleic acid bases. Importantly, benefited by its multiplexing capacity, the developed MEC biosensor showed excellent high efficiency, which was time-saving and cost less.



Deoxyribonucleic acid (DNA) methylation studies have been traditionally concerned with 5-methylcytosine (5mC), mostly in the CpG islands, which plays important roles in regulation of gene expression, genomic imprinting, and X-chromosome inactivation.[1] Recently, 5mC was found to be oxidized by Tet (ten eleven translocation) family dioxygenases to form 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC), and 5-carboxylcytosine (5caC).[2,3] Among the three 5mC oxidative derivatives, 5hmC is the most abundant form in vivo and can be detected in all mammalian tissues and cells.[4] 5hmC has been proposed to be an intermediate in an active DNA demethylation pathway and may be a stable epigenetic modification in its own right, contributing unique regulatory functions to the epigenome.[5] It is also noted that the level of 5hmC significantly decreases in various cancers, including the tumors of lung, brain, liver, kidney, skin, prostate, breast, and colon, suggesting that 5hmC plays an important role in cancer development.[4,6,7] In addition, aberrant 5hmC distribution has also been found in myelodysplastic syndrome, Huntington's disease, and Alzheimer's disease.[8,9] All of these findings suggest that 5hmC might be an emerging biomarker for disease diagnosis, treatment, and prognosis. However, the most prominent techniques for analysis of DNA methylation modification, such as bisulfite sequencing and methylation-sensitive enzyme-based assays, can not distinguish 5hmC from 5mC due to their similar structures.[10−13] More importantly, the abundance of 5hmC is much lower than that of 5mC, which increases the difficulty for sensitive detection of 5hmC.

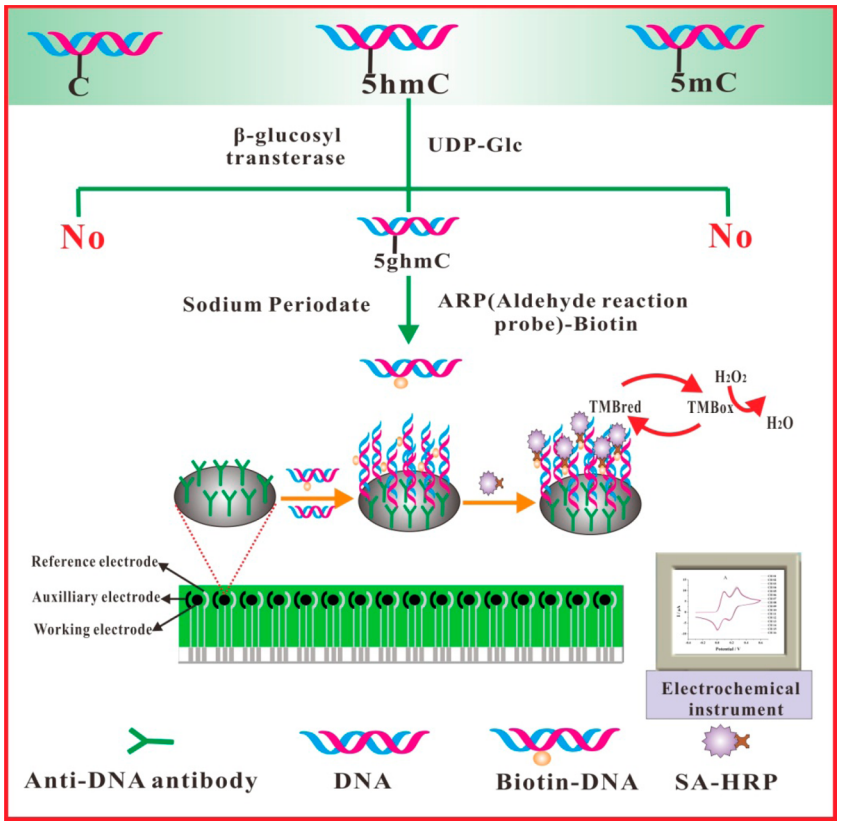
Recently, several specific technologies for global 5hmC analysis have been developed, including thin layer chromatography (TLC),[14] liquid chromatography mass spectrometry (LC-MS),[15] high-performance liquid chromatography (HPLC),[16] and immunoassays.[17] These methods can effectively distinguish 5hmC from 5mC, but they are suffering from disadvantages due to their inherent technical deficiencies. The TLC method requires radioactive substrates, which is harmful to the operator and environment. Meanwhile, the detection accuracy of TLC needs to be improved further. Those methods based on the LC-MS and HPLC are complex and exorbitantly priced, requiring skilled personnel and presently unsuitable for high throughput analyses. The immunoassay-based methods lack sensitivity and are extremely expensive.

Currently, chemical modification of 5hmC has been used as a reliable method for identifying 5mC and 5hmC. For instance, Song et al. used the T4 bacteriophage β-glucosyltransferase to transfer an engineered glucose moiety containing an azide group onto the hydroxyl group of 5hmC; then, the N3 group

Received: January 19, 2016
Accepted: March 4, 2016
Published: March 4, 2016

DOI: 10.1021/acs.analchem.6b00230
Anal. Chem. 2016, 88, 3476−3480

Scheme 1. Schematic Diagrams of MEC Biosensor for Genomic 5-hmC Detection[a]



[a]A portable ECH16 and disposable 16-channel SPCE array were employed as the assay system. The dsDNA antibodies on the surface of electrodes and biotinlated modified DNA samples formed an immunocomplex on the working electrodes, followed by incubation of SA-HRP with biotinlated DNA, leading to the binding of HRP to the electrode that can be transformed to the catalytic amperometric readout.

was further chemically modified with biotin.[18] Pastor et al. performed another strategy with an inexpensive process by the oxidation of glucose moiety with sodium periodate, which converts the vicinal hydroxyl groups to aldehydes, and further modified with aldehyde-reactive probe, which adds two biotin molecules to each 5hmC.[19]

Inspired by the previous works for 5hmC detection based on chemical modifications, we developed a simple and sensitive multiplexing electrochemical (MEC) biosensor for 5hmC detection in the genomic DNA sequence based on T4 β-glucosyltransferase catalytic glycosylation modification of 5hmC, followed by Pastor's strategy and avidin-HRP catalytic signal amplification. The developed method showed excellent detection sensitivity and selectivity.

EXPERIMENTAL SECTION

Materials. Casein, Tween 20, tetramethylbenzidine (TMB), ethylenediaminetetraacetic acid disodium salt (EDTA), methoxyamine-HCl, sodium sulfite, sodium phosphate, DMSO, trihydroxymethyl aminomethane, and $NaIO_4$ were purchased from Sigma-Aldrich. Horseradish-peroxidase (HRP), streptavidin (SA), and aldehyde reactive probe (ARP) were purchased from Invitrogen. T4 β-glucosyltransferase, UDPG-glucose, Plasmids pBR322, Dde I, klenow fragment (exo$^-$), and Taq DNA polymerase were ordered from NEB. 5-hmCTP (where 5-hmC is hydroxymethylation), dATP, dCTP, dGTP, dTCP, and dsDNA antibody were purchased from Zymo Research. A 498bp DNA fragment was amplified by PCR using forward 5′-ACCGCACAGATGCGTAAGGAG-3′ and reverse 5′-AACGACCTACACCGAACTGAGATA-3′ primers from pBR322. All DNA sequences were offered by TAKARA (Dalian, China; see Supporting Information for more details). The synthesized oligonucleotides were dissolved in 10 mM Tris-HCl and 1 mM EDTA (pH 8.0) to obtain the desired concentration and stored at −20 °C.

DNA Isolation from Tissue Samples and Cell Lines. The following DNA samples were obtained from genomic DNA-Tissue MiniPrep Kit (Zymo Research). All tissues of mouse included brain, lung, heart, liver, kidney, and spleen. The following DNA samples were prepared using Cultured Cell DNA Extraction Kit (Epigentek). All cancer cell lines included Hela cervical cancer cell line, PC-3, and MCF. While a known content of 5-hmC modified sample DNA (0.03% of 5-hmC in total DNA) was offered by Zymo Research. The concentrations of all DNA samples are measured with the spectrophotometer and confirmed with a PicoGreen dsDNA quantitation kit (invitrogen). A 260/280 ratio of all DNA samples is greater than 1.8.

Preparation of Biotinylated 5-hmC DNA Products. The GLIB (glucosylation, periodate oxidation, biotinylation) method was employed as reported.[19] The 498 bp dsDNA was amplified by PCR at the conditions of 95 °C for 3 min, followed by 30 cycles of 30 s at 95 °C, 30 s at 55 °C, and 1 min at 72 °C, with a final extension cycle of 72 °C for 5 min. At the end of PCR, DNA was quenched with 5 mM methoxyamine-HCl in 1× PBS for 1 h at 37 °C, purified by a Qiagen PCR purification column (genomic DNA needs quenching). Then, the DNA fragment was digested with the enzyme Dde I,

DOI: 10.1021/acs.analchem.6b00230
Anal. Chem. 2016, 88, 3476−3480

producing a fragment with a 3 bp 5′TGA overhang and a 3 bp 5′TCA overhang. The overhangs were filled in using klenow enzyme and dATP, dGTP, dTTP, and dhmCTP, yielding a 460bp blunt ended DNA fragment with a single 5hmC on the plus strand (0.1087% 5-hmC of total DNA). After modification, 1 μg of DNA was glucosylated for 3 h at 30 °C according to the T4 β-glucosyltransferase manufacturer's instructions, purified by the Qiagen PCR purification column. Then, DNA was incubated with 23 mM $NaIO_4$ (final concentration) in 100 mM sodium phosphate, pH 7.0, at 22 °C for 16 h, followed by incubation with sodium sulfite (46 mM final concentration) for 10 min at room temperature. After oxidation, G50 columns (GE) were used to buffer-exchange. Biotins were added by aldehyde reactive probe (ARP) with a final concentration of 2 mM for 1 h at 37 °C and then purified over a Qiagen PCR Purification column. However, for genomic DNA, PCR and enzyme cutting processes were not necessary.

Fabrication of MEC Immunosensor and Electrochemical Detection. Screen-printing is a thick-film technology that can be applied for mass production of a screen-printed carbon electrode (SPCE) array with very low cost and high reproducibility.[20,21] The disposable 16-channel MEC biosensor was fabricated as in our developed techniques.[22] The electrochemical performance of the MEC biosensor was evaluated for the redox activity of TMB by CV and amperometric measurement with a homemade 16-channel electrochemical detector (ECH16).[22] The measurement for TMB was performed by cyclic voltammetry scanning for 2 segments at the potential window of −0.3 to 0.6 V and amperometric measurement at the voltage of −0.1 V for 50 s. First, the carbon electrode was coated with 320 ng of dsDNA antibody diluted in carbonate buffer (pH 9.5). The electrodes were washed with PBST (pH 9.5) and blocked with 1% casein. After washing, biotinlated DNA samples were added and incubated at 37 °C for 1 h. After washing for three times, 10 μL of SA-HRP (1:1000 dilution in 0.01 M PBS (pH 9.5) containing 1% casein) was added and incubated at 37 °C for 1 h in each electrode, followed by washing with PBST (pH 9.5) for five times and rinsing with ddH_2O. Then, 50 μL of TMB substrate was applied onto each electrode of the MEC immunosensor. After that, the reactions in the 16 channels were detected simultaneously with high efficiency by CV measurement at a scan rate of 0.1 V/s and a potential range of −0.3 to 0.6 V. The voltage of amperometric measurement was fixed at −0.1 V. The electroreduction current was measured at 100 s after the HRP redox reaction reached steady state.

Data Analysis. Data are reported below as mean ±SD. Slope of the standard curve is determined using liner regression, and the percentage of 5-hmC in total DNA is calculated using formula:

$$\text{5-hmC\%} = \frac{\text{sample current} - \text{background current} + 15.923\,(\text{nA})}{\text{slope}} \times 100\% \quad (1)$$

RESULTS AND DISCUSSION

Multiplexing Electrochemical Biosensing Strategy. Herein, the sensitive MEC biosensor was developed for 5-hmC detection based on T4 β-glucosytransferase catalytic glycosylation reaction and HRP catalytic signal amplification. As shown in Scheme 1, after genomic DNA was extracted from samples, the glycosylation reaction was performed by T4 β-glucosyltransferase and UDP-glucose, where the glucosyl group in UDP-glucose can be transferred to the hydroxymethyl of 5-hmC. On the contrary, the 5-mC and C could not react with T4 β-glucosytransferase. As a result, only the glucose group on the 5-hmC of genomic DNA was covalently modified. Then, the glucosyl-modified DNA was oxidized by sodium periodate, following incubation with ARP. Finally, the biotin labeled genomic DNA was captured by dsDNA antibody modified on screen-printed carbon electrodes, followed by the binding of SA-HRP to form a sandwich complex. The multiplexing electrochemical signals were obtained by HRP catalytic signal amplification and harvested by the homemade multichannel electrochemical workstation. Remarkably, 16 samples can be detected simultaneously with high-efficiency, which was time-saving and cost less.

Optimum of Detection Conditions. In order to improve the detection sensitivity, some key parameters were optimized, including incubation time and pH value for the binding of DNA (0.5 nM dsDNA hybridization of template DNA and 5-hmC modified complementary DNA) and dsDNA antibody. As seen in Figure 1a, the peak current values increased when the incubation time was extended from 15 min to 1 h, and then, the peak current remained unchanged when the incubation time was further prolonged, indicating the saturated binding on the surface of electrodes. Thus, 1 h was chosen as the optimal incubation time. As shown in Figure 1b, the peak current increased when the pH values were increased from 8.4 to 9.5.

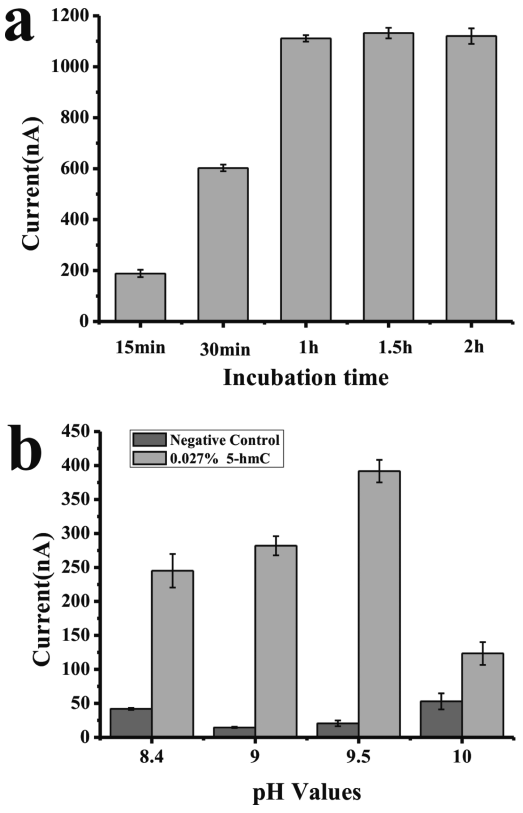


Figure 1. Optimal conditions for MEC biosensor detection. (a) Incubation time of DNA with dsDNA antibody to affect amperometric responses. Herein, the DNA was modified with 5-hmC at sequence end and without dilution. (b) The effect of pH values on signal-to-background results. The original 5-hmC modified DNA was diluted with unmodified DNA for 4 times; then, the 5-hmC content in total DNA was 0.0271%. The data are average values ± standard deviation from 3 different assays.

DOI: 10.1021/acs.analchem.6b00230
Anal. Chem. 2016, 88, 3476−3480

However, the current tended to decrease when the pH value was higher than 9.5, so pH 9.5 was used as the optimal pH condition in this work.

Sensitivity and Specificity of 5-hmC MEC Biosensor. To test the capacity of the MEC biosensor for the relative quantitative analysis of 5-hmC, the 5-hmC modified DNA fragments were serially diluted with unmodified DNA fragments (0.1087%, 0.0543%, 0.0271%, 0.0108%, 0.0054%, 0.0025%, and 0% of 5-hmC in total DNA) and detected by the MEC biosensor. As shown in Figure 2a, the current values

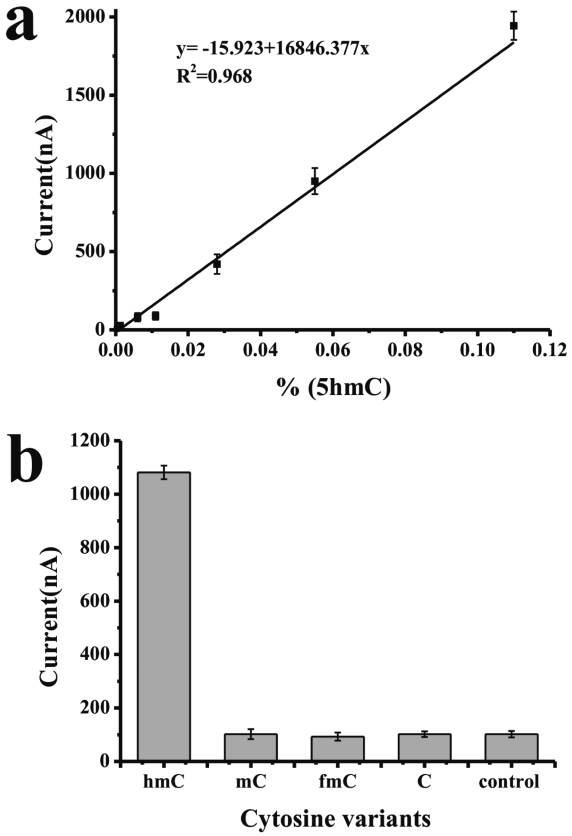


Figure 2. Sensitivity and specificity determination of 5-hmC by the MEC biosensor. (a) Linear relationship between the current value and the percentage of 5-hmC in total DNA. (b) Multiple cross-reactivity tests to determine the specificity of proposed biosensor. The data are average values ± standard deviation from 3 different assays.

were increased with the amount of 5-hmC DNA. The dose–response curve is linear in the concentration range of 0 to 0.1087%. The linear regression equation can be expressed as function (1) with the correlation of 0.968. The limit of detection (LOD) was calculated to be 0.0012% (0.489 pg), which is 20 times lower than that obtained by commercial methods (10 pg, Table 1). To testify the specificity of the MEC biosensor, the same amounts of umC DNA, 5-mC DNA, 5-hmC DNA, and 5-fmC DNA were used in this MEC biosensor. The results (Figure 2b) showed that the current value of the 5-hmC DNA was 10-fold higher than others (0.5 nM dsDNA hybridization of template DNA and 5-mC, 5-hmC, and 5fmC modified complementary DNA). In contrast, the current value of 5-mC DNA is still at the background level. This demonstrates that the MCE biosensor allows discrimination between 5-hmC and the other variants of cytosine (5-hmC, 5-fmC, and normal cytosine).

Detection for Distribution of 5-hmC in Different Mouse Tissues and Cell Lines. Utilizing the MEC biosensor, we determined the content of 5-hmC in different mouse tissues and cell lines. DNA was isolated from 5 normal mouse tissues of individual weight from 20 to 25 g. These tissues included brain, lung, heart, kidney, liver, and spleen. Also, DNA was isolated from 3 cell lines including Hela cervical cancer, PC-3 (epithelial cell line from a human prostatic adenocarcinoma), and MCF (breast cancer cell line). As shown in Figure 3, a

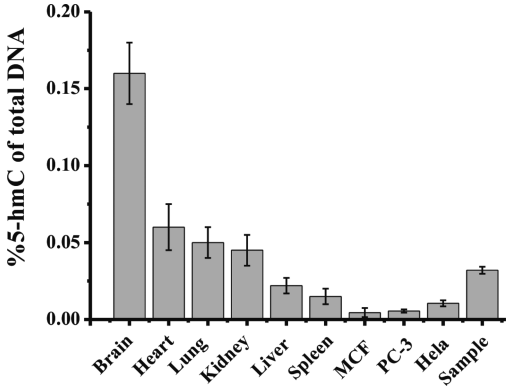


Figure 3. Quantification of 5-hmC in genomic DNA isolated from mouse tissues and 3 cancer cell lines. Data represent values for each tissue of at least three mice with standard deviation (SD).

significant difference of 5-hmC content in different tissues was observed. For example, the percentage of 5-hmC measured in brain is 0.163%, which is about 10.8-fold higher than that in the spleen (0.015%). It was also observed that 5-hmC was abundant in kidney (0.045%), heart (0.06%), and lung (0.05%) tissues. In contrast, 5-hmC content was relatively low in liver (0.022%) and spleen (0.015%) tissues. Interesting, little content of 5-hmC was detected in all 3 cell lines (<0.02%). We also measured the 5-hmC amount of one known 5-hmC content sample (0.03%), which would provide supporting evidence to indicate that the 5-hmC contents measured with the MCE biosensor are accurate.

The role of 5-mC in epigenetic regulatory processes is today quite well understood.[25] However, the removal of this epigenetic mark from genetic code is still controversially

Table 1. Comparison of MEC Biosensor with LC-MS/MS and Commercial Kits in Terms of the DNA Input, Limit of Detection (Single 5-hmC), and Cost

assay	time[a] (h)	DNA input (ng)	LOD (pg)	equipment needed	cost ($/sample)	ref
LC-MS/MS	~20	1000–5000	1.411	HPLC, mass spectrometer	15	23
commercial kits	1.3	>200	10	colorimeter	4–6.8	24
MEC biosensor	0.6	90	0.489	electrometer	1.5	

[a]Average detection time per sample.

DOI: 10.1021/acs.analchem.6b00230
Anal. Chem. 2016, 88, 3476–3480

discussed.[26] Thus, it is not surprising that the discovery of 5-hmC and the modifying TET enzymes caused significant excitement. To elucidate the function of 5-hmC in mammalian tissue, the knowledge of its distribution could provide valuable information. Our quantification results in different tissues show that 5-hmC is present in every tissue investigated. The highest level of 5-hmC is detected in DNA isolated from brain (0.163%). DNA extracted from kidney, heart, and lung has medium 5-hmC values from 0.045% to 0.06%. DNA from liver and spleen possess the lowest amount of 5-hmC. Remarkably, these values obtained by MEC biosensing are in agreement with the former detection levels measured using other methods.[4,27,28] As expected, DNA from all cell lines possessed a very low amount of 5-hmC, which is in agreement with most reports.[28,29]

CONCLUSION

In summary, a sensitive and selective MEC biosensor was developed for 5-hmC detection in total genomic DNA based on chemical modification of hydroxyl in 5-hmC triggered enzymatic signal amplification. The developed method presented high detection specificity because the chemical reaction for 5-hmC modification can not happen at any other unhydroxymethylated nucleic acids bases. Meanwhile, multiply samples can be detected simultaneously with high-efficiency, which was time-saving and cost less. As a proof of concept, this method also showed high detection sensitivity with low LOD of 0.489 pg (0.0012%) for 5-hmC in total DNA. It is particularly interesting to find that 5-hmC is significantly reduced in cancerous tissues and cancer cell lines. Meanwhile, it would also be necessary to determine 5-hmC in other epigenetic-associated diseases such as neurodegenerative disorders. The MEC biosensor for 5-hmC determination would help to further understand methylation/demethylation regulation in the formation and development of epigenetic-associated diseases, thereby benefiting diagnostics and therapeutics at early stages of these diseases.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.6b00230.

Template DNA and modified complementary DNA sequences (PDF)

AUTHOR INFORMATION

Corresponding Authors

*E-mail: spsong@sinap.ac.cn (S.S.).
*E-mail: fchh@sinap.ac.cn (C.F.).

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank the National Basic Research Program of China (973 program, 2012CB932600 and 2013CB932800), the National Science Foundation of China (21373260 and 31571014), and Distinguished Scientist Fellowship Program of King Saud University for the financial support.

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DOI: 10.1021/acs.analchem.6b00230
Anal. Chem. 2016, 88, 3476−3480