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Label-Free and Template-Free Chemiluminescent Biosensor for Sensitive Detection of 5-Hydroxymethylcytosine in Genomic DNA

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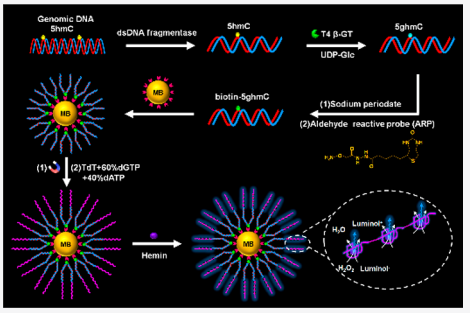
ABSTRACT: 5-Hydroxymethylcytosine (5hmC) is a modified base present at low levels in various mammalian cells, and it plays essential roles in gene expression, DNA demethylation, and genomic reprogramming. Herein, we develop a label-free and template-free chemiluminescent biosensor for sensitive detection of 5hmC in genomic DNAs based on 5hmC-specific glucosylation, periodate (IO_4^+) oxidation, biotinylation, and terminal deoxynucleotidyl transferase (TdT)-assisted isothermal amplification strategy, which we term hmC-GLIB-IAS. This hmC-GLIB-IAS exhibits distinct advantages of bisulfite-free, improved sensitivity, and genome-wide analysis of 5hmC at constant reaction temperature without the involvement of either specially labeled nucleic acid probes or specific templates for signal amplification. This method can sensitively detect 5hmC with a detection limit of 2.07×10^{-13} M, and it can detect 5hmC in the whole genome DNA with a detection limit of 3.92×10^{-5} ng/μL. Moreover, this method can distinguish 5hmC from 5-methylcytosine (5mC) and cytosine (C) and even discriminate 0.1% 5hmC in the mixture of 5hmC-DNA and 5mC-DNA. Importantly, this hmC-GLIB-IAS strategy enables genome-wide analysis without the involvement of either isotope-labeled substrates or specific antibodies, providing a powerful platform to detect 5hmC in real genomic DNA with high reproducibility and accuracy.



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Epigenetic inheritance is a heritable change in gene expression without the involvement of DNA sequence change.[1] In the mammalian genomes, 5-methylcytosine (5mC) and 5-hydroxymethylcytosine (5hmC) are the two predominant epigenetic markers. 5hmC is an oxidative intermediate in ten-11 translocation (TET)-mediated oxidation of 5mC.[2,3] 5hmC is involved in many key cell functions, including gene regulation, embryonic development, embryonic stem cell differentiation, normal bone marrow generation, and zygotic development.[4−6] Aberrant DNA hydroxymethylation has been associated with various diseases[7,8] and is a predictive biomarker for cancers, neurological abnormalities, and perilous diseases.[9] Thus, the accurate detection of 5hmC in genomic DNAs is essential for the research related to the epigenetic regulation of disease development and early cancer diagnosis.[10]

The conventional methods for the identification of 5hmC modification include oxidative bisulfite sequencing (oxBS-seq)[11] and TET-assisted bisulfite sequencing (TAB-seq).[12] oxBS-seq relies on the selective chemical oxidation of 5hmC to 5-formylcytosine prior to bisulfite treatment, while TAB-seq involves the enzymatic blocking of 5hmC and the oxidation of 5mC to 5-carboxycytosine by TET proteins prior to bisulfite conversion. However, these bisulfite treatment-based methods suffer from significant DNA degradation resulting from depyrimidination under acidic and thermal conditions and pronounced sequencing biases resulting from the selective and context-specific DNA degradation.[13,14] Alternatively, some new methods have been developed, such as liquid chromatography−mass spectrometry (LC-MS) (for urinary deoxynucleosides[15] and isotope-labeled nucleosides[16]), thin layer chromatography (TLC),[17] and immunoassays.[18,19] Even though these methods can efficiently discriminate 5hmC from 5mC, they involve expensive and complicated instruments for LC-MS,[15] costly radioactive substrates,[16,17] and specific antibodies (e.g., anti-5hmC antibodies[18] and anticytosine 5-methylenesulfonate antibodies[19]) with relatively poor sensitivity for the immunoassays.[18] Recently, some amplification strategies have been introduced to improve the sensitivity, including boronic acid-mediated polymerase chain reaction (PCR),[20] peroxotungstate oxidation-mediated ligation PCR,[21] and ligation-mediated rolling circle amplification,[22] but they inevitably suffer from nonspecific amplification and even false positivity[20,21] and require the careful design of primers, labeled nucleic acid probes, and specific templates.[21,22] Moreover, these methods enable only locus-specific[21] and fragment-specific detection of

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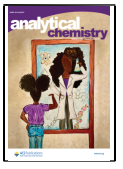

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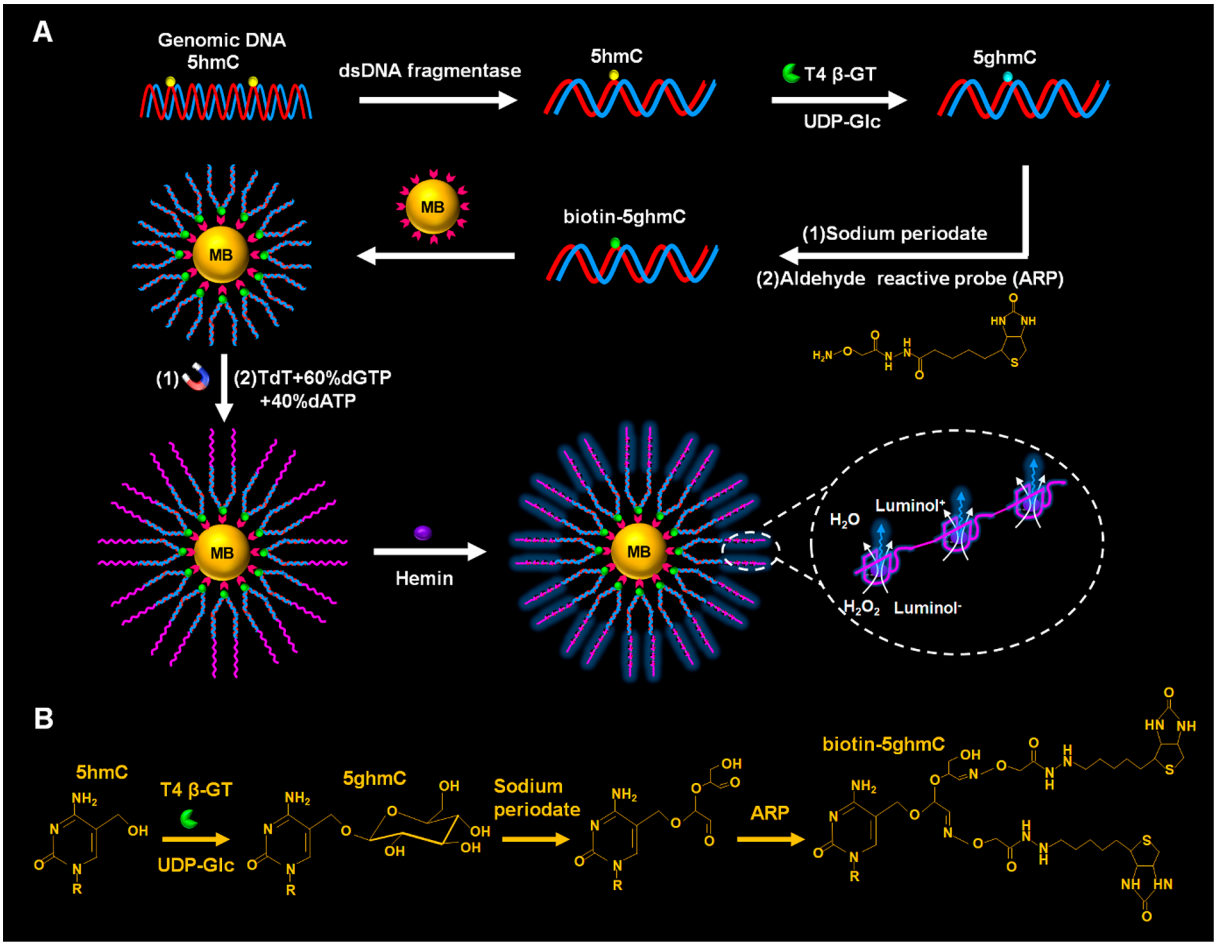

Figure 1. (A) Schematic illustration of the chemiluminescence detection of 5hmC in genomic DNA based on the hmC-GLIB-IAS strategy. (B) Molecular mechanism for the generation of biotinylated 5hmC-DNA using GLIB treatment.

5hmC.[20,22] Therefore, new strategies for 5hmC detection are highly desirable.

Since 5hmC exists in lower abundance and has a similar structure to 5mC and cytosine (C),[5] the methods for the 5hmC assay should possess high sensitivity and good specificity with the capability of eliminating the interference from 5mC and C. Chemiluminescence is the optical radiation produced by chemical reactions, and its signal readout is less disturbed by autofluorescence, scattered light, and photobleaching, enabling the achievement of high sensitivity and high signal-to-noise ratio.[23,24] Herein, we develop a label-free and template-free chemiluminescent biosensor for the sensitive detection of 5hmC in genomic DNAs based on 5hmC-specific glucosylation, periodate (IO_4^+) oxidation, biotinylation, and terminal deoxynucleotidyl transferase (TdT)-assisted isothermal amplification strategy, which we term hmC-GLIB-IAS (Figure 1A). This assay involves four steps including (1) glucosylation of 5hmC to glucosyl-modified 5hmC (5ghmC) by T4 β-glucosyltransferase (T4 β-GT), (2) selective oxidation of the newly generated 5ghmC and direct chemoselective biotinylation using aldehyde reactive probe (ARP), (3) terminal deoxynucleotidyl transferase (TdT)-mediated isothermal amplification for the generation of long G-rich DNAs, and (4) G-rich DNAzyme-driven chemiluminescence measurement. When 5hmC-DNA (Figure 1A, red color) is present, the glucosyl group in uridine diphosphate glucose (UDP-Glc) is transferred to the hydroxymethyl of 5hmC by T4 β-GT to carry out the glycosylation reaction.[25] In contrast, neither C nor 5mC can react with T4 β-GT. Consequently, only the 5hmC of genomic DNA can be covalently modified by the glucosyl group to form 5ghmC. Subsequently, 5ghmC is oxidized by sodium periodate, enabling vicinal hydroxyl groups to be converted to aldehydes.[26] 5ghmC is further modified with ARP, allowing the addition of biotin to 5hmC to form biotin-5ghmC. The detailed molecular mechanism is shown in Figure 1B. The hydroxyl group of 5hmC can be glucosylated by T4 β-GT to form 5ghmC with UDP-Glc as the cofactor, and then, 5ghmC is oxidized by sodium periodate to yield aldehydes, followed by the reaction with ARP to yield two biotins at the site of each 5hmC. After magnetic separation, the biotinylated DNAs are enriched and then incubated with 60% dGTP + 40% dATP + TdT to produce large amounts of long G-rich DNA sequences (Figure 1A, purple (magenta) wavy line). The G-rich products can bind the cofactor hemin to form the hemin-G-quadruplex nanostructures for the generation of a distinct chemiluminescence signal in the presence of H_2O_2 and luminol.

Notably, this hmC-GLIB-IAS strategy builds on selective chemoenzymatic reactions without the involvement of either isotope labeling or specific antibody, eliminating the potential radioactive hazards and data misinterpretation in antibody-based assays.[27] This strategy is PCR-free without the involvement of varying reaction temperature, and it does not involve either specially labeled nucleic acid probes or specific

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templates for signal amplification.[28] Moreover, the strategy is bisulfite-free without any noticeable DNA degradation, and the integration of the enzyme with chemical steps can isolate even a single 5hmC from 5mC and C,[19] enabling genome-wide analysis of 5hmC in limited clinical and biological samples.

This hmC-GLIB-IAS strategy is dependent on the successful generation of long G-rich DNAs by the TdT-mediated amplification reaction. We used polyacrylamide gel electrophoresis (PAGE) to analyze the amplification products with 5hmC-DNA containing a 5hmC modification (Table S1) as a model. After GLIB treatment, the obtained biotinylated DNAs are enriched to initiate the TdT-mediated amplification, resulting in the observation of a long DNA band containing various molecular weights (Figure 2A, lane 1). In contrast,

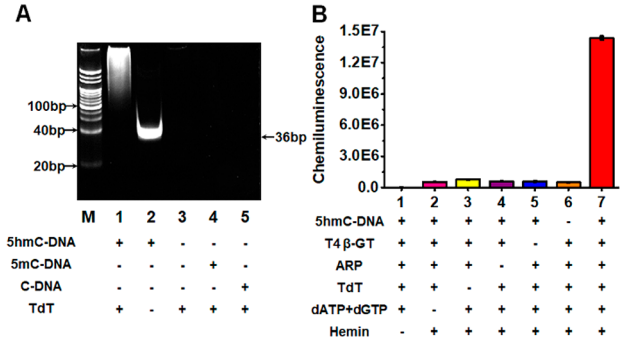

Figure 2. (A) Analysis of TdT-catalyzed amplification products by PAGE with SYBR gold staining. Lane M, DNA marker; lane 1, 5hmC-DNA with TdT; lane 2, 5hmC-DNA without TdT; lane 3, in the absence of 5hmC-DNA; lane 4, 5mC-DNA + TdT; lane 5, C-DNA + TdT. (B) Measurement of chemiluminescence intensity in the absence of one of the reaction reagents (columns 1−6) and in the presence of 5hmC-DNA-initiated GLIB-IAS (column 7). Error bars show the standard deviations of three experiments.

when TdT is absent, only a 36-bp dsDNA band is detected without any amplification bands being observed (Figure 2A, lane 2). In the absence of 5hmC-DNA, no DNA band is observed (Figure 2A, lane 3). Moreover, no DNA band is observed in the presence of either 5mC-DNA (Figure 2A, lane 4) or C-DNA (Figure 2A, lane 5). This can be explained by the fact that biotin cannot be added to either 5mC-DNA or C-DNA via GLIB treatment, and consequently, no magnetic bead-mediated DNA enrichment occurs. The GLIB-treated 5hmC-DNA (i.e., biotinylated DNA) with a yield of 63.5% can be confirmed by the agarose gel electrophoresis analysis (Figure S1). Notably, 5hmC-DNA-initiated GLIB-IAS can induce significant chemiluminescence enhancement (Figure 2B, column 7). In the absence of one of the reaction reagents (Figure 2B, columns 1−6), no distinct chemiluminescence signal is detected due to the lack of 5hmC-DNA-initiated GLIB-IAS. These results demonstrate that only 5hmC-DNA can generate a distinct chemiluminescence signal in the presence of the complete reagents.

Under the optimal conditions (Figure S2), we measured the chemiluminescence intensity induced by different concentrations of hydroxymethylated DNA (Figure 3A). Significant chemiluminescence enhancement is detected in response to the increasing concentration of 5hmC-DNA. Moreover, the chemiluminescence intensity (I) exhibits a good linear relationship with the logarithm of 5hmC-DNA concentration (C) in the range of 238 fM to 23.8 nM. The regression

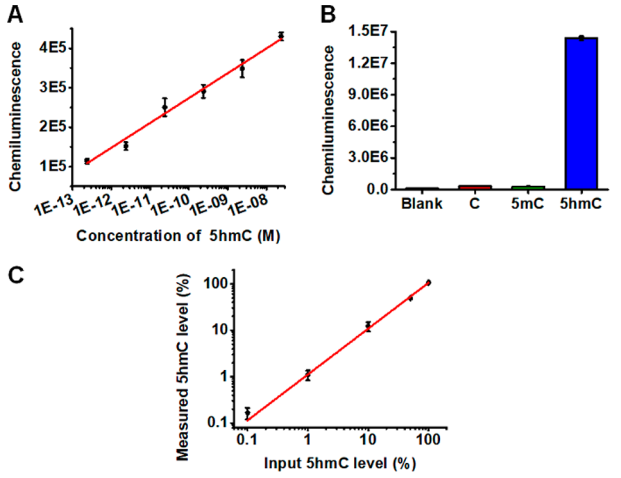

Figure 3. (A) Chemiluminescence intensity shows a linear relationship with the logarithm of 5hmC-DNA concentration. (B) Chemiluminescence intensities in response to C-DNA (red column), 5mC-DNA (green column), 5hmC-DNA (blue column), and the reaction buffer (blank, black column). The concentration of each DNA is 238 nM. (C) Correlation between the actual input and the measured 5hmC level in the mixtures of 5mC-DNA and 5hmC-DNA. The total concentration of 5mC-DNA and 5hmC-DNA is 28.6 nM.

equation is $I = 9.04 \times 10^5 + 6.30 \times 10^4 \log_{10}C$ with a correlation coefficient (R^2) of 0.991. The limit of detection is calculated to be 2.07×10^{-13} M, and the dynamic range is over 5 orders of magnitude. The sensitivity of this hmC-GLIB-IAS has improved by 806-fold, 434-fold, 154-fold, and 78.7-fold compared with those of the fluorescent assay (0.167 nM),[29] capillary electrophoresis-based fluorescent assay (90 pM),[30] electrochemical immunosensor (0.032 nM),[31] and electrogenerated chemiluminescent assay (16.3 pM),[32] respectively.

We further used three oligonucleotides including 5hmC-, 5mC-, and C-containing sequences (i.e., 5hmC-DNA, 5mC-DNA, and C-DNA) to evaluate the specificity of this strategy. As shown in Figure 3B, the chemiluminescence intensities of 5mC-DNA and C-DNA remain at the background level, and the signal in response to 5hmC-DNA is 50.0-fold and 43.9-fold higher than that in response to 5mC-DNA and C-DNA, respectively, suggesting the high specificity of this method toward 5hmC with even single-base discrimination capability.

To investigate the feasibility of this method for the accurate analysis of the hydroxymethylation level in the mixture, we prepared a series of artificial mixtures by mixing 5hmC-DNA and 5mC-DNA at different ratios. The measured 5hmC-DNA level (Y) exhibits a good linear relationship with the input 5hmC-DNA level (X) (Figure 3C). The regression equation is $Y = 0.0479 + 0.988X$ ($R^2 = 0.993$). Even as low as a 0.1% 5hmC level can be distinguished by this chemiluminescent biosensor.

To evaluate the reproducibility of the chemiluminescent biosensor, we performed 10 measurements of the same concentration of 5hmC-DNA. The relative standard deviation (RSD) is measured to be 2.6% (Figure S3), suggesting good reproducibility of this biosensor. To demonstrate the proof-of-concept of clinical application, we carried out the recovery experiments by spiking different concentrations of 5hmC-DNA into serum samples. The measured recovery of 5hmC-DNA is in the range of 93.1−106.0% with an RSD of 0.92−1.42% (Table S2).

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To validate the feasibility of this strategy for genome-wide analysis of 5hmC, we measured 5hmC in three cancer cell lines including human lung adenocarcinoma cell line (A549 cells), human glioblastoma cell line (U-118 MG cells), and human cervical carcinoma cell line (HeLa cells). On the basis of previous research, the 5hmC content varied drastically among different tissues, with the highest level in brain, while 5hmC is significantly reduced in various human tumors.[33] Especially, there is no evidence for the presence of 5hmC in HeLa cells and A549 cells probably due to the limited sensitivity of the reported methods.[5,30] In this research, we used the hmC-GLIB-IAS method to analyze 300 ng of genomic DNA extracted from the above cancer cells and successfully quantified the accurate 5hmC content in different types of cells (Figure 4A). The 5hmC level of U-118 MG cells is

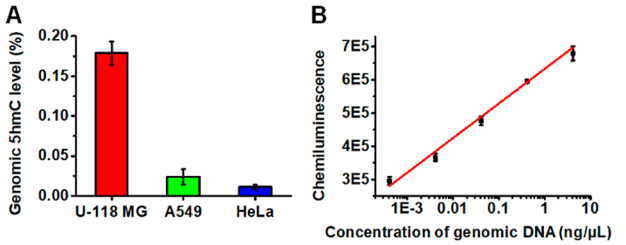

Figure 4. (A) Detection of 5hmC in genomic DNA extracted from HeLa cells (blue column), A549 cells (green column), and U-118 MG cells (red column). (B) Chemiluminescence intensity shows a linear relationship with the logarithm of genomic DNA concentration in the range of 414 fg/μL to 4.14 ng/μL.

measured to be 0.179% of the total nucleotides. Notably, the presence of 5hmC is successfully detected in A549 cells and HeLa cells with much lower abundance (0.0240% for A549 cells and 0.0111% for HeLa cells) than that in U-118 MG cells. Notably, the 5hmC level obtained by our method (0.179% for U-118 MG cells, Figure 4A) is consistent with that obtained by using the commercial colorimetric hydroxymethylated DNA quantification kit (0.166%, Figure S4). However, the commercial colorimetric kit cannot be used to quantify the 5hmC level in A549 cells and HeLa cells, because the absorbances of A549 cells and HeLa cells are too small to be distinguished from the control group without genomic DNA.

We further used U-118 MG cells as a model to investigate the relationship between the chemiluminescence intensity and concentration of genomic DNA. As shown in Figure 4B, the chemiluminescence intensity (I) improves with the increasing concentration of genomic DNA (C), and it shows a linear correlation with the logarithm of genomic DNA concentration in the range of 414 fg/μL to 4.14 ng/μL. The regression equation is $I = 6.33 \times 10^5 + 1.04 \times 10^5 \log_{10}C$ ($R^2 = 0.992$). The detection limit is calculated to be 39.2 fg/μL. These results demonstrate that this method can be applied for genome-wide analysis of 5hmC with good accuracy and reliability.

In conclusion, we have developed a hmC-GLIB-IAS-based chemiluminescent biosensor for the sensitive detection of 5hmC in genomic DNA. This strategy is bisulfite-free without severe DNA degradation and PCR-free without the involvement of varying reaction temperature, and it does not involve specially labeled nucleic acid probes and specific templates for signal amplification,[28] enabling genome-wide analysis of 5hmC without the need of either isotope/fluorescence-labeled substrates or specific antibodies. Especially, this strategy is very sensitive with a detection limit of 2.07×10^{-13} M, and its sensitivity has been improved by at least 2 orders of magnitude compared with those of optical and electrochemical methods.[29] It can accurately quantify the 5hmC content in A549 cells and HeLa cells, which cannot be achieved by the conventional methods due to their limited sensitivity.[5,30] Moreover, this method exhibits good selectivity toward 5hmC, and it can measure even a 0.1% 5hmC level in the mixture. Furthermore, this method can accurately measure 5hmC in whole genome DNA extracted from U-118 MG cells with a detection limit of 39.2 fg/μL. This hmC-GLIB-IAS strategy will facilitate the study of 5hmC modification at molecular and cellular levels.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.analchem.0c05419.

Experimental details, sequences of the oligonucleotides (Table S1), characterization of biotinylated DNA (Figure S1), optimization of the experimental conditions (Figure S2), reproducibility of the chemiluminescent biosensor (Figure S3), recovery assay of 5hmC DNA in serum samples (Table S2), and detection of the 5hmC level by using the standard method (Figure S4) (PDF)

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Notes

The authors declare no competing financial interest.

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