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Spectroscopic quantification of 5-hydroxymethylcytosine in genomic DNA using boric acid-functionalized nano-microsphere fluorescent probes☆

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ABSTRACT

5-hydroxymethylcytosine (5hmC) is the sixth base of DNA. It is involved in active DNA demethylation and can be a marker of diseases such as cancer. In this study, we developed a simple and sensitive 2-(4-boronophenyl) quinoline-4-carboxylic acid modified poly (glycidyl methacrylate (PBAQA-PGMA) fluorescent probe to detect the 5hmC content of genomic DNA based on T4 β-glucosyltransferase-catalyzed glucosylation of 5hmC. The fluorescence-enhanced intensity recorded from the DNA sample was proportional to its 5-hydroxymethylcytosine content and could be quantified by fluorescence spectrophotometry. The developed probe showed good detection sensitivity and selectivity and a good linear relationship between the fluorescence intensity and the concentration of 5 hmC within a 0–100 nM range. Compared with other fluorescence detection methods, this method not only could determine trace amounts of 5 hmC from genomic DNA but also could eliminate the interference of fluorescent dyes and the need for purification. It also could avoid multiple labeling. Because the PBAQA-PGMA probe could enrich the content of glycosyl-5-hydroxymethyl-2-deoxycytidine from a complex ground substance, it will broaden the linear detection range and improve sensitivity. The limit of detection was calculated to be 0.167 nM after enrichment. Furthermore, the method was successfully used to detect 5-hydroxymethylcytosine from mouse tissues.

1. Introduction

5-hydroxymethylcytosine (5hmC) is the sixth base of DNA after 5-methylcytosine (5mC). These bases are all variants of cytosine (C) in the DNA sequence (Kothari and Shankar, 1976; Penn and Nathar, 1976). 5hmc was originally presumed to be a DNA damage product (Penn and Nathar, 1976; Kriaucionis and Heintz, 2009; Song et al., 2011b), but is now known to be generated from 5mC by the enzymatic activity of Ten-eleven translocation Fe(II)-dependent dioxygenases (Iyer et al., 2009). DNA methylation at the 5'-position of cytosine is associated with epigenetic mechanisms of gene regulation (He and Cole, 2015). In mammals, 5 hmC is an intermediate of DNA demethylation (Wang et al., 2015), which influences normal and disease states in cells (Kian et al., 2011; Wossidlo et al., 2011). Changes in 5 hmC status have been linked to leukemia (Chowdhury et al., 2015), autism spectrum disorders (Papale et al., 2015), hypertension, Alzheimer's disease, neuronal maintenance (Mastroeni et al., 2010; Liu et al., 2013; Rudenko et al., 2013; Field et al., 2015), genetic diseases, and various types of cancer (Robertson, 2001; Yang et al., 2016), and plays important roles in epigenetic reprogramming and regulation of tissue-specific gene expression (Branco et al., 2012; Zeng et al., 2015). Although the abundance of 5mC in tissues is stable, its distribution varies substantially across cell and tissue types (Hong et al., 2013). For example, it is most abundant in the brain but is present at low levels in the lungs, heart, breast, and spleen (Globisch et al., 2010; Münzel et al., 2010; Wu and Zhang, 2011; Nestor et al., 2012; Hasan et al., 2013; Yin and Mo, 2015). These findings suggest that 5hmC can serve as a biomarker for disease diagnosis, treatment, and prognosis (Chen et al., 2016).

Various experimental tools including thin-layer chromatography (Ito et al., 2010) and high-performance liquid chromatography coupled with mass spectrometry (HPLC-MS) have been used detect cytosine, 5mC, and 5hmC levels in Purkinje and granule cells (Kriaucionis and Heintz, 2009), human cell lines, and yeast strains (Tang et al., 2013). Anti-5hmC antibodies have also been widely used for dot blot, immunoprecipitation, and enzyme-linked immunosorbent assay (Ficz

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et al., 2011; Jin et al., 2011; Williams et al., 2011; Wu et al., 2011; Huang et al., 2012; Pastor et al., 2012). Enzymatic labeling of 5hmC is a more recent approach (Höbartner 2011) in which T4-β-glucosyl-transferase (β-GT) transfers glucose from uridine diphosphate glucose (UDP-Glu) to 5hmC in T4 bacteriophages (Szwagierczak et al., 2010; Song et al., 2011a). J-binding protein 1 can also bind to glucosylated 5hmC (Robertson et al., 2011) and can be used to isolate 5hmC-containing DNA following β-GT treatment (Song and He, 2011). In addition, phenylboronic acid, which recognizes glucose (Shen and Xia, 2014) and 1,4-phenyldiboronic acid, has been used to detect 5hmC in DNA sequences by β-GT-mediated glucosylation and alkaline phosphatase-mediated signal amplification (Zhao et al., 2014; Zhou et al., 2015).

Thin-layer chromatography requires labeling with radioactive isotopes, and the accuracy is not comparable to those of other available methods. Immunohistochemical staining is tedious and less quantitative. HPLC analysis is a powerful method for the detection of 5 hmC in genomic DNA. However, large amount of DNA sample is typically required for the quantification of a low content of 5-hmC which limited its application (Tang et al., 2013). For $KRuO_4$ oxidation combined with fluorescence labeling method, it omits the influence of 5fC in DNA, and the process requires multiple purifications which make the detection more tedious and prone to interference (Mao et al., 2013]. Thus, highly selective and efficient methods are required to detect 5hmC.

We therefore developed a new method for 5hmC detection in DNA (Fig. 1). First, a PBAQA-PGMA nano-microsphere fluorescent probe was prepared by the emulsion polymerization method. β-GT catalyzes the conversion of 5hmC to glucosylated 5hmC DNA (G-5hmC-DNA) using UDP-Glu. Finally, the glucose moiety of glycosyl-modified 5hmC DNA was reacted with the boric acid group of the PBAQA-PGMA probe to form boric acid ester, which enhanced the fluorescence signal. The changes in fluorescence intensity of the DNA sample were directly proportional to its 5hmC content. In addition, the PBAQA-PGMA probe enriched G-5hmC-DNA from a complex ground substance containing low levels of 5hmC, thereby improving the sensitivity of the method.

2. Materials and methods

2.1. Reagents and instruments

GMA (99.0%), proteinase K from *Tritirachium album*, ribonuclease (RNase) A fr-om bovine pancreas, N-hydroxysuccinimide (NHS), and 1-(3-dimethylaminopropyl)−3-ethylcarbodiimide hydrochloride (EDC) were purchased from Sigma-Aldrich (Shanghai, China). PBAQA and phenol/Sevag=25:24:1 were from J & K Scientific Ltd. (Beijing, China). β-GT (including 10× NEB Buffer 4 and UDP-Glu) and 5hmC- and 5mC-containing oligodeoxynucleotides [5'-CTT AAG CCG (5hmC)AG GTA CCT TCC-3' and 5'-ATC GTT GAT (5mC)AC GTC TAG CTG-3', respectively] and normal DNA (5'-CGG TAC CTG CGG CTT AAG CC-3') sequences were purchased from Takara Biotechnology (Dalian, China). The QIAquick Nucleotide Removal kit was from Qiagen (Hilden, Germany). Mice were provided by the Xi'an Jiaotong University Health Science Center. All other chemicals were of analytical grade and were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). All solutions were prepared in Milli-Q water (18 mΩ; Millipore, Billerica, MA, USA).

Ultraviolet-visible light (UV–vis) absorption and fluorescence spectra were recorded using a Lambada35 instrument (PerkinElmer, Waltham, MA, USA) and an F-7000 fluorospectrophotometer (Hitachi, Tokyo, Japan), respectively. Fourier transform infrared (FT-IR) spectra were recorded with a Tensor 27 instrument (Bruker Daltonics, Bremen, Germany). Laser desorption/ionization and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectra (Bruker Daltonics) were acquired in LP-66 kDa mode.

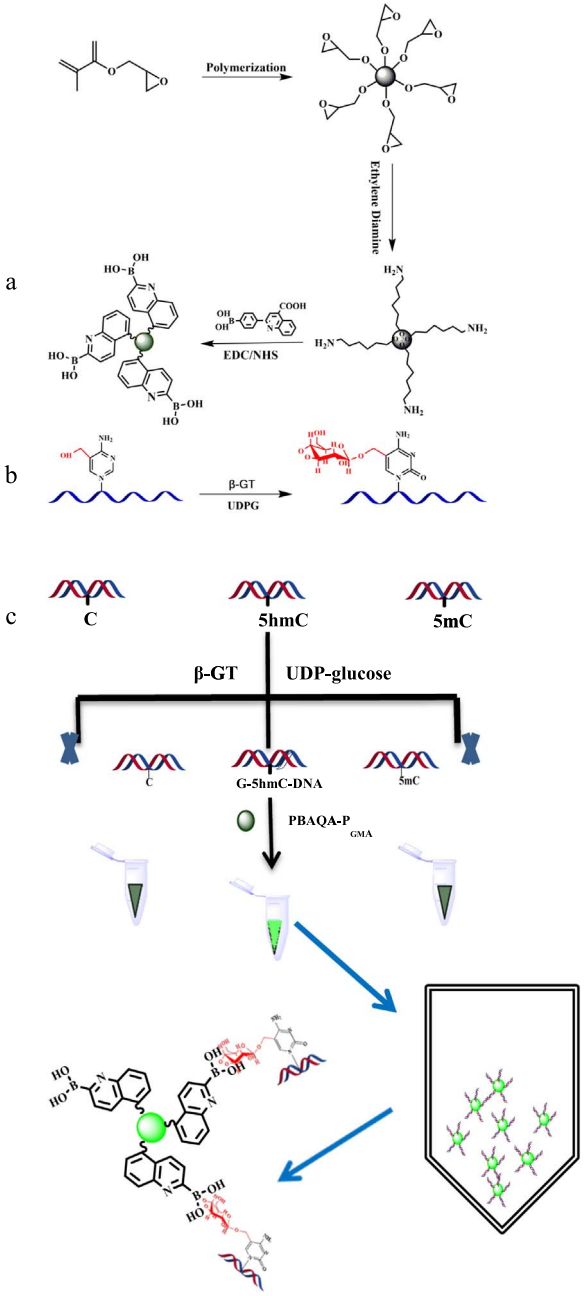

Fig. 1. (a) Preparation of PBAQA-PGMA fluorescent composite nano-microsphere biosensor. (b) The hydroxyl group of 5hmC in single-stranded DNA was glucosylated by β-GT to form G-5hmC-DNA using UDP-Glu as a cofactor. (c) Schematic illustration of the quantification of 5hmC content in genomic DNA using the PBAQA-PGMA probe.

2.2. PBAQA-PGMA synthesis

PGMA microspheres were prepared by the emulsion polymerization method using 2 ml of GMA solution and 175 ml of water, as previously described (Tapeinos et al., 2013). Amino-modified PGMA microspheres (NH_2-PGMA) were obtained by grafting ethanediamine to the PGMA surface (Wang et al., 2008; Li et al., 2012; Xiao et al., 2013). A mixture of 0.058 g NH_2-PGMA, 0.12 g PBAQA, EDC, NHS, and 20 ml ethanol was immersed in a preheated oil bath at 40 °C and stirred for 7 h after sonication for 40 min. The product was vacuum-dried at 60 °C after consecutive washes with ethanol, water, and acetonitrile, yielding the PBAQA-functionalized fluorescent probe (PBAQA-PGMA).

5hmC detection.

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