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Label-free methylation specific sensor based on silicon microring resonators for detection and quantification of DNA methylation biomarkers in bladder cancer

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ABSTRACT

Detection and quantification of DNA methylation is important to provide an opportunity to resolve clinical issues such as cancer early detection, progression, and drug treatments. In spite of myriad current technologies for DNA methylation detection that have been developed in last decade, they are still complicated and inadequate to be used as in vitro clinical diagnostic device. This work presents the first methylation specific sensor based on the silicon microring resonators which achieves fast, simple and specific label-free detection of DNA methylation after the bisulfite conversion. The methylated promoter regions of three genes (*DAPK*, *E-cadherin* and *RARβ*), which have been widely studied as biomarkers for human cancers including bladder cancer, are used as the target DNA sequences. We show that the methylated targets are strongly captured by methylated probe compared to unmethylated probe or vice versa. The discrimination between methylated and unmethylated DNA sequences is achieved within 5 min after hybridization between target and probe. Additionally, we have quantified DNA methylation density using various proportions (100, 75, 50, 25, and 0% of methylation sites) of methylation sequences of *DAPK* gene. Finally, we confirmed that the sensor can clearly detect the methylation of *RARβ* gene by using amplified target from genomic DNA of cancer cells. Therefore, our technique can be used to detect and quantify the methylation density in cancer biomarkers.

1. Introduction

Epigenetics have been raised as a hallmark of cancers over the last decades [1]. It plays a key role in the function and regulation of gene without the alteration of nucleotide sequence. Epigenetic changes (i.e., DNA methylation, histone modification, chromatin remodeling, and non coding RNAs) have come to be associated with cancer early detection, diagnosis, prognosis, and treatments. DNA methylation, especially a well-studied type of the field of epigenetics, refers to the gain of methylated group at cytosine that is mainly in promoter CpG islands of gene. Unlike in normal cells, cytosines in CpG islands of promoter region of tumor suppressor gene are methylated in many human cancer cells. The DNA methylation directly blocks the transcription process through inhibition of the binding of transcription factors, and finally causes silencing of expression of tumor suppressor genes in human cancers [2–4]. Therefore, the detection and quantification of the alteration of DNA methylation could provide one of most promising means for the early cancer diagnosis and prognosis in clinical cancer research.

One of well-established methods for DNA methylation analysis is methylation specific polymerase chain reaction (MS-PCR) after bisulfite modification in which methylated cytosines are blocked from modification, while unmethylated cytosines are converted to uracil [5,6]. Albeit the method has many drawbacks such as the need of chemical modifications and gel electrophoresis after PCR, MS-PCR is still widely used because of no additional requirement for expensive and complex equipments [3,7]. Recently, several label-free biosensors such as surface plasmon resonance (SPR) [8], opto-fluidic ring resonators (OFRR) [9], and nanowire-transistor (Nano-FET) [10] have been employed in the detection of DNA methylation. The studies show label-free detection methods without bisulfite modification, while they have focused on utilizing the interaction between methylated DNAs and proteins (methyl binding protein) or anti-5-methylcytosin antibody. Label-free biosensors offer a direct and quantitative detection of analytes without fluorescent labeling. However, the previous studies for the detection of DNA methylation based label-free biosensor without bisulfite modification have been so far demonstrated only with synthetic oligonucleotides. The direct detection of native methylated DNAs in genomic DNA in bodily fluids such as blood, urine, or saliva would be difficult due to their extremely low concentration. Although the DNA concentration in blood or urine of cancer

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Table 1
Sequences of target and probe oligonucleotides.

Gene	Name	Sequence
DAPK (*Death assoicated protein kinase*)	Methylated Probe	5′-NH_2-$(CH_2)_{12}$-GGA GGA TAG T**mC**G GAT **mC**GA GTT AA **mC**GT**mC**-3′
	Methylated Target (100%)	5′-GAC GTT AAC TCG ATC CGA CTA TCC TCC-3′
	Methylated Target (75%)	5′-AAC GTT AAC TCG ATC CGA CTA TCC TCC-3′
	Methylated Target (50%)	5′-AAC ATT AAC TCG ATC CGA CTA TCC TCC-3′
	Methylated Target (25%)	5′-AAC ATT AAC TCA ATC CGA CTA TCC TCC-3′
	Unmethylated Probe	5′-NH_2-$(CH_2)_{12}$-GGA GGA TAG TTG GAT TGA GTT AA**T** GT**T**-3′
	Unmethylated Target	5′-AAC ATT AAC TCA ATC CAA CTA TCC TCC-3′
E-cadherin	Methylated Probe	5′-NH_2-$(CH_2)_{12}$-TAA TTT TAG GTT AGA GGG TTA T**mC**G **mC**G-3′
	Methylated Target	5′-CG CGA TAA CCC TCT AAC CTA AAA TTA-3′
	Unmethylated Probe	5′-NH_2-$(CH_2)_{12}$-TAA TTT TAG GTT AGA GGG TTA T**T**G **T**G-3′
	Unmethylated Target	5′-CA CAA TAA CCC TCT AAC CTA AAA TTA-3′
RARβ(*Retinoic acid receptor β*)	Methylated Probe	5′-NH_2-$(CH_2)_{12}$-GGT TAG TAG TT**mC** GGG TAG GGT TTA T**mC**-3′
	Methylated Target	5′-GAT AAA CCC TAC CCG AAC TAC TAA CC-3′
	Unmethylated Probe	5′-NH_2-$(CH_2)_{12}$-GGT TAG TAG TT**T** GGG TAG GGT TTA T**T**-3′
	Unmethylated Target	5′-AAT AAA CCC TAC CCA AAC TAC TAA CC-3′

mC denotes 5-methylcytosine.

patients has known to be higher than that of healthy control (a mean of 180 ng/mL vs a mean of 13 ng/mL), the number of a specific gene in total DNA is extremely low [11]. For instance, Su et al. have reported that about 2 copies of mutated tumor Kristin-*ras* DNA can be found in 50–200 μL of urine or blood samples of cancer patients [12]. The sensitivity of the reported label-free biosensors is not good enough to detect such a low concentration of native DNA biomarkers. Therefore, these label-free techniques are inadequate to be used as in vitro diagnostic (IVD) device without amplification of target DNAs.

To date, silicon microring resonators have been applied to many bio-sensing applications by leveraging the advantages of highly sensitive, label-free, real-time, multiplexed detection within a single device, and high quality, low-fabrication cost due to CMOS compatibility [13]. Silicon microring resonators are used for sensing by monitoring a shift in the resonant wavelength when targets, such as proteins, bacterial and nucleic acids (i.e.; single nucleotide polymorphisms, miRNAs) [13–19] bind with the capture ligands on modified sensor surface. Prior reports of silicon microring resonators have demonstrated the ability to measure a quantification of targets in real time. Moreover, due to the microring resonator's extremely small footprint, they provide highly multiplexed detection capability within a single device and can be integrated into a compact array [20,21]. The combination of low-cost fabrication, high sensitivity and high multiplexing capability through small dimensions makes microring resonator a good candidate for disposable biosensor chips for point of care diagnostic test (POCT).

This work is the first study to develop a highly sensitive label-free methylation specific sensor for detection and quantification of DNA methylation of cancer biomarkers using silicon microring resonators. In order to implement the sensor device in real clinical setting as a POCT with bodily fluids, it would be necessary to combine an amplification step such as MS-PCR with biosensors. In this work, we first investigated the possibility of label-free based DNA methylation detection based on bisulfite modification. We used DNA methylated probes for capturing the methylated targets, which are the DNA biomarkers of bladder cancer related genes (*DAPK*, *E-cadherin*, and *RARβ*) as the detection model. Bladder cancer is the fourth most common malignancy among men in the Western world, following prostate, lung, and colon cancers and causes approximately 3% of all cancer-related deaths [22]. There have been many efforts led by research groups to develop molecular biomarkers for diagnosis and prognosis of bladder cancer, one of the urothelial cell carcinomas (UCC), which typically often recurs after initial diagnostics (50–70%) and needs to have a long term follow-up with repeated test for monitoring [23]. Recently, DNA methylation profiles have been analyzed in various bladder cancer samples such as blood [24], voided urine and tissues [23,25], and reported the molecular biomarkers including *DAPK* (*Death-associated protein kinase*), *E-cadherin*, and *RARβ* (*Retinoic acid receptor β*), which were all well-known genes for pattern of methylation in human cancers including bladder cancer. *DAPK*, a serine/threonine kinase, plays a role in tumor suppression. The loss of *DAPK* expression is associated with the recurrence and metastasis of several human cancers [26,27]. *E-cadherin*, a calcium dependent cell adhesion molecule, plays an important role in the growth and development of the cells. The *E-cadherin* methylation has been suggested in several human cancers including bladder cancer as a causative factor of loss of expression [28]. *RARβ*, a thyroid-steroid hormone receptor, controls the growth of many cell types by regulating gene expression. Methylation of *RARβ* was reported in breast [29], lung [30], and bladder cancers [23]. The developed DNA methylation based silicon microring resonators showed that the methylated targets (synthesized oligonucleotides as well as extracted genomic DNA from cancer cells) can be detected with high sensitivity and specificity without additional step such as protein binding. Therefore, we present that our methylation specific sensor is providing sufficient possibility for label-free detection of DNA methylation of biomarkers in real time manner.

2. Materials and methods

2.1. Materials

Based on bisulfite modification methods, we used methylated probes that contain methylated cytosine, and unmethylated probe that contain thymine instead of uracil. The methylate and unmethylated targets are the complementary sequences of methylated and unmethylated probes, respectively. A 5′ end amine modified 27 nucleotides probe with either methlyated cytosine (mC) or unmethylated thymine (T) was designed based on previous study [23]. C12 alkyl group was used as a linker group between the terminal functional amine group and the nucleotides. The linker group is necessary to spatially extend the probe from the surface to increase its accessibility by the target in the solution [31]. All sequences of probes and targets (Table 1) were custom synthesized and 3-aminopropyltriethoxysilane (APTES), glutaraldehyde (GAD) solution (50% wt in water), sodium cyanoborohydride solution (5.0 M in 1 M NaOH) were purchased from Sigma–Aldrich (St. Louis, MO). Other chemicals were analytical reagent grade and used as received. All samples and buffers were prepared using deionized water obtained from a Milli-Q water purification system.