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Electrochemical detection of methylated DNA on a microfluidic chip with nanoelectrokinetic pre-concentration

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ABSTRACT

DNA methylation is considered to be a promising marker for the early diagnosis and prognosis of cancer. However, direct detection of the methylated DNAs in clinically relevant samples is still challenging because of its extremely low concentration (~fM). Here, an integrated microfluidic chip is reported, which is capable of pre-concentrating the methylated DNAs using ion concentration polarization (ICP) and electrochemically detecting the pre-concentrated DNAs on a single chip. The proposed chip is the first demonstration of an electrochemical detection of both level and concentration of the methylated DNAs by integrating a DNA pre-concentration unit without gene amplification. Using the proposed chip, 500 fM to 500 nM of methylated DNAs is pre-concentrated by almost 100-fold in 10 min, resulting in a drastic improvement of the electrochemical detection threshold down to the fM level. The proposed chip is able to measure not only the DNA concentration, but also the level of methylation using human urine sample by performing a consecutive electrochemical sensing on a chip. For clinical application, the level as well as the concentration of methylation of glutathione-S transferase-P1 (*GSTP1*) and EGF-containing fibulin-like extracellular matrix protein 1 (*EFEMP1*), which are known to be closely associated with prostate cancer diagnosis, are electrochemically detected in human urine spiked with these genes. The developed chip shows a limit of detection (LoD) of 7.9 pM for *GSTP1* and 11.8 pM for *EFEMP1* and is able to detect the level of methylation in a wide range from 10% to 100% with the concentration variation from 50 pM to 500 nM.

1. Introduction

DNA methylation is the addition of a methyl group (-CH_3) to the C5 position of the cytosine pyrimidine ring through a covalent bond (Robertson, 2005). Abnormal DNA methylation is closely associated with the occurrence of human diseases, particularly cancer (Feinberg and Tycko, 2004; Jones and Takai, 2001; Wu and Zhang, 2010). Hypermethylation continuously inhibits the expression of cancer suppressor genes, cell cycle regulator genes, and DNA repair genes. As the expression of these genes is suppressed, cells abnormally proliferate, promoting cancer and failure to maintain genetic stability (McCabe et al., 2009; Hamilton, 2011). Thus, the detection of methylation in CpG islands is considered a promising biomarker for cancer prognosis and diagnosis.

The most commonly used method is the detection of DNA methylation by bisulfite assay, polymerase chain reaction (PCR), and the pyrosequencing (PSQ) method (Zhang et al., 2011, 2015; Plongthongkum et al., 2014; Taleat et al., 2015). These methods can accurately and quantitatively measure DNA methylation, but are time-consuming (minimum of 10–20 h after sample collection), complicate, requiring expensive equipment and highly skillful operator (Ehrich et al., 2005; Wang et al., 2009).

To overcome these limitations, new methods for the detection of DNA methylation with simple and short assay time, high sensitivity, and non-invasive are being developed. Colorimetric methods and surface plasmon resonance methods have been studied (Ge et al., 2012; Kurita et al., 2012), but the most actively studied field is an electrochemical biosensor detection. Electrochemical biosensors have advantages over other analytical transducing systems, such as relatively high sensitivity (about 1 nM), good quantification, fast detection time,

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and the possibility of miniaturization for point-of-care testing (POCT). Recently reported DNA methylation electrochemical biosensors (Hong et al., 2016; Zhang et al., 2016; Koo et al., 2014; Haque et al., 2017a; Daneshpour et al., 2016) have the advantage of low detection limit using labeled method, but an additional electrochemical labeler is needed. Additionally, there is a limit to the complex detection of concentration of methylated genes and levels of methylation associated with a cancer diagnosis, and studies on clinically relevant samples such as blood or urine are lacking.

Consequently, in this study, we aimed to develop a method to detect methylated DNA concentration and levels of methylation with a simple and relatively rapid (2 h after sample collection) label-free method. Two additional techniques were introduced to enhance the low sensitivity of the existing label-free detection method. First, a nanostructured gold electrode was used to increase the electrochemically active surface area. Using a electrochemical deposition method, a nano-sized flower-like or tree-like surface was formed on the gold electrode. Having a larger surface area per unit area increases the area on which biomolecules can be immobilized, resulting in detection of even lower concentrations of the target material. Verification of the electrochemical sensor using this nanostructured gold surface has been confirmed in previous studies (Farrow et al., 2013; Hong et al., 2015). Secondly, ion concentration polarization (ICP) phenomenon has been conducted to pre-concentrate the methylated DNA. ICP refers to the phenomenon by which, when an electrolyte is injected into a microchannel connected to a cation-selective nanochannel and a DC electric field is applied, only cations pass through the nanochannel, while anions accumulate on the anodic side (Son et al., 2016; Kim et al., 2010a, 2010b). In the meantime, both anions and cations are all pushed toward the anode of the channel, and an ion depletion zone (IDZ) having a very low concentration is formed at the anodic side of the nanochannel. As a low concentration should be maintained with the electro neutrality condition and because of the fast flow (Kim et al., 2007), the flow of charged materials, which can increase the internal concentration of IDZ, is blocked. As a result, charged materials such as proteins, viruses, and DNA cannot pass through the IDZ. Therefore, using this property, charged materials are pre-concentrated at the boundary of the IDZ to detect a trace amount of the substance (Ko et al., 2012, 2011; Cheow et al., 2014, 2010; Lee et al., 2009, 2008). In addition, the development of a seawater desalination device by continuous withdrawal a fluid having a low electrolyte concentration in a depletion layer has been actively pursued (Kim et al., 2010a, 2010b; Park et al., 2016; Martins et al., 2015).

Prostate cancer (PCa) is a malignant tumor that occurs in the prostate gland. If PCa is diagnosed at a late stage, the patients' survival rate is less than 30% (Antonarakis et al., 2012; Siddiqui et al., 2014). Therefore, early diagnosis of PCa is essential. Generally, PCa is diagnosed based on prostate tissue biopsy, including measurement of serum prostate specific antigen (PSA) concentration or digital rectal examination (DRE). However, new noninvasive tests are required because of the low sensitivity and selectivity of the conventional methods as well as the pain, discomfort, complications associated with the biopsy procedure itself (Schroder and Kattan, 2008; Costa et al., 2007). Among new non-invasive PCa diagnostic markers, DNA methylation has the advantage of high sensitivity (> 90%) and selectivity (> 95%). Methylated genes associated with PCa are not only present in biopsy specimens, but also partially released through the urine (Bastian et al., 2008; Hoque et al., 2005). However, since the concentrations of methylated genes are not high in the urine, a pre-concentration process is essential to detect.

In this study, by maximizing the surface area of the electrochemical sensor and performing pre-concentration by introducing the ICP method, we electrochemically detected methylated DNA at low concentrations using a microfluidic chip. The proposed microfluidic chip consists of an ICP-based pre-concentrating unit for nanoelectrokinetic pre-concentration and an electrochemical sensing unit for methylated DNA detection. In addition, to verify the applicability of the electrochemical biosensor in clinical settings, we detected the methylation of glutathione-S transferase-P1 (*GSTP1*) and EGF-containing fibulin-like extracellular matrix protein 1 (*EFEMP1*), two known DNA methylation markers of PCa, spiked in human urine samples (Almeida et al., 2014; Yoon et al., 2012a, 2012b; Kim et al., 2011). Our findings suggest the developed chip as a promising tool for the diagnosis of PCa using human urine samples.

2. Experimental section

2.1. Materials and reagents

All chemicals and solvents used in this study were of analytical grade and were used as received. All buffer solutions were prepared in Milli-Q water (Millipore, Billerica, MA, USA). The buffer solutions employed in this study were as follows: Buffer for pre-concentration: Phosphate buffer saline (0.1× PBS, concentration channel), (10 mM KCl, buffer channel); Buffer for other chemicals: Tris buffered saline (1× TBS); Rinse buffer used to remove all non-specific binding: 1× TBS with 0.05% Tween.

2.2. Urine sample preparation

For urine experiments, normal human urine was purchased from Innovative Research and stored at 4 °C until used. To change the pH of the urine sample, sodium hydroxide (NaOH) and sulfuric acid (H_2SO_4) were used and purchased from Sigma-Aldrich.

2.3. Electrochemical sensor preparation

The bare gold electrode (diameter: 400 μm) was fabricated by a photolithography process. A detailed procedure is provide in Supplemental information. To make the nanostructured gold electrode, a gold solution was prepared for the electrochemical deposition. In brief, gold (III) chloride hydrates was immersed in 0.5 M H_2SO_4. To induce the electrochemical deposition of the nanostructured gold, a potential of −400 mV was applied to a bare gold electrode against an Ag/AgCl reference electrode under 400 rpm stirring (Supplemental information Fig. S3). After preparing the nanostructured gold electrode, cation perm-selective membrane, Nafion was patterned on a glass substrate by the surface patterning method (Choi et al., 2015). A detailed procedure is provide in Supplemental information. A 50 μM solution of thiol-modified ssDNA in DI water was cast-drop on the Nafion patterned gold electrode and incubated 6 h at 4 °C. The ssDNA-modified electrode was further treated with 10 μL of cast-drop 1 mM mercaptohexanol (MCH) for 2 h at 25 °C followed by washing with DI water. Sequence information of -SH ssDNA (Integrated DNA Technology, Coralville, IA, USA) and cDNA-cy3 (Bioneer, Seoul, South Korea) is provided in Table S1.

2.4. Microfluidic chip fabrication

The microfluidic chip consists of two layers, a working layer and a valve layer. The working and valve layer were fabricated through a standard photolithography process. A detailed fabrication procedure is provided in the Supplemental information.

2.5. Chip preparation

The final chip consists of a Nafion patterned electrode layer and PDMS block layer (valve layer + working layer). The bonding between the glass (bottom substrate) and the PDMS block was achieved via an O_2 plasma process. The detailed process and results are provided in Fig. S4.