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# Quantification of gene-specific DNA methylation in oesophageal cancer via electrochemistry

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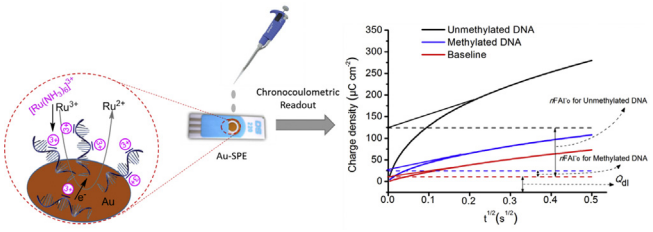
## HIGHLIGHTS

- A method for gene-specific DNA methylation in oesophageal cancer is described.
- Affinity interaction between DNA bases and gold is used to distinguish methylated and unmethylated sequences.
- The methylation level is quantified by measuring saturated amount of redox molecules in the surface-attached DNAs.
- The method is sensitive to detect 10% methylation differences.
- The assay is applied to analyze and validate various degree of methylation in cell lines and fresh tissues samples.
- The method is validated with methylation specific-high resolution melting curve analysis and Sanger sequencing methods.

## GRAPHICAL ABSTRACT

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## ABSTRACT

Development of simple and inexpensive method for the analysis of gene-specific DNA methylation is important for the diagnosis and prognosis of patients with cancer. Herein, we report a relatively simple and inexpensive electrochemical method for the sensitive and selective detection of gene-specific DNA methylation in oesophageal cancer. The underlying principle of the method relies on the affinity interaction between DNA bases and unmodified gold electrode. Since the affinity trend of DNA bases towards the gold surface follows as adenine (A) > cytosine (C) > guanine (G)> thymine (T), a relatively larger amount of bisulfite-treated adenine-enriched unmethylated DNA adsorbs on the screen-printed gold electrodes (SPE-Au) in comparison to the guanine-enriched methylated sample. The methylation levels were (*i.e.*, different level of surface attached DNA molecules due to the base dependent differential

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adsorption pattern) quantified by measuring saturated amount of charge-compensating $[Ru(NH_3)_6]^{3+}$ molecules in the surface-attached DNAs by chronocoulometry as redox charge of the $[Ru(NH_3)_6]^{3+}$ molecules quantitatively reflects the amount of the adsorbed DNA confined at the electrode surface. The assay could successfully distinguish methylated and unmethylated DNA sequences at single CpG resolution and as low as 10% differences in DNA methylation. In addition, the assay showed fairly good reproducibility (% RSD= <5%) with better sensitivity and specificity by analysing various levels of methylation in two cell lines and eight fresh tissues samples from patients with oesophageal squamous cell carcinoma. Finally, the method was validated with methylation specific-high resolution melting curve analysis and Sanger sequencing methods.



## 1. Introduction

DNA methylation is one of the clinically relevant epigenetic biomarkers that regulates gene expression *via* controlling transcriptional alteration, genomic stability, X chromosome inactivation, genomic imprinting and mammalian cell development [1]. Recent studies on epigenetic research demonstrate that aberrant DNA methylation plays a critical role in the pathophysiology of human cancers including oesophageal squamous cell carcinoma (ESCC) [2,3]. For example, gene-specific promoter hypermethylation is an important driver in the development and progression of many human cancers *via* transcriptional inactivation and suppressing of gene function [4–8]. More recently, it has also been demonstrated that DNA methylation can be used as tumour-specific therapeutic targets in ESCC [2]. Therefore, sensitive and specific profiling of gene-specific DNA methylation in ESCC could have potential implication for prediction of prognosis as well as therapy response monitoring in clinical settings.

Until recently, gene-specific DNA methylation in ESCC is generally detected *via* methylation specific PCR approaches along with bisulfite sequencing [9,10]. Over the past several decades, a variety of molecular biological approaches including methylation-sensitive single nucleotide-primer extension, methylight, methylation-sensitive high resolution melting, enzyme-linked immunosorbent assay (ELISA) based methylation assays, mass spectroscopy and fluorescence readout based methods have been conspicuously exploited to quantify the level of the DNA methylation in many human cancers [11–16]. However, most of these approaches are relatively simpler and robust but typically require large sample volumes, sophisticated instruments, multi-step procedures, hazardous radiolabeling, complex fabrication, expensive antibodies, etc. Furthermore, these assays are affected by multiple external controls for quantitative analysis, background fluorescence interference, high labour and bioinformatics costs which limit their use in routine clinical applications.

In recent years, much attention has been focused on the development of sensitive, specific, relatively simple and inexpensive method for the analysis of DNA methylation using electrochemistry, colorimetry, surface plasmon resonance and Raman scattering readouts [17–22]. While most of these readout methods have their own merits and demerits, electrochemical readout offers additional advantages in clinical diagnostics applications due to their relatively higher sensitivity and specificity, cost-effectiveness and compatibility with the miniaturization [23–25]. In these assays, sensor requires a surface-attached capture probe to hybridize the complementary target sequence, and to form duplex DNA that intercalatively bind with a redox-active transition-metal cations (e.g., $[Ru(NH_3)_6]^{3+}$) for the generation of electrochemical signals [26–29]. As described in many conventional electrochemical assays [30–33], the saturated amount of charge-compensation $[Ru(NH_3)_6]^{3+}$ complex (RuHex) on the electrode surface is electrochemically determined, which is directly proportional to the number of negatively charged phosphate residues and thereby the surface density of the target DNA.

Previously, we demonstrated the use of direct adsorption of bisulfite treated and asymmetric PCR-amplified DNA sequences onto an unmodified gold electrode (without the use of complementary capture probe and hybridization step) to quantify the level of DNA methylation present in the sequence *via* measuring the total adsorbed DNA on to the electrode surface [34,35]. Since the adsorption (*i.e.*, physisorption) trend of the DNA bases to gold surfaces follows as adenine (A) > cytosine (C) > guanine (G)> thymine (T) [36,37], two DNA sequences with different methylation patterns (*i.e.*, bisulfite treated adenine-enriched unmethylated and guanine-enriched methylated DNA sequences) should have different adsorption affinity towards gold surface. Indeed, a relatively large amount of unmethylated DNA was adsorbed on the gold electrode in comparison to the methylated DNA. In this system, we showed that monitoring the Faradaic current generated by the $[Fe(CN)_6]^{3-/4-}$ system alone could be used for the interrogation of DNA methylation level present in the bisulfite treated samples [34,35]. While this assay is relatively simple, it follows an electron transfer kinetic-based mechanism, where density of the DNA sequences at the electrode surface should be sufficiently low [31]. Additionally, the risk of false-positive responses at low concentration of target is well known when using a detection technique based on attenuation of the interfacial electron transfer reaction of a redox process (i.e., “signal-off” approach).

In order to avoid this complexity, in the current study, we explored whether simply monitoring the total charge generated by the electrostatically-attached RuHex onto the adsorbed DNA could report on the level of DNA methylation present in the samples, where generated total redox charge is the function of adsorbed DNA sequences on the electrode surface [26–29]. Since in this “signal-on” approach, the charge of the RuHex complex qualitatively reflects the amount of the adsorbed DNA at the electrode surface [30], the electrochemical signal generated by the chronocoulometric (CC) interrogation of DNA-bound RuHex will give the level of methylation present in the amplified samples. It is also important to note that unlike RuHex based conventional methods [30], the current method detects DNA methylation by simply monitoring the adsorbed target DNA on an unmodified SPE-Au electrode. Since we use direct adsorption of target DNA on an unmodified electrode rather than the conventional biosensing approach of using recognition and transduction layers, this method substantially simplifies the detection system by avoiding the complicated chemistries underlying each step of the sensor fabrication.

In this method, we first optimized the adsorption parameters (*i.e.*, adsorption time, pH of the solution, and concentration of DNA) for the direct adsorption of target DNA onto the unmodified SPE-Au surface. Then, we detected the level of promoter methylation present in *FAM134B* gene in a panel of ESCC cell lines and tissue