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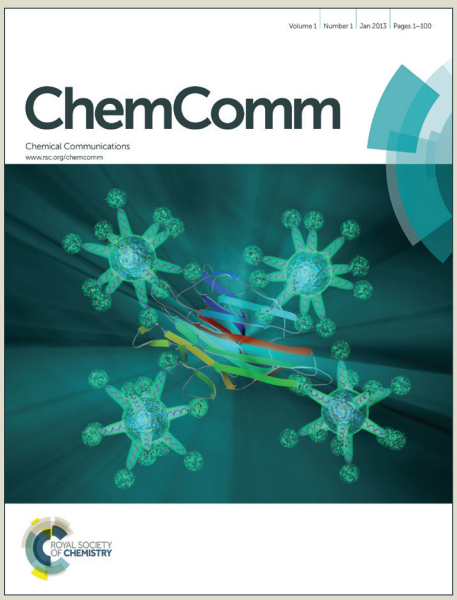

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eMethylsorb: Electrochemical quantification of DNA methylation at CpG resolution using DNA-gold affinity interactions

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We report a simple electrochemical method referred to as "eMethylsorb" for the detection of DNA methylation. The method relies on the base dependent affinity interaction of DNA with gold. The methylation status of DNA is quantified by monitoring electrochemical current as a function of relative adsorption level of bisulphite treated DNA samples onto a bare gold electrode. This method can successfully distinguish methylated and unmethylated epigenotypes at single CpG resolution.

The nature of adsorption affinity of unmodified DNA (e.g., without thiol-binding groups) on bare gold surfaces has long been regarded as "non-specific" and "difficult to control".[1,] However, Tarlov *et al.*[2] has reported that this process is finely governed by the composition of DNA bases and strictly follows the following affinity trend: adenine (A) > cytosine (C) > guanine (G) > thymine (T).[2] Since then base dependent DNA adsorption emerged as one the most promising solutions to achieve controlled immobilization of unmodified DNA probes onto gold surfaces.[3] Because this adsorption is highly sequence (base) dependent,[2] it can also be used to distinguish two different DNA sequences (*e.g.,* bisulphite treated sequences representing methylated and unmethylated DNA). However, to date no one has used this direct adsorption process to detect methylation events at a single CpG level of resolution. Herein, we report a simple electrochemical method for the direct detection of DNA methylation on bisulfite treated samples by simply measuring their relative adsorption level on a gold electrode.

DNA Methylation is one of the key epigenetic signatures that usually occur at the 5 position of cytosine within CpG dinucleotides located at certain regulating regions,[4] and is considered to be a potential biomarker for many diseases including cancer.[5] It does not involve changes in DNA sequences, but it can easily be translated into base changes by bisulphite treatment, which converts unmethylated cytosines into uracils keeping methylated one unchanged.[6] To date, several methods based on methylation specific PCR,[7] combined bisulphite restriction analysis,[8] bisulphite sequencing,[9] MALDI-TOFF[10] or capillary-electrophoresis[11] have been developed to detect DNA methylation on bisulfite treated samples. However, these methodologies require long analysis periods, labels and/or expensive instrumentations, which restrict their practical applications in clinical settings. In recent years some attempts have also been made to achieve cost-effective methodologies for detecting DNA methylation.[12-13] These methodologies are also somewhat complex and involve multistep analysis procedure. To provide a better solution to these issues, we hypothesized that a detection method based on the electrochemical quantification of adsorbed DNA (bisulphite treated DNA adsorbs on gold via gold-DNA affinity interaction) onto a bare gold electrode might be adapted to methylation detection. Previously, a number of electrochemical methods have also been developed for DNA methylation detection[14], most of which are based on hybridization of a target sequence with a surface bound receptor probe and the use of the redox system (e.g., $[Ru(NH_3)_6]^{3+}$/$[Fe(CN)_6]^{3-}$). In this study, we show that simply monitoring of the Faradaic current generated by the $[Fe(CN)_6]^{3-/4-}$system alone could report on the presence or absence of the methylation events on bisulfite treated DNAs derived from cancer cell lines (referred to as "eMethylsorb"), where generated current is the function of adsorbed DNAs on the electrode surface.

The scheme 1 illustrates the basic principle of eMethylsorb approach. Briefly, DNA samples extracted from cell lines were treated with bisulfite to convert unmethylated cytosines into uracils while methylated cytosines remained unchanged. These samples were then converted to ss-DNA amplicons by an asymmetric PCR step, where methylated cytosines were copied into guanines, and uracils into adenines. The resulting ss-DNA amplicons were directly adsorbed onto a gold electrode. The amount of the adsorbed DNA

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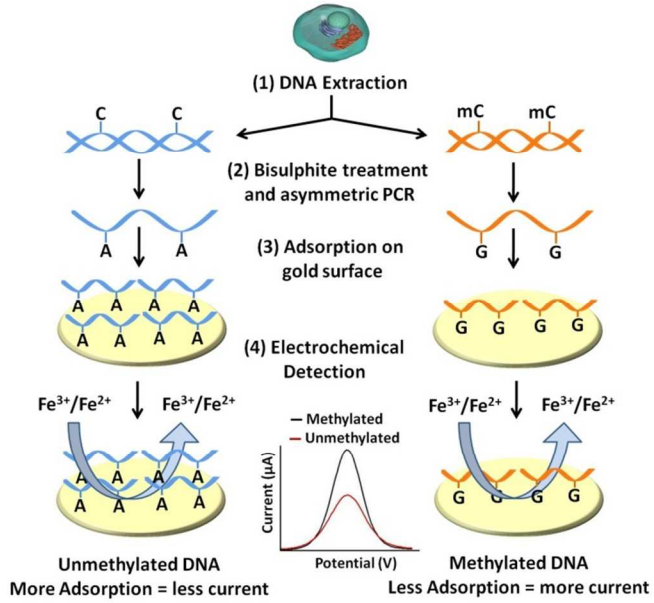

Scheme1: Schematic of the eMethylsorb approach for the detection of DNA methylation. Inset, the DPV current generated at the electrode modified with methylated and unmethylated DNA samples. The coulombic repulsion between $Fe(CN)_6^{3-}$ and negatively charged DNA (adsorbed) strands for unmethylated samples (*i.e.,* adenine enriched) is much stronger than that of the methylated one, and hence generates lower current for unmethylated samples.

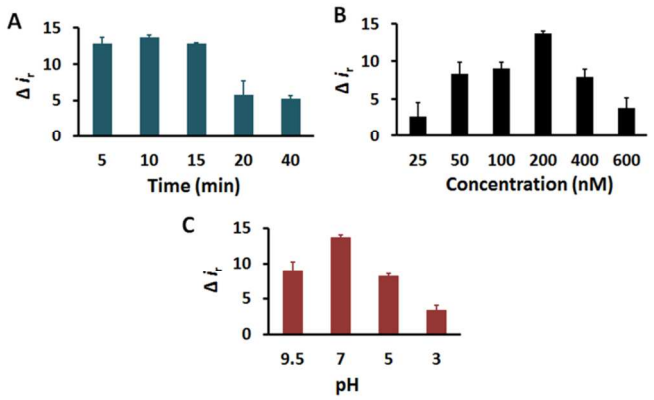

Figure 1: Mean values of relative current difference (Δi_r) obtained for the adsorption of methylated and unmethylated DNA at (A) different time, (B) concentration, and (C) pH of the solution. Other parameters for (A): DNA concentration, 200 nM, solution pH 7; for (B): adsorption time, 10 min; solution pH 7; for (C): adsorption time, 10 min, DNA concentration 200 nM. Each bar in Figs (A), (B), and (C) represents the average of three separate trials (n=3). Error bars represent the standard deviation of measurements (relative standard deviation (%RSD) was found to be < 6% for n= 3).

was quantified by the differential pulse voltammetry (DPV) in the presence of the $[Fe(CN)_6]^{3-/4-}$ system (see ESI† for details). Although, this system is usually coupled to the $[Ru(NH_3)_6]^{2+/3+}$ redox system to improve detection sensitivity (*i.e.,* electrocatalytic enhancement of the signal),[15] Zhang *et al.* has recently reported that this system alone can be used for quantification of surface-bound DNAs at gold electrodes.[16] They propose an electron transfer kinetic-based mechanism, where density of the DNA strands at the electrode surface should be sufficiently low. Under this condition, the coulombic repulsion between $Fe(CN)_6^{3-}$ and negatively charged DNA strands (low coverage) at the electrode surface is not effective enough to fully repel $Fe(CN)_6^{3-}$ molecules to approach the electrode surface, and therefore generates a significant Faradaic current. In our approach, the level of adsorbed adenine- or guanine-enriched DNA amplicons correlates with the Faradaic signal generated by the $Fe(CN)_6^{3-/4-}$ system at the electrode surface. Since DNA-gold affinity interaction follows the trend of A>C>G>T, the adenine-enriched unmethylated DNA leads to a larger level of adsorbed DNA on the electrode in compare to the guanine-enriched methylated DNA. This provides less Faradaic current due to the strong coulombic repulsion between $Fe(CN)_6^{3-}$ and negatively charged adenine-enriched DNA strands.

To demonstrate the applicability of eMethylsorb, we designed synthetic DNA in such a way that represents the same methylated and unmethylated sequence derived from the engrailed homeobox1 (*EN1)* genomic region after bisulphite treatment and asymmetric PCR (Table S1, ESI†). This region encompasses eight CpG sites located within a span of 53 bases downstream of a transcription start site, which is highlighted as a potential DNA methylation biomarker in different cancer types.[17] The affinity interaction between these samples and gold depends on several experimental conditions, such as adsorption time, solution pH, and DNA concentration.

We first optimized the time required to achieve maximum relative current difference between the DPV signals (Δi_r, see ESI† for details) obtained for 200 nM methylated and 200 nM unmethylated samples under the solution pH of 7. As shown in Fig 1A, only five min of adsorption time was sufficient to generate a significant Δi_r (12.8 ± 0.82) between these two samples. The difference was maximum (Δi_r = 13.6 ± 0.36) for 10 min of adsorption, and started to decrease rapidly after 15 min. This can be explained by considering the fact that the longer adsorption time allows to achieve the saturation of electrode surfaces with the methylated and unmethylated samples. This provides a very similar level of DPV signals for both the samples due to the similar levels of coulombic repulsion between the bulk $Fe(CN)_6^{3-}$ and negatively charged DNA samples adsorbed at the electrode surface. We then investigated the effect of the concentration of DNA (25-600 nM) on the Δi_r value (Figure 1B) between the methylated and unmethylated samples under the solution pH of 7 for 10 min of adsorption. The maximum Δi_r value between these two samples (*i.e.,* Δi_r = 13.6 ± 0.36) was achieved for 200 nM DNA, which was gradually decreased with increasing the concentrations. This is again related to the increasing level of adsorbed DNA (*i.e.,* stronger repulsion of $Fe(CN)_6^{3-}$) on the electrode surface with concentrations. At higher DNA concentrations, the amount of the adsorbed DNA on the electrode surface for both the methylated and unmethylated samples were increased as evident by the gradual decrease of their individual DPV responses (data not shown). Clearly, the DPV currents for the unmethylated samples decreased more dominantly than that of the methylated one, which results a gradual decrease of the Δi_r value. Finally, we optimized the solution pH by adsorbing 200 nM DNA for 10 min over the pH range of 3-9. As shown in Fig. 1C, the methylated and unmethylated samples could be differentiated at all tested pHs, however maximum Δi_r value was achieved at neutral pH. This is presumably due to the repulsion of negatively charged phosphate backbone with gold at pH 7 that hinders adsorption of methylated DNA while still allowing unmethylated sequences with higher adenine content to be adsorbed effectively. On the other hand, eMethylsorb shows its lowest ability

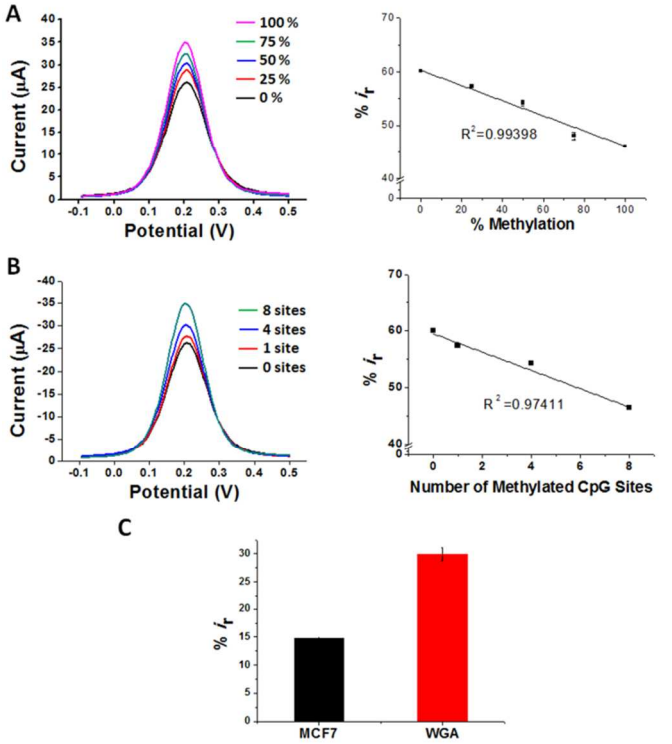

Figure 2: (A) Left: DPV signals for the designated mixture of synthetic methylated and unmethylated samples. Right: corresponding calibration plot. (B) Left: DPV signal for the synthetic samples with the designated number of methylated CpG sites. Right: corresponding calibration plot. (C) Detection of DNA methylation in *EN1* region of cell derived DNA samples. DPV responses for MCF7 DNA (black) and WGA DNA (red). Each data point represents the average of three separate trials, and error bars represent the standard deviation of measurements (% RSD = < 6% for $n = 3$).

to distinguish between methylated and unmethylated DNA at pH 3. This is due to the protonation of cytosines and adenines at pH 3, which favors faster adsorption for both the methylated and unmethylated samples. As a result, both targets reached the saturation point very quickly leading to a minimal Δi_r value.

Next, we tested the applicability of our approach to analyse DNA methylation in heterogeneous samples (*i.e.,* heterogeneous DNA methylation). Quantification of heterogeneous methylation is particularly important for cancer diagnosis since biopsy samples often contains heterogeneous methylation profile.[18] To mimic this scenario, we mixed designated proportions of methylated and unmethylated DNA (*i.e.*, Meth/Unmeth at 0%/100%, 25%/75%, 50%/50%, 75%/25%, and 100%/0%) and detected their voltammetric responses under the optimized experimental conditions. Fig. 2A shows the DPV signals generated by these heterogeneously methylated synthetic samples. Clearly, relative current response (right panel, see ESI† for details) decreases linearly (R^2= 0.99398) with increasing methylation percentage in the mixture. This is possibly due to the decrease in adenine contents with increasing methylation percentage. It is also noted that our approach is capable of analysing heterogeneous samples containing low methylation percentage (*e.g.,* 25%) with good reproducibility (RSD <5%).

To assess the applicability of our approach for the detection of low number of CpG methylation, we designed synthetic DNAs containing four and one A/G base changes corresponding to four and one methylated CpG sites within the same EN1 region respectively (Table S1, ESI†). As shown in figure 2B (right panel), the correlation between the number of methylated CpG sites and the relative DPV current response is fairly linear (R^2 = 0.97411). The current decreases with the increasing number of methylated CpG sites, probably due to the decreasing adenine numbers with increasing methylated CpG sites. The level of current responses shown in figure 2B clearly indicates that our approach can successfully detect DNA methylation events at a single CpG level of resolution.

Finally, we tested the applicability of our approach for detecting the same *EN1* region in genomic DNA derived from MCF7 breast cancer line. As an internal standard for unmethylated source, we used whole genome amplified (WGA) DNA. To avoid any PCR bias during sample preparation, we normalised the gene copy numbers prior to PCR amplification (See ESI† for details). Only 20 µL of sample spiked in 200 µL of buffer solution was enough to get a substantial difference between the DPV signals generated by methylated MCF7 cell derived DNA and unmethylated WGA DNA. Similar to synthetic DNA experiments, the relative current difference between methylated and unmethylated DNA was significantly large (Δi_r = 15.1 ± 0.96) indicating that the evaluated cell line was fully methylated (*i.e.* 100%). This finding is in agreement with our previously reported methylation status for this region on the MCF7 cell line. [12]

In conclusion, we have developed an entirely new method to detect DNA methylation by using differential adsorption affinity of DNA nucleotides towards a bare gold electrode. The simplicity and functionality of our method involves i) use of differential adsorption affinity of DNA nucleotides as a tool for cancer biomarker detection, (ii) adoption of a simple and inexpensive read-out (i.e., electrochemistry), iii) demonstration of an extremely simple and rapid detection method without use of complicated surface modifications, and (iv) potential multiplexing capability. Since eMethylsorb approach distinguish methylated and unmethylated DNA depending on the base changes generated at the CpG sites by bisulphite treatment, we assume that this approach may also find its application to detect global hypomethylation. We believe that this simple and rapid method can potentially be applicable for clinical diagnostics.

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