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eMethylsorb: rapid quantification of DNA methylation in cancer cells on screen-printed gold electrodes

Kevin M. Koo,[a] Abu Ali Ibn Sina,[a] Laura G. Carrascosa,*[a] Muhammad J. A. Shiddiky*[a] and Matt Trau*[ab]

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Simple, sensitive and inexpensive regional DNA methylation detection methodologies are imperative for routine patient diagnostics. Herein, we describe eMethylsorb, an electrochemical assay for quantitative detection of regional DNA methylation on a single-use and cost-effective screen-printed gold electrode (SPE-Au) platform. The eMethylsorb approach is based on the inherent differential adsorption affinity of DNA bases to gold (*i.e.* adenine > cytosine ≥ guanine > thymine). Through bisulfite modification and asymmetric PCR of DNA, methylated and unmethylated DNA in the sample becomes guanine-enriched and adenine-enriched respectively. Under optimized conditions, adenine-enriched unmethylated DNA (higher affinity to gold) adsorbs more onto the SPE-Au surface than methylated DNA. Higher DNA adsorption causes stronger coulombic repulsion and hinders reduction of ferricyanide $[Fe(CN)_6]^{3-}$ ions on the SPE-Au surface to give a lower electrochemical response. Hence, the response level is directly proportional to the methylation level in the sample. The applicability of this methodology was tested by detecting the regional methylation status in a cluster of eight CpG sites within the engrailed (*EN1*) gene promoter of the MCF7 breast cancer cell line. A 10% methylation level sensitivity with good reproducibility (RSD = 5.8%, $n = 3$) was achieved rapidly in 10 min. Furthermore, eMethylsorb also has advantages over current methylation assays such as being inexpensive, rapid and does not require any electrode surface modification. We thus believe that the eMethylsorb assay could potentially be a rapid and accurate diagnostic assay for point-of-care DNA methylation analysis.

Introduction

DNA methylation is an epigenetic modification of DNA for controlling gene expression and maintaining genomic stability.[1–3] In mammals, DNA methylation typically occurs by an addition of a methyl group onto the fifth carbon of the cytosine base within a CpG dinucleotide.[4] Aberrant DNA methylation of the CpG-rich regions in gene promoters has been regarded as a hallmark in cancer.[5] Therefore, an assay for the rapid detection of DNA methylation could be of aid in early cancer diagnosis and predisposition.

While many assays have been developed for the early detection of DNA methylation[6–13] on bisulfite treated samples, the majority of these methylation detection techniques usually involve complex surface chemistries, chemical labels, long experimental time or relatively tedious experimental procedures. These limitations restrict their use to research settings rather than for clinical usage. Hence, we believe the development of a simple, rapid, sensitive and low cost approach for DNA methylation detection will be ideal for point-of-care or public health diagnostics.

In order to address the above-mentioned limitations, we have recently described a novel DNA methylation approach termed as 'Methylsorb'[14] to quantitatively detect DNA methylation on bisulfite treated DNA. Previous studies have shown that various DNA bases interact with gold at different adsorption affinities following the adenine > cytosine ≥ guanine > thymine trend.[15–19] Methylsorb is the first methylation assay to exploit the higher adenine-gold adsorption affinity (*vs.* guanine-gold) for a simple and rapid measure of methylation status of bisulfite processed samples without any surface modification. Through coupling with a surface plasmon resonance (SPR) readout, methylsorb was able to accurately measure the regional methylation status in genomic DNA samples down to a sensitivity of 25% methylation in real-time. Since the underlying principle of methylsorb is only dependent on DNA-gold affinity, we hypothesized that changing to an electrochemical readout could enable higher detection sensitivity, lower assay running cost and shorter assay time for a more robust diagnostic assay.

[a]*Centre for Personalized Nanomedicine, Australian Institute for Bioengineering and Nanotechnology (AIBN), The University of Queensland, Qld 4072, Australia. E-mail: lgcarrascosa@uq.edu.au; m.shiddiky@uq.edu.au; m.trau@uq.edu.au*

[b]*School of Chemistry and Molecular Biosciences, The University of Queensland, QLD 4072, Australia*

Following this line, we engaged the use of inexpensive single-use screen-printed gold electrodes (SPE-Au) which were fabricated by screen-printing gold inks onto a ceramic substrate. Screen-printed electrodes have been shown to be ideal for developing point-of-care assays due to their low cost, disposability and design flexibility as compared to traditional electrode materials.[20–22] While various assays have been shown to integrate SPE-Au into electrochemical biosensors,[23] its application in DNA methylation detection has yet to be demonstrated.

Herein, we describe a cost-effective electrochemical assay (referred to as eMethylsorb) for detecting DNA methylation on a SPE-Au platform. eMethylsorb combines the differential DNA base-gold adsorption (affinity) principle with an electrochemical readout. Following bisulfite modification of DNA samples to replace unmethylated cytosines with uracils, asymmetric PCR is used to amplify the DNA sequences. The asymmetric PCR amplicons consist of adenine-enriched ss-DNA (unmethylated DNA) and guanine-enriched ss-DNA (methylated DNA). Unmethylated adenine-enriched amplicons display higher binding affinity towards the SPE-Au surface as compared to the guanine-enriched amplicons, thus leading to higher amount of adsorbed DNA after a fixed time period. A higher amount of negatively-charged DNA on the electrode surface results in greater coulombic repulsion with bulk ferricyanide $[Fe(CN)_6]^{3-}$ ions to give a lower current response during electrochemical detection. Hence, the magnitude of electrochemical response in our assay is directly proportional to DNA methylation level (*i.e.*, the higher the methylation level, the higher the current response). In this work, experimental parameters (sample concentration, adsorption time, and pH) affecting the dynamic range of electrochemical response were evaluated and optimized. Under optimized conditions, we successfully used the eMethylsorb approach to measure the methylation status of eight CpG sites in the engrailed (*EN1*) gene promoter of the MCF7 breast cancer cell line.

Experimental

Reagents and materials

All reagents were of analytical grade and purchased from Sigma Aldrich (Australia). UltraPureTM DNase/RNase-free distilled water (Invitrogen, Australia) was used throughout the experiments. DNA oligonucleotides were purchased from Integrated DNA Technologies (USA) and sequences are shown in Table 1. Screen-printed gold electrodes, DRP-C220BT (diameter = 4 mm), were acquired from Dropsens (Spain).

Preparation of genomic DNA samples

MCF7 breast cancer cell line was purchased from ATCC (USA) and grown in RPMI 1640 growth media supplemented with 10% fetal bovine serum in a humidified incubator containing 5% CO_2 at 37 °C. Genomic DNA was extracted using the DNeasy Blood and Tissue DNA extraction kit (Qiagen, Australia). In addition, 50 ng of human genomic DNA (Roche, Germany) was amplified using REPLI-g Whole Genome Amplification kit (Qiagen, Australia) to generate Whole Genomic Amplified (WGA) DNA samples as 0% methylated DNA standards.

Bisulfite treatment and asymmetric PCR

Bisulfite modification of extracted genomic DNA was performed using MethyEasyTM Xceed kit (Human Genetics Signatures, Australia) according to the manufacturer's instructions. In order to generate ss-DNA amplicons, asymmetric PCR of the bisulfite-treated DNA was performed using AmpliTaq PCR kit (Applied Biosystems, Australia) with minor modifications to manufacturer's protocol. The asymmetric PCR reaction mix consisted of 1.5 U AmpliTaq DNA polymerase, 0.7X AmpliTaq PCR buffer,0.2 mM of dNTPs, 125 nM of forward primer (Table 1), 375 nM of reverse primers (Table 1) and 0.1% Tween. Thermocycling was carried out under the following conditions: 94 °C for 10 min, followed by 50 cycles of 94 °C for 30 s, 58 °C for 45 s and 72 °C for 30 s. Finally, agarose gel electrophoresis was done to verify amplification.

DNA adsorption onto gold electrode surface

For optimization of DNA adsorption conditions and quantitative DNA methylation detection studies, synthetic oligonucleotide sequences (Table 1) were used. The synthetic oligonucleotides represent fully methylated and unmethylated bisulfite treated and asymmetrically PCR-amplified DNA sequences of a region containing 8 CpG sites within the *EN1* gene promoter. For real samples analysis, asymmetric amplicons derived from the same region of the *EN1* gene in MCF7 genomic DNA were used. Experiments with synthetic oligonucleotides were performed by diluting the samples in 5X SSC buffer (0.75 M in NaCl, 0.075 M in sodium citrate, pH 7.0, unless otherwise stated) to give various designated concentrations. For experiments with real samples, 10 μL of PCR amplicons of genomic DNA were diluted to 30 μL in 5X SSC buffer (pH 7.0). Then, 30 μL of each sample (synthetic oligonucleotides or PCR amplicons of genomic DNA) was directly dropped onto the working electrode surface of a SPE-Au and allowed to adsorb for 10 min (unless otherwise stated) with gentle shaking at room

Table 1 Sequences of DNA oligonucleotides and primers. CpG sites being interrogated are highlighted in bold and underlined font

Oligos	5′-Sequence-3′
Methylated sequence	GATAACGACGACAATAAAAACGACGCGAAAAA CCCCGAAACGCAAAACACCAA
Unmethylated sequence	AATAACAACAACAATAAAAACAACACAAAAA CCCCAAAACACAAAACACCAA
Asymmetric PCR fwd. primer	ATTCAGTCCACAACAAYGTTGGTTGAGTTTATAA GTAGGATAGT
Asymmetric PCR rev. primer	ACRACCRCAACAACCAAACCCT

temperature. The electrodes were then washed with 10 mM phosphate buffer (137 mM sodium chloride, 2 mM potassium chloride, pH 7.4) before electrochemical measurements.

Electrochemical measurements

All electrochemical measurements were performed on a CH1040C potentiostat (CH Instruments, USA) with the three-electrode system (gold working and counter electrodes, silver reference electrode) on each SPE-Au. The electrolyte buffer consisted of 10 mM phosphate buffer solution (pH 7.4) containing 2.5 mM $[Fe(CN)_6]^{3-}/[Fe(CN)_6]^{4-}$ (1 : 1) and 0.1 M KCl. Differential pulse voltammetry (DPV) signals were recorded from −0.1–0.5 V with a pulse amplitude of 50 mV and a pulse width of 50 ms. The relative change in DPV signal (% i) was normalized with the response of 0% methylated DNA and was calculated as follows:

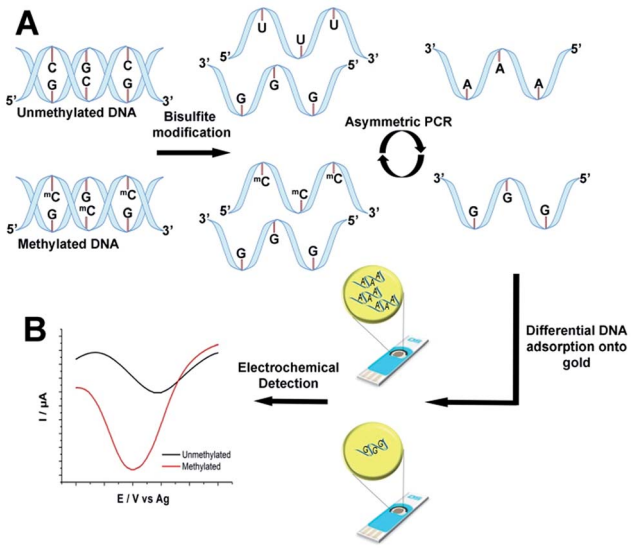
$$\text{Relative DPV signal change } (\%\ i) = [(i_M - i_{UM})/i_{UM}] \times 100$$

where i_M and i_{UM} are current densities for adsorbed methylated and unmethylated DNA samples respectively.

Results and discussion

eMethylsorb principle

As shown in Scheme 1A, extracted genomic DNA is firstly bisulfite-treated to convert unmethylated cytosines to uracils. Next, asymmetric PCR is used to exponentially generate ss-DNA sequences at the gene of interest. This method of amplification critically provides only ss-DNA which have been shown to adsorb faster onto gold surface than ds-DNA.[24] Asymmetric PCR also allows conversion of initial methylated and unmethylated DNA into guanine-enriched and adenine-enriched DNA sequences respectively. Adenine-rich oligonucleotides have been observed to display the highest binding affinity to gold surfaces over other oligonucleotide sequences.[15–19] Due to adenine-gold adsorption affinity being higher than that of guanine-gold, a higher amount of adenine-enriched ss-DNA will adsorb onto the working surface of the SPE-Au. The amount of adsorbed DNA is detected electrochemically by measuring the interfacial electron transfer reaction of $[Fe(CN)_6]^{3-/4-}$ ions on the SPE-Au surface. Although, this system is usually coupled to the $[Ru(NH_3)_6]^{3+/4+}$ redox system to improve detection sensitivity, it has been shown previously[25] that $[Fe(CN)_6]^{3-}$ ions could still overcome coulombic repulsion from negatively-charged DNA monolayers to access the electrode surface if the DNA surface density is adequately low. Our eMethylsorb assay fulfills this condition, as DNA adsorption for both methylated and unmethylated samples lead to significant but not complete coulombic repulsion of $[Fe(CN)_6]^{3-}$ ions. However, due to higher adsorption levels of adenine-enriched (unmethylated) samples as compare to guanine-enriched (methylated), this sample type generates the highest coulombic repulsion (*i.e.*, the lowest current response level). Hence, the methylation status of the DNA sample directly correlates with the magnitude of the electrochemical response level.



Scheme 1 Schematic representation of eMethylsorb. (A) Methylated and unmethylated DNA in a sample undergoes bisulfite-modification and asymmetric PCR to produce guanine-enriched and adenine-enriched ss-amplicons respectively. Both ss-amplicons adsorb onto the SPE-Au surface with different adsorption affinities and the amount of DNA adsorption is detected electrochemically. (B) Higher amount of unmethylated ss-amplicons adsorption will repel $[Fe(CN)_6]^{3-}$ ions approaching the electrode surface, and therefore generates a low Faradaic current at the electrode surface. Therefore, the methylation level of the sample (increase of % methylation) is directly proportional to the magnitude of the electrochemical responses generated at the electrode.

To demonstrate the eMethylsorb assay, we detected methylation levels in synthetic DNA samples which were designed to be similar to bisulfite-treated and asymmetric PCR-amplified target sequences of the *EN1* gene. The *EN1* gene has been described as a potential biomarker in several types of cancer[26,27] and the eMethylsorb assay was used to quantify the methylation status at a cluster of eight CpG sites within a span of 53 bases downstream of the transcription start site of the *EN1* gene. As an initial study, eMethylsorb was performed on synthetic DNA sequences which were designed to be either 100% methylated with guanines or 0% methylated with adenines at the eight CpG sites. As shown on Scheme 1B, the electrode being adsorbed with 50 nM of guanine-enriched methylated DNA displayed an approximately 95% larger DPV current response than the adenine-enriched unmethylated DNA to clearly distinguish methylated from unmethylated DNA. This result indicates that, under similar conditions, the amount of adenine-enriched unmethylated DNA being adsorbed onto the working SPE-Au surface is higher, thus reducing surface electron transfer to give a lower DPV current response. Our finding is also in good agreement with our previous study which used a SPR readout[14] as well as other studies which observed base-dependent oligonucleotide adsorption (*i.e.*, adenine > cytosine ≥ guanine > thymine) onto gold surfaces.[15,17–19]

Optimization of eMethylsorb experimental conditions

In order to maximize the signal/noise ratio to achieve maximal response difference between methylated and unmethylated

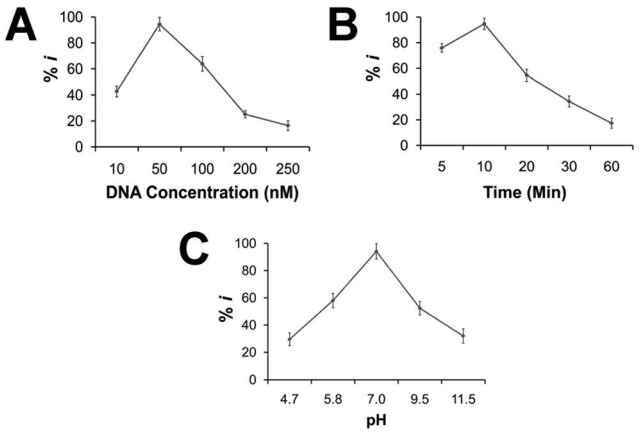


Fig. 1 Optimization of DNA adsorption conditions. Variance of % *i* (current response difference) between methylated and unmethylated DNA with (A) DNA concentration used for adsorption, (B) adsorption time, (C) pH of adsorption solution. Each data point represents the average of the three separate trials ($n = 3$) and error bars represent standard error within each experiment.

DNA samples (*i.e.* largest % *i*), we optimized the DNA concentration, time and pH of the DNA adsorption process (Fig. 1). The optimization of DNA concentration was carried out by adsorbing methylated or unmethylated synthetic oligonucleotides of various concentrations (10–250 nM) onto separate SPE-Au surfaces for 10 min at pH 7.0. As shown on Fig. 1A, a 42% change in DPV signals between methylated and unmethyated DNA (% *i*) was observed even at the lowest tested DNA concentration (*i.e.*, 10 nM). This result highlights the good sensitivity of the eMethylsorb assay in discriminating the more highly-adsorbed unmethylated sequences from methylated sequences even at low concentration. A further enhancement to 92% *i* was observed by using 50 nM concentration but any higher concentration result in sharp decrease of % *i*, with the 250 nM concentration only generating a 17% *i*. These data clearly indicate that concentration levels above 50 nM lead to similar current response levels for both methylated and unmethylated sequences, thus resulting in difficulty distinguishing methylated from unmethylated sequences. This is possibly due to saturation of both sequences on the electrodes surfaces at higher concentrations which subsequently induce similar level of coulombic repulsion between the bulk $[Fe(CN)_6]^{3-}$ ions and surface-bound DNA (*i.e.* similar current response levels). Thus, 50 nM DNA concentration was used for subsequent adsorption time and pH experiments.

The effect of time on DNA adsorption was studied by incubating 50 nM of methylated or unmethylated synthetic oligonucleotides onto separate SPE-Au surfaces over a range of different time periods (5–60 min) at pH 7.0. As shown in Fig. 1B, a 78% *i* between methylated and unmethylated sequences was observed after only 5 min of adsorption time. The highest level of 94% *i* was observed after 10 min adsorption time and subsequent extended adsorption time resulted in reduced % *i*, with 60 min adsorption time giving the lowest level of 18% *i*. The % *i* decrease after prolonged adsorption time could also be due to adsorption saturation of both methylated and unmethylated sequences (*i.e.*, similar level of coulombic repulsion between the bulk $[Fe(CN)_6]^{3-}$ ions and surface-bound DNA). Therefore, for better discrimination between methylated and unmethylated current responses, 10 min was selected as the optimal adsorption time for the eMethylsorb assay.

Finally, the effect of buffer pH on the DNA adsorption process was studied by adsorbing 50 nM of methylated or unmethylated DNA oligonucleotides onto separate gold electrodes for 10 min over a range of pH (4.7–11.5). Fig. 1C shows that the % *i* increased from 30% at pH 4.7 to a maximal 92% at pH 7.0 and then began to decrease for pH above 7. Our data suggested that buffer pH affects the competition between DNA-gold electrostatic forces and inherent DNA bases-gold binding affinities. At basic pH (*i.e.*, above pH 7.0), the gold surface would be more negatively-charged and electrostatic repulsion with the negatively-charged phosphate backbone of DNA could occur to reduce overall DNA adsorption (*i.e.* lower % *i*). In contrast, at low acidic pH, adenines and cytosines in the oligonucleotide sequences would be protonated and display higher adsoption affinity for gold.[28,29] This issue, coupled with the less negatively-charged gold surface at low pH, could favor higher DNA adsorption of both methylated and unmethylated sequences to give lower difference between their respective electrochemical responses. However, at pH 7.0, the superior adsorption affinity of unmethylated sequences could still occur due to sufficient protonation of adenines and screening of negative charges by positive ions in the buffer to reduce electrostatic repulsion. Therefore, we reasoned that neutral pH 7.0 represents the most suitable condition for obtaining higher adsorbed amount of adenine-enriched unmethylated sequences over methylated sequences to maximize response change and selected pH 7.0 as the ideal adsorption pH for eMethylsorb.

Sensitivity of eMethylsorb for heterogeneous methylation detection

Heterogeneous DNA methylation[30] is a common event in cancer. During cancer development, DNA methylation level across CpG regions occurs as a gradual process[31] with high methylation levels usually observed at the advanced stages. Furthermore, tissue biopsies usually consist of a mix of diseased and healthy cells, thus leading to a mixture of methylated and unmethylated DNA within a patient sample. Taken together, for early cancer detection, it is essential for an assay to be highly sensitive for detecting a low amount of methylated DNA in a high background of unmethylated DNA. In order to simulate this situation for our eMethylsorb assay, we mixed different volume ratios of methylated and unmethylated synthetic sequences to create samples of different % methylation (0%, 10%, 25%, 50%, 75%, 90%, and 100%). These samples were adsorbed onto surfaces of SPE-Au under optimized conditions and subjected to DPV measurements. As shown in Fig. 2A, DPV current responses displayed an increasing trend with higher DNA methylation levels. This supports the eMethylsorb principle of using adsorption of different amount of adenine-enriched DNA sequences to estimate DNA methylation level. In addition, the

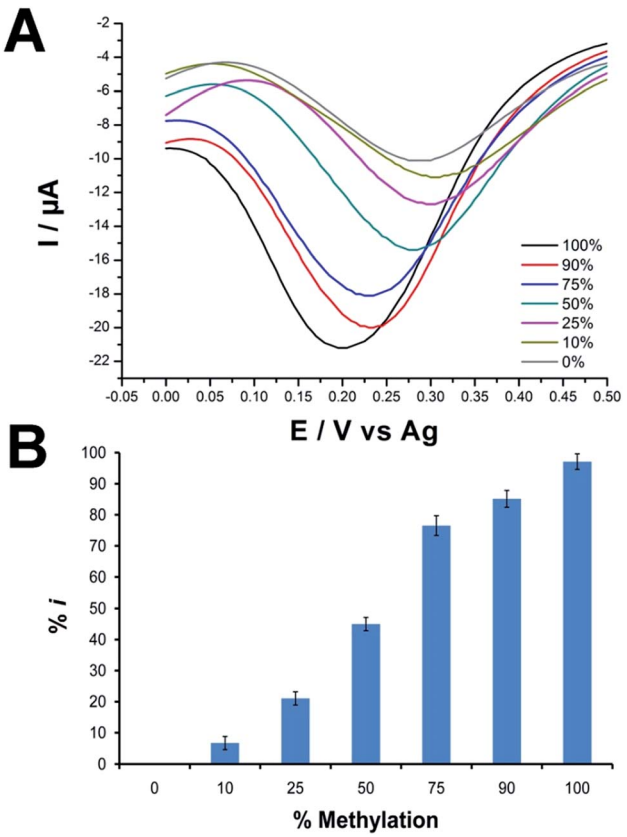


Fig. 2 (A) Differential pulse voltammograms corresponding to different % methylated synthetic oligonucleotide sequences. (B) % *i* (current response difference) for synthetic oligonuceotides at 0%, 10%, 25%, 50%, 75%, 90% and 100% methylation. Each bar graph represents the average of the three separate trials ($n = 3$) and error bars represent standard error within each experiment.

eMethylsorb assay was sensitive to 10% methylation, showing an associated response change of 6.9% above background signal (Fig. 2B). Considering that only a total of eight CpG sites were being interrogated, this detection limit demonstrates high sensitivity of the eMethylsorb assay in accurately quantifying DNA methylation. It is worthy to highlight the good sensitivity of eMethylsorb was achieved without costly fluorescence labels used in majority of current methylation detection techniques.[8,32–34]

Furthermore, eMethylsorb does not involve any time-consuming electrode surface modification and does not require any complex data analysis. Our eMethylsorb detection technique is also not limited to availability of methylation-sensitive restriction enzyme sites used in other methylation assays.[6,35,36] Moreover, the RSD over three independent eMethylsorb assays with single-use disposable SPE-Au is 5.3% (Fig. 2B), showing good assay reproducibility. As the electrodes are of single usage, there is no need for time-consuming cleaning of electrodes after each round of experiments to reduce surface modification variability and subsequently, data reproducibility. In all, the low detection limit and good reproducibility shows that eMethylsorb is a potential methylation assay for clinical applications.

eMethylsorb analysis of tumour cell line methylation

To test the application potential and analytical reproducibility of the eMethylsorb assay on real samples, we investigated the methylation status of the eight CpG sites within the *EN1* gene of human breast cancer cell line MCF7 which have been reported to be methylated in MCF7 cells.[9] DNA amplicons generated from WGA were used as unmethylated DNA (*i.e.* adenine-enriched) standards. In a similar manner to previous experiments with synthetic DNA samples, we also attempted to test the eMethylsorb assay's potential in detecting different levels of DNA methylation in a mixture of methylated and unmethylated DNA. After bisulfite conversion of the MCF7 and WGA genomic DNA, samples of different methylation levels (0%, 10%, 25%, 50%, 75% and 100%) were prepared through mixing both at different ratios. The genomic DNA samples were then asymmetrically amplified and analyzed by eMethylsorb under the optimized conditions. As shown in Fig. 3A, there is a corresponding increase in DPV current response with increasing methylation level in the DNA samples. This trend is identical to the eMethylsorb assay results using different % methylated oligonucleotides (Fig. 3) and shows that the assay can be used for quantitative methylation detection in cancer cells. Fig. 3B

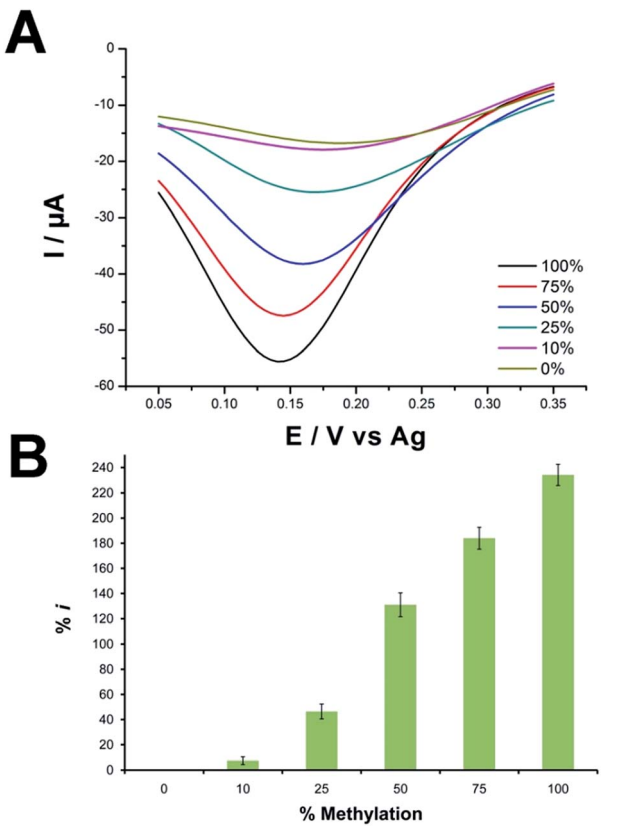


Fig. 3 (A) Differential pulse voltammograms corresponding to different % methylated DNA samples derived from MCF7 and whole genome amplified (WGA) genomic DNA. (B) % *i* (current response difference) for MCF7- and WGA-derived genomic DNA samples at 0%, 10%, 25%, 50%, 75% and 100% methylation. Each bar graph represents the average of the three separate trials ($n = 3$) and error bars represent standard error within each experiment.

shows that the difference in electrochemical response between the 100% methylated MCF7 genomic DNA and 0% methylated WGA DNA is approximately 230%. This response change is more than two-fold improvement from the response change obtained using fully methylated and unmethylated synthetic sequences (Fig. 3B). This could be explained by PCR amplicons of MCF7 DNA and WGA DNA (140 bp) being longer in length than synthetic sequences (53 bp). Longer sequences could promote adsorption of adenines in unmethylated sequences while concurrently decrease lower-affinity guanine adsorption in methylated sequences. This would lead to a higher and better dynamic range of electrochemical response during detection. The detection sensitivity is 10% methylation with a RSD of 5.8% ($n = 3$), demonstrating sensitivity and reproducibility as a potential diagnostic assay for detecting heterogeneously methylated DNA in real samples.

Conclusions

We have developed a simple, economical and label-free approach for the detection and quantification of DNA methylation in extracted genomic DNA from a breast cancer cell line. The eMethylsorb assay is based on the differential adsorption (affinity) of nucleotides on a gold surface and for the first time, combined with the SPE-Au platform to rapidly interrogate regional DNA methylation within a gene. We have demonstrated the feasibility of this technique to sensitively (10% methylation across eight CpG sites) and specifically detect methylated DNA in a heterogeneous cancer cell line sample. We believe the eMethylsorb assay has potential for diagnostics applications in the early detection of DNA methylation in diseases.

Conflict of interest

The authors declare no competing financial interest.

Acknowledgements

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