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Rapid electrochemical assessment of tumor suppressor gene methylations in raw human serum, and tumor cells and tissues using immuno-magnetic beads and selective DNA hybridization

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Dedication ((optional))

Abstract: We report a rapid and sensitive electrochemical strategy for the detection of gene-specific 5-methylcytosine DNA methylation. Magnetic beads (MBs) modified with an antibody specific for 5-methylcytosines (5-mC) are employed for the selective capture of any 5-mC methylated single-stranded (ss)DNA sequence. A flanking region next to the 5-mCs of the captured methylated ssDNA is recognized by selective hybridization with a synthetic biotinylated DNA sequence, further labeled with an HRP streptavidin conjugate. Amperometric transduction at disposable screen-printed carbon electrodes (SPCEs) is employed. The developed biosensor exhibits a dynamic range from 3.9 to 500 pM and a detection limit of 1.2 pM for the methylated synthetic sequence of the tumor suppressor gene O-6-methylguanine-DNA methyltransferase (*MGMT*) promoter region. The applicability of this strategy is demonstrated through the 45 min-analysis of specific methylation in the *MGMT* promoter region directly in raw spiked human serum samples and in genomic DNA extracted from U-87 glioblastoma cells and paraffin-embedded brain tumor tissues without any amplification and pretreatment step.

Epigenetic modifications, such as specific DNA methylation changes, have great emerging potential as biomarkers for early detection, diagnosis, prognosis and therapeutic stratification of cancer [1-3]. DNA methylation, catalyzed by specific DNA methyltransferases (DNMTs), involves the covalent addition of a methyl group to the carbon-5 position of a cytosine within CpG dinucleotides in clusters called CpG islands to yield methylcytosine (5-mC), while keeping the original DNA sequences unchanged [4,5]. 5-mC hypermethylation by altered activity of DNMTs is closely associated with the transcription level of a gene and considered an early event in the development of cancer [6-8]. While most CpG islands of promoter regions are unmethylated in normal cells, and their downstream genes are transcriptionally active, the CpG island promoters are methylated in cancer cells and their downstream genes, such as Ras association domain-containing protein 1 (*RASSF1A*) and *MGMT* tumor suppressor genes, are consistently silenced leading to cancer initiation [9-11]. Therefore, monitoring of altered DNA methylation patterns in the promoter region of cancer related genes is considered as a promising tool for early diagnosis and monitorization of cancer evolution following medical treatments [12-16].

Despite the potential utility of methylation assays in cancer relevant processes, their adoption in the clinic is still far. Methods for measuring DNA methylation often rely on bisulfite treatment or digestion with restriction enzymes in connection to subsequent DNA amplification or sequencing techniques. These methods are effective in studying DNA methylation patterns, but they are not quantitative, exhibit relatively high false positive rates and require expensive instruments and complex and time-consuming protocols thus hindering their implementation in standard laboratories [4]. It is also important to mention that some attractive approaches to detect DNA methylation without bisulfite treatment have been proposed involving optical detection and osmium-DNA complexation coupled with PCR [17] as well as SPR detection [18]. Other strategies involving affinity/immuno recognition of 5-mC in double-stranded (ds)DNAs by methyl-binding domain (MBD) proteins and of 5-mC in ssDNAs by antibodies, are limited due to their difficulty in quantifying methylation levels and, therefore, they are used also in connection to PCR or sequencing. Moreover, there are several enzyme-linked immunosorbent assay (ELISA) kits available for global DNA methylation profiling, but they are prone to high variability and only suitable for a rough estimation of DNA methylation [19].

In this context, electrochemical biosensors offer unique advantages to overcome the mentioned limitations for the detection of DNA methylation status [4,5,8,11,19-23]. However, the electrochemical biosensors reported so far for this purpose, using both PCR [14,24-27] and PCR independent approaches [13,28-30], have been mostly limited to synthesized DNA targets and scarcely explored in clinical samples. Moreover, most of the developed PCR-free electrochemical biosensors require multiple reagents and complex and long protocols involving bisulfite treatment [28,29] and amplification strategies using

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nanomaterials [13] to comply with the required sensitivity. Therefore, novel electrochemical biosensing strategies to perform reliable quantification of DNA methylation in clinically relevant and minimally treated samples with simple and rapid protocols, are highly demanded.

This work reports an immuno-DNA based method for the fast assessment of specific methylations at *MGMT* tumor suppressor gene promoter, considered a useful predictor of therapy responsiveness in patients with glioblastoma [31,32]. The designed biosensing strategy is schematized in Figure 1a. HOOC-MBs were modified with a specific 5-methylcytosine (anti-5-mC) monoclonal antibody, through covalent immobilization by EDC/Sulfo-NHS chemistry, to capture any 5-mC-methylated ss-DNA sequence. The methylated captured ss-DNA is recognized by hybridization with a synthetic biotinylated DNA probe complementary with a flanking region of the methylated region. Further labeling with Strep-HRP allows amperometric determination using the H_2O_2/HQ system after magnetic capture of the modified MBs on the SPCE surface. A detailed description of the optimization of the experimental variables involved in the biosensor preparation is provided in the Supporting Information.

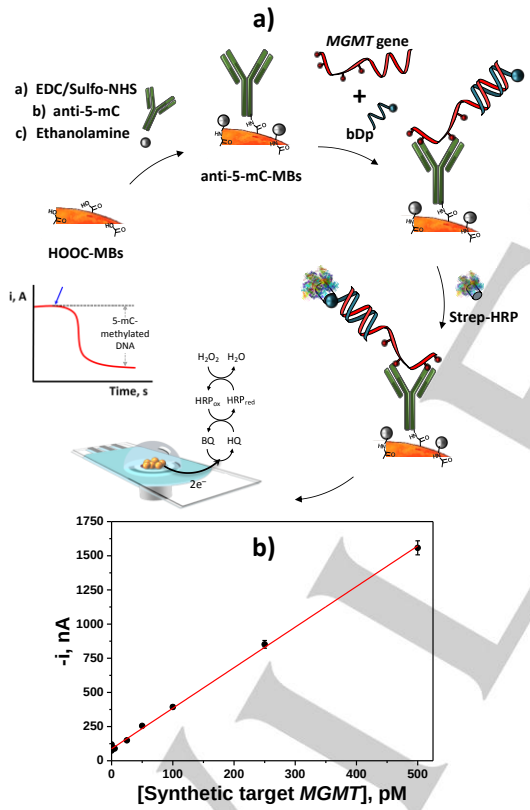

Figure 1. (a) Schematic display of the method developed for the determination of specific 5-mC methylations at *MGMT* tumor suppressor gene promoter. (b) Calibration plot constructed with the immuno-DNA sensor for the methylated synthetic sequence of the *MGMT* promoter region. Error bars estimated as triple of the standard deviation of three replicates.

The calibration plot obtained with the immuno-DNA sensor for the determination of the methylated *MGMT* promoter region (Figure 1b) shows a linear dependence (r = 0.999) with the target *MGMT* concentration between 3.9 and 500.0 pM, with slope and intercept values of (2,969 ± 30) nA nM^{-1} and (87 ± 6) nA, respectively. The limit of detection (LOD) and limit of quantification (LOQ) values were estimated as 1.2 and 3.9 pM (30 and 97 amol in 25 µL of sample), respectively (according to the $3\times s_b/m$ and $10\times s_b/m$ criteria, where s_b corresponded to the standard deviation (n = 10) for measurements made with no target DNA and m was the slope value of the calibration plot).

Table S3 compares the analytical performance of the developed biosensor with that of the few reported electrochemical affinity biosensors for the determination of gene specific methylation. Only the biosensor reported by Daneshpour et al. [13] provides a better LOD. However, the preparation of this biosensor involved the use of nanomaterials for amplification purposes and required much longer time. On the contrary, the methodology described here implies a significantly shorter assay time (45 min against 2 h 40 min) and a simple biosensor construction. Importantly, we show below that the achieved sensitivity is adequate to detect the status of the target gene promoter in complex samples.

The amperometric responses for 0.25 nM of the synthetic sequence of the promoter region of *MGMT* measured with 6 different immuno-DNA sensors yielded a RSD value of 3.3 % confirming the suitability of the sensor fabrication and the amperometric transduction protocols. In addition, the storage stability of the anti-5-mC-MBs was evaluated by storing the modified MBs at 4 °C in microcentrifuge tubes containing 50 µL of filtered PBS. Different immuno-DNA sensors prepared on different days with the stored MBs provided non-statistically different S/N ratios for the amperometric responses for 0.0 and 0.25 nM *MGMT* for at least 35 days.

The selectivity of the biosensor was evaluated by comparing the amperometric responses measured in the absence and in the presence of the synthetic methylated sequences of the promoter regions of the target *MGMT* and the *RASSF1A*. A significantly different amperometric response with respect to the blank was only observed when a biotinylated DNA probe complementary to a region flanking the methylated region of the target gene was used (Figure S3).

The ability of the anti-5-mC to capture any methylated ssDNA in a sequence independent manner can be exploited for the development of strategies that allow simultaneous detection of methylation events at different genes or different gene-specific methylation loci [29] simply by hybridization of the ss-DNA captured onto the anti-5-mC-MBs with appropriate complementary biotinylated probes. We explored this possibility by performing the simultaneous detection of synthetic methylated DNA sequences of the *MGMT* and *RASSF1A* promoter regions in one single experiment making use of dual SPCEs. The fundamentals and the amperometric measurements recorded in the absence and in the presence of 0.25 nM of the synthetic target methylated DNAs are illustrated in Figure S4. The results, discussed in SI, demonstrated that the methodology can be applied to the simultaneous detection of multiple methylation events at the same or different genes through a combination of multiplexed detection and appropriate bDps.

The applicability of the immuno-DNA sensor was evaluated by analyzing raw spiked human serum samples as well as genomic DNA extracted from human primary glioblastoma cell line U-87 MG and brain tumor tissues from patients diagnosed with glioblastoma.

There is a considerable interest in developing simple and highly sensitive methods to be applied in complex biological matrices like serum, where the target DNA is present along with a large amount and variety of biomolecules which may cause serious interferences in the analytical readout. Figure 2 compares the amperometric responses obtained in the absence and in the presence of 0.25 nM of the synthetic target *MGMT* in buffer solution and in human serum diluted at different percentages. No significant difference was found in the S/N ratio calculated in these media. In addition, the slope values of the calibration graphs constructed in undiluted human serum (3,017 ± 39 nA nM^{-1}) and in buffered solutions (2,969 ± 30) nA nM^{-1}) were also not significantly different. These findings supported the absence of significant matrix effect and allowed quantification in raw undiluted human serum samples. Recoveries calculated for two different concentration levels of the synthetic target *MGMT* (25 and 100 pM) in undiluted human serum were (100 ± 5) and (105 ± 3), respectively, for a confidence level of 95 (n =3). These results demonstrated the potentiality of the methodology for the accurate determination of the methylated *MGMT* directly in raw human serum samples at amounts of 0.625-2.5 amol.

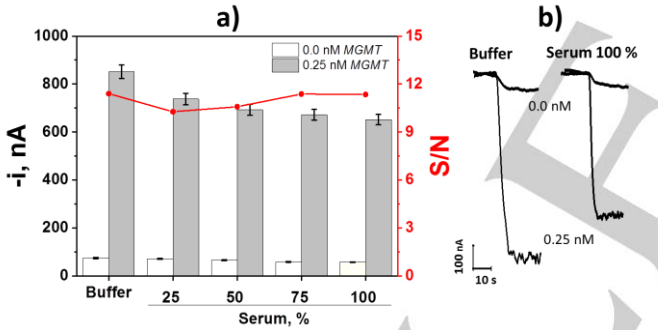

Figure 2. Amperometric responses measured with the immuno-DNA sensor in the absence and in the presence of 0.25 nM of the synthetic methylated *MGMT* in buffer solution and in human serum diluted at different percentages (a). Amperometric traces recorded in buffered solution and undiluted human serum (b). Error bars estimated as triple of the standard deviation of three replicates.

The applicability of the implemented methodology to analyze the endogenous *MGMT* status in cells was also checked. Figure 3 shows the amperometric responses obtained with 100 ng genomic DNA extracted from glioblastoma U-87 cells (positive to *MGMT* promoter methylation) [33,34] and HeLa cells (as negative control). The sensor response is consistent with the specific hypermethylation of the *MGMT* gene of the U-87 cell line. Figure 3 shows as better results were achieved with fragmented genomic DNA which is due to higher efficiencies of the anti-5-mC immunorecognition and hybridization rates for shorter DNA [35]. It is worth to mention at this point that a lower sensitivity was observed in the analysis of fragmented genomic DNA when compared with the assays in spiked serum. This is most likely due to the length of the DNA fragmented by sonication is approximately 10-times longer than the synthetic methylated target (300-500 bp [36] vs 49-mer). As it is known, long targets produce hindered hybridization efficiency due to steric hindrance [35,37,38].

The immuno-DNA sensor was also used for the detection of specific methylation in the *MGMT* gene promoter region in especially challenging biological samples such as genomic DNA extracted from paraffin-embedded brain tumor tissues (FFPE) from patients diagnosed with glioblastoma (grade IV). Figure 3 shows as the biosensor response was significantly larger in 2 out of the 4 analyzed samples. Interestingly, this agrees with the presence of methylation in the promoter region of this gene identified by RT-PCR only in these 2 samples. The different methylation levels of the positive samples and the heterogeneity of tumor tissues explain the differences in the amperometric signals [14]. It is important to remark that the measurements with the immuno-DNA sensor lasted only 45 min and used 100 ng of genomic DNA which is a similar amount than that required by the commercial ELISA kits [17] and only slightly larger than the minimal amount recommended for PCR methods (4-40 ng) to prevent the generation of false-negative results [15]. Most interestingly, the reported results show that the developed biosensor can be successfully used to interrogate methylation status in FFPE samples. This is especially relevant because these samples provide an invaluable source for research and their analysis is challenging because the fixation process and long term storage of tissue may lead to DNA damage [39].

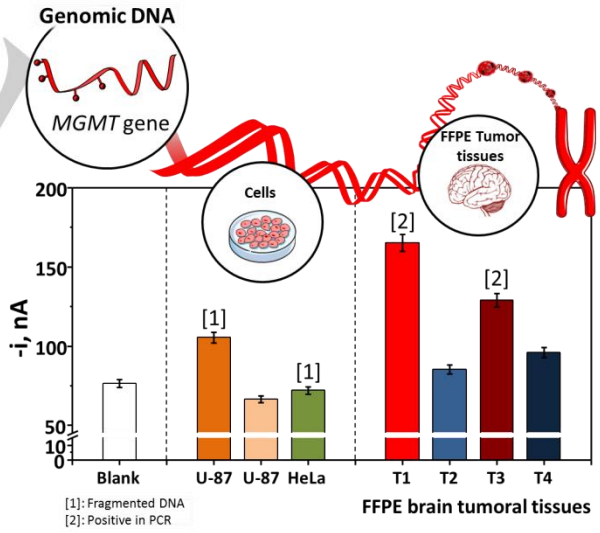

Figure 3. Amperometric measurements obtained with the immuno-DNA sensor for 100 ng of fragmented and non-fragmented genomic DNA extracted from U-87 and HeLa cells and from FFPE brain tumor tissues of 4 patients diagnosed with glioblastoma. Error bars estimated as triple of the standard deviation of three replicates.

This communication describes the development of a novel immuno-DNA sensor for the simple, sensitive and rapid detection of gene-specific DNA methylation events. The method,

illustrated for the detection of *MGMT* promoter methylation, exhibits a great analytical performance, demonstrating competitiveness with the conventional methodologies and the few electrochemical biosensors reported so far. Under the optimal conditions, a 1.2 pM LOD is achieved for the synthetic sequence without prior amplification or laborious pretreatment steps in only 45 min. The methodology is useful for universal analysis of any methylated DNA sequence, to read-out different methylated targets in a single experiment and for direct determination, without PCR amplification, bisulfite or labeling processes, in undiluted human serum samples. The pioneering applications reported for DNA extracted from human primary glioblastoma cells and FFPE tumor tissues from glioblastoma patients without previous amplification of the genetic material, as required by conventional methodologies, illustrate the attractive features of this novel electrochemical biosensing strategy. Therefore, it can be positioned as a promising and affordable tool for high-throughput of single or multiple methylation events and for detecting other relevant epigenetic modifications of cytosines such as 5-hmC (simply by changing the capture antibody). These applications can be of great interest for better management and outcome of cancer patients and other relevant diseases, such as neurodegenerative, metabolic and cardiovascular diseases, in different settings or at the point-of-need.

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Keywords: methylated DNA • Immuno-DNA sensor • biofluids • brain tissues • dual detection

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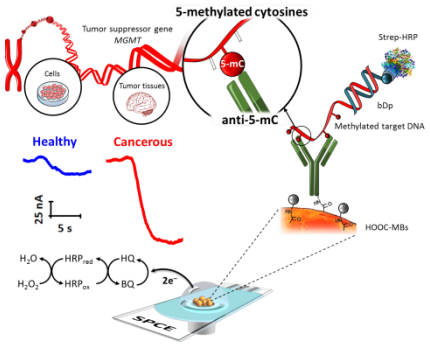
Immuno-DNA electrochemical sensor for rapid assessment of tumor suppressor gene methylations in raw human serum, tumor cells and cancer tissues



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