A simple electrochemical biosensor for the detection of methylated DNA and for methyltransferase activity monitoring

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Abstract: DNA methylation is one of the well-known epigenetic mechanism which plays a crucial role in the development of various diseases, such as cancer, cardiovascular diseases or diabetes. Electrochemical biosensors promise excellent results for clinical diagnostics, especially in term of their sensitivity, stability, selectivity, portability, they are cost-effective, easy-to-use and provides a fast response. In this study, simply, reliably and selective DNA based electrochemical biosensor for detection of methylated DNA was fabricated. The proposed biosensor was modified with the synthesised reduced graphene oxide combined with gold nanoparticles (rGO-AuNPs). This nanocomposite has shown a strong affinity to the DNA probe and demonstrated promising analytical characteristics. The electrochemical impedance spectroscopy (EIS) was used for the characterization of interface properties of the gold electrode (GE). Additionally, the sensitivity of the developed biosensor was performed by differential pulse voltammetry (DPV) to investigate the activity of enzyme methyltransferase *M.SssI* (MTase). Fabricated biosensor offers quite a low detection limit (LOD), which was 3.2 U/ml and limit of quantification (LOQ) was 3.3 U/ml.

Key Words: electrochemical biosensor, methylated DNA, reduced graphene oxide, gold nanoparticles

INTRODUCTION

Methylation of cytosines in DNA is one of the most studied epigenetic process regulating gene expression and thus plays an important role in cell differentiation or proliferation. Among others, methylated DNA can be used as a new generation of biomarkers in modern diagnostic for detection of various diseases, prognosis or prediction of therapeutic response (Krejcova et al. 2017, Hossain et al. 2017). Methylated DNA can be detected quantitatively by many methods at the entire genome or specific gene loci in various samples of biological origin, such as urine, plasma and serum. The main advantage is that those samples can be obtained by non-invasive procedures (Gao et al. 2018, Li et al. 2012). Current conventional methods, such as polymerase chain reaction (PCR), colorimetric methods or high-performance liquid chromatography (HPLC) are effective, nevertheless, they have several disadvantages, such as expensive instrumentation and often involve complicated protocols or require skilled operators. On the other hand, electrochemistry provides sensitive, selective, cost-effective, reliable, fast and simple detection of MTase activity and methylated DNA assays (Wang et al. 2013, Chen et al. 2019).

This study was aimed to develop an efficient electrochemical DNA-based biosensor for the detection of DNA methylation. Furthermore, the rGO-AuNPs nanocomposite was used for quantification of methylated DNA to enhance the electrochemical signal and increase the sensitivity of the developed biosensor. The GE with a 1.6 mm diameter was used as a working electrode. As a recognition element was used thiolated single-stranded DNA (ssDNA) probe, because of strong sulphur affinity to gold (Au-S). Firstly, the ssDNA probe was immobilised on the GE surface via hybridization with ssDNA target, resulting in double-stranded DNA (dsDNA). The non-specific

binding sites were blocked by 6-mercapto-1-hexanol (MCH) and the characterization of the fabricated biosensor was carried out by EIS (Keighley et al. 2008). Afterwards, the dsDNA was methylated by enzyme *M.SssI* MTase. Then it was immersed in Methylene Blue solution (MB), which is used as an electrochemical indicator. Therefore, the sensitivity of the proposed biosensor was investigated by DPV. Then the methylated dsDNA was digested by the restriction endonuclease *Hpa*II and then cleaved at a specific site sequence 5'-CCGG-3'. Obtained electrochemical signals of the reporter were decreased or disappeared (Bao et al. 2018).

MATERIAL AND METHODS

Reagents

The synthetics oligonucleotides of specific sequence, such as probe: (ssDNA_1) HS-$(CH_2)_6$-5'-CCT CGT GCG GGA TCA TTG TTA TTA GGCA-3'and target: (ssDNA_2) 3'-GGA GCA CGC CCT AGT AAC AAT AAT CCGT-5', chemicals for Piranha solution: nitric acid (HNO_3), hydrogen peroxide (H_2O_2), MB, enzymes: CpG MTase *M.SssI* supplied with 10× NEBuffer 2, 200× S-adenosylmethionin (SAM, 32 mM) and restriction endonuclease *Hpa*II with 10× CutSmart buffer (ThermoFisher), chemicals for buffers preparation: sodium chloride (NaCl), magnesium chloride ($MgCl_2$), Trizma® base, hydrochloric acid HCl (37%), sodium hydroxide (NaOH), potassium sulfate (K_2SO4), potassium hexacyanoferrate(III) ($K_3[Fe(CN)_6]$), potassium hexacyanoferrate(II) ($K_4[Fe(CN)_6]$), sodium dihydrogen phosphate (NaH_2PO_4) and sodium phosphate dibasic (Na_2HPO_4). Composition of used buffers is shown in Table 1. Reagents for the synthesis of GO and rGO-AuNPs: Tetrachloroauric acid ($H[AuCl_4]$), 37% HCl, sodium citrate ($Na_3C_6H_5O_7$), potassium permanganate ($KMnO_4$) and H_2O_2.

Chemicals used in this study, unless otherwise stated, were purchased from Sigma-Aldrich (St. Louis, MO, USA). The deionized water was prepared using reverse osmosis equipment Aqual 25 (Aqua Osmotic, Tisnov, Czech Republic). The deionized water was further purified by using the apparatus Milli-Q Direct QUV, equipped with the UV lamp (Aqua Osmotic, Tisnov, Czech Republic). The resistance was 18.2 MΩ·cm. The pH was measured using pH meter WTW InoLab (Weilheim, Germany).

Table 1 Buffer composition

Buffer	Composition
Phosphate buffer	1.0 M at pH 7.0: 0.5 M NaH_2PO_4 + 0.5 M Na_2HPO_4
Immobilization buffer	0.8 M PB at pH 7 + 1.0 M NaCl + 5.0 mM 50 mM PB at pH 7 + $MgCl_2$ + 1.0 mM EDTA
Hybridization buffer	0.1 M PB + 0.1 M K_2SO_4
Measuring buffer	50 mM PB at pH 7 + 5 mM $K_3[Fe(CN)_6]$, $K_4[Fe(CN)_6]$ + 0.1 M K_2SO_4

Synthesis of GO and rGO/ AuNPs

GO was prepared by chemical oxidation of 5.0 g graphite flakes (100 mesh, ≥75% min) in a mixture of concentrated H_2SO_4 (670 ml) and 30 g $KMnO_4$ according to the simplified Hummer's method (Hummers 1958) The reaction mixture was stirred vigorously. After 4 days, the oxidation of graphite was terminated by the addition of H_2O_2 solution (250 ml, 30 wt%). Formed GO was washed 3 times with 1.0 M HCl (37 wt%) and several times with Milli-Q water (total volume used 10 l) until constant pH value (3 – 4) was achieved. The amount of 3.75 mg of GO was added to 50 ml $H[AuCl_4]$ (0.24 mM) and the mixture was aged for 30 mins. Then, the mixture was heated at 80 °C and 940 µl of $Na_3C_6H_5O_7$ (0.085 M) was added dropwise. The mixture was maintained at 80 °C for 1 h (Goncalves et. al 2009).

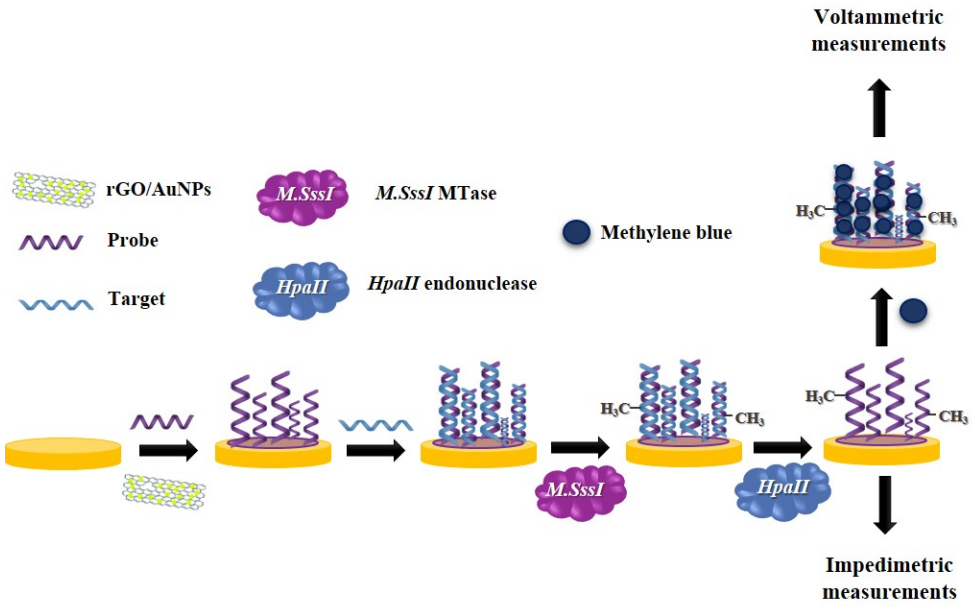
Cleaning procedures of electrodes

Prior to biosensor fabrication, the bare GE with a radius 1.6 mm (Basi, West Lafayette, USA) was cleaned mechanically, chemically and electrochemically. The chemical cleaning of the bare GE was performed in Piranha solution (96% H_2SO_4 to 30% H_2O_2, ratio 3:1) for 15 mins with constant stirring and afterwards, the GEs were rinsed with Milli-Q and the procedure continued with mechanical polishing. The three bare GEs were first sonicated in ultrasonic cleaner Elmasonic P60H (Singen am Hohentwiel, Germany) for 15 mins in ethanol (EtOH) and then mechanically polished on a polishing pad (Buehler) with water-based Diamond suspension (0.5 µm). Followed by another sonication in EtOH and polished with and Alumina slurry (1.0 µm) and sonicated in EtOH for another 15 mins. Afterwards, the last polishing was carried out with 0.3 µm alumina slurry. As the last step were electrodes cleaned electrochemically in 0.5 M H_2SO_4 by 50 scans of CV by potential scanning between −0.05 V and +1.1 V. The GEs were rinsed with Milli-Q water and air dry out.

Biosensor fabrication

For the biosensor construction (Figure 1), a 10 µl of rGO-AuNPs was dropped on bare GE and let it dry in the oven at 70 °C for 1 h. The procedure continues with incubation of ssDNA_1 with 100 µM MCH diluted in immobilization buffer (50 µl in total) overnight (14 – 16 hours) at 4 °C. Then the GE was backfilled in 1.0 mM MCH for 1.5 h and followed with hybridization of ssDNA_2 for 1 h at 37 °C. After that, the 20 µl of enzyme *M. SssI* was dropped on electrode surface and let it incubated for 2 h at 37 °C. As the last step, the biosensor sensitivity via accumulation of MB on the electrode surface was investigated. The MB was accumulated by the immersing of GE containing various concentrations of *M.SssI* (c = 0; 50; 100; 150; 200; 300; 400 and 500 U/ml) in 20 mM Tris–HCl buffer (pH 7.0) containing 20 mM NaCl and 20 µM MB for 5 min. After incubation in the MB, was the GE rinsed with 0.1 M PB three times to remove the non-accumulated MB.

Figure 1 Illustration of the developed biosensor for the detection of methylated DNA and the M.SssI MTase activity



Electrochemical experiments

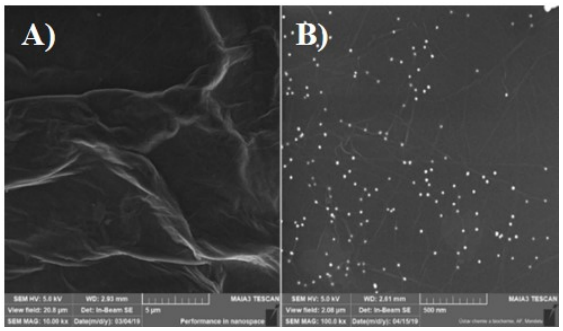
Electrochemical techniques, DPV and EIS were performed with an Autolab PGSTAT302N (Metrohm, Herisau, Switzerland). For the measurement was used a standard three-electrode connection in the electrochemical cell. As a reference electrode was used Ag/AgCl connected via a salt bridge (50 mM PB + 0.1 M K_2SO_4) and as auxiliary was set a platinum electrode. The parameters of EIS were the following: the frequency range from 100 kHz to 100 mHz, with a 10 mV a.c. voltage applied on a bias d.c. voltage of 0.2 V vs. reference electrode (corresponding to the formal potential of the redox couple). The sensitivity of biosensor was verified by DPV and the parameters were following: applied potential from 0.0 V to −0.6 V, amplitude 0.025 V and scan-rate 0.01 V/s.

RESULTS AND DISCUSSION

Synthesis of GO and rGO-AuNPs

The SEM pictures (see Figure 2) confirmed the preservation of the fine structure of the large area GO. This method also enabled determining the degree of exfoliation, which is crucial for nanomaterial. The SEM micrographs also allowed the rating of AuNPs adhesion to GO which acts as a carrier of AuNPs.

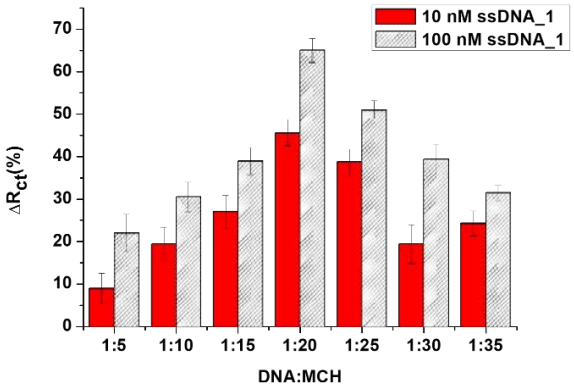
Figure 2 SEM pictures of A) GO and B) rGO-AuNPs



Optimization of self-assembled monolayer (SAM)

For the modification of biosensor, the ssDNA_1 was mixed with 100 µM MCH for the creation of the SAM layer. The MCH used as a spacer molecule which allows better control of the lateral density of the DNA structure as well as to both reduce the non-specific interactions and regulate the charge transfer (R_{ct}) for EIS experiments. Additionally, MCH molecules help the ssDNA_1 structure to stand in the upright position enabling better capturing properties towards the target. To establish complete thiol coverage of the GE surface, the electrodes were backfilled with 1.0 mM MCH diluted in Milli-Q water. Therefore, the optimisation of the EIS sensor must be done compromising the surface conditions that provide the maximum target binding with a ratio of 1:20 of ssDNA_1 to 100µM MCH fraction as is seen in Figure 3. There were tested two concentration of the DNA probe, while the 100 nM showed a more significant change in the R_{ct}. This ratio was used in the following electrochemical experiment as an optimal value.

Figure 3 The percentage increase of R_{ct} for a ratio of DNA: MCH concentrations for the determination of ssDNA_1 (10 nM and 100 nM) in measuring buffer

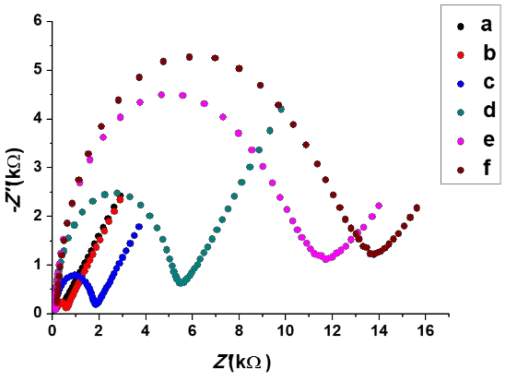


Characterization of developed biosensor

For investigation and characterisation of every single layer in the presence of the measuring buffer (Table 1) was used EIS (Figure 4). The EIS method has been confirmed as an effective technique by evaluating of R_{ct}. The effects of various modifications are presented by Nyquist plots in measuring buffer (Figure 3A). The bare GE has shown the lowest R_{ct} (354 Ω) with a small semi-circle due to the free-electron transport process. After modification by rGO-AuNPs, the value of R_{ct} (621 Ω) increased a little bit because of enhanced electron transport caused by the strong affinity between Au-S. When the ssDNA_1 was immobilised on a layer of rGO-AuNPs the value R_{ct} (1.8 kΩ) increase.

And after hybridization with ssDNA_2, while the double structure of DNA (dsDNA) was formed and the R_{ct} (5.5 kΩ) was more than three times higher. This increase is caused by electrostatic repulsion effect of $[Fe(CN)_6]^{3-/4-}$ due to the negative charge of phosphate groups from DNA structure. The process continues with treatment by *M.SssI* MTase, which also led to increasing of R_{ct} (13.7 kΩ), which indicates full methylation of dsDNA. As the last step, there was applied *Hpa*II, which caused the decreasing of R_{ct} (11.8 kΩ). The main reason is in blocking of the cleavage of a specific site by *Hpa*II. This result indicates a successful biosensor fabrication.

Figure 4 EIS characteristic of different modifications in measuring buffer (Table 1). (a) The bare GE, (b) GE/rGO-AuNPs, (c) GE/rGO-AuNPs/probe DNA_S1, (d) GE/rGO-AuNPs/probe DNA_1/ target DNA_2, (e) GE/rGO-AuNPs/probe DNA_1/ target DNA_2/ M.SssI MTase (500 U/ml)/ HpaII (20 U/mL) and (f) GE/rGO-AuNPs/probe DNA_1/ target DNA_2/ M.SssI MTase (500U/ml)

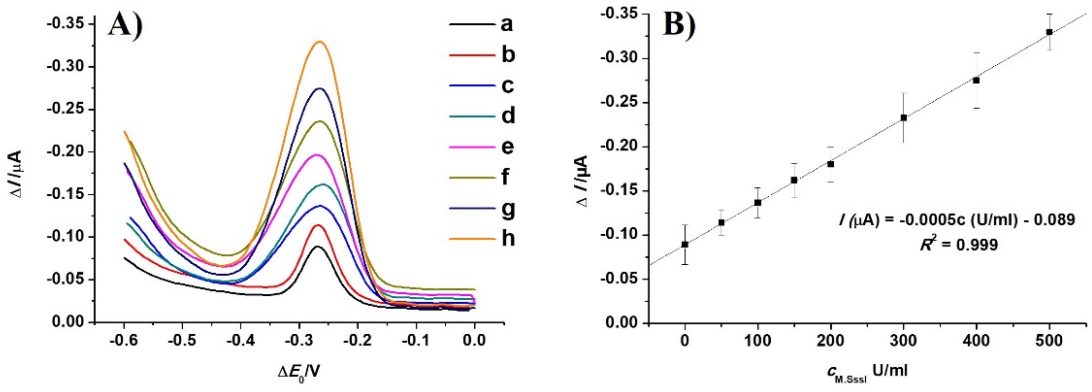


Sensitivity of biosensor

In the term of biosensor sensitivity, the DPV technique was performed to investigate the level of *M.SssI* activity by increasing concentration of *M.SssI*, from 0 to 500 U/ml (Figure 5). As Figure 5A) shows, the signal grows with a higher concentration of *M.SssI*. It depends on the incorporation of MB into the grooves of the double helix structure, and therefore the more dsDNA, the more MB can be integrated and it enhances the signal. Nevertheless, it confirms a fact, that the highest concentration of *M.SssI* prevents *Hpa*II to cleave the target specific sequence of dsDNA.

Figure 5B) presents the calibration curve with a linear signal increasing concentration of *M.SssI* from 0 to 500 U/ml. The values of linear equation are: $I(\mu A) = -0.006c$ (U/ml) − 0.089; ($R^2 = 0.999$). The LOD was estimated to 3.2 U/ml and the LOQ was 3.3 U/ml. The LOD was calculated from 3σ rule (S/N = 3) and error bars are presented as a standard deviation from measurement (n = 3).

Figure 5 A) DPV curves of modified electrode. The dsDNA was methylated by 0; 50; 100; 150; 200; 300; 400 and 500 U/ml M. SssI. B) Calibration curve corresponding to the accumulated MB in the groove of helix structure. Error bars were created in dependence on standard deviations from the measurement



CONCLUSION

In summary, simple, reliable and selective DNA based electrochemical biosensor for the detection of methylated DNA was fabricated. There were tested DNA methylation process via EIS and the level of methylation by DPV, which indicates good sensitivity as well. Compared with conventional methods, the proposed technique is simple, easy, without expensive instrumentation, PCR amplification and does not require multiple steps of the procedure. It has shown a great potential for detection on real samples which will be held in the future. Moreover, this biosensor promises to transfer to screen printed electrodes which enable mass and low-cost production of this biosensor which can be part of smart nanodevices for personal use.

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