ELECTROCHEMICAL BIOSENSOR BASED ON MODIFIED REDUCED GRAPHENE OXIDE WITH SILVER NANOPARTICLES FOR DETECTION OF METHYLATED DNA

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Abstract

DNA methylation is one of the most studied and basic epigenetic process related to several diseases such as diabetes, neurodegenerative or cardiovascular diseases and even cancer. Methylated DNA presents a new generation of biomarkers which can be used for point-of-care detection. Electrochemical biosensors provide simple, fast, cost-effective, easy-to-use, reliable and efficient detection in contrast with conventional diagnostic methods. These biosensors can be used for early diagnosis of mentioned diseases and increase patient recovery by early clinical interventions. In this study, methylated DNA was detected electrochemically by the developed biosensor. The bare gold electrode was modified by drop-casting reduced graphene oxide with silver nanoparticles which enhance electrochemical signal due to their strong affinity to thiol modified DNA probe via a disulfide bridge. Afterwards, the sensitivity and selectivity of the nanocomposite were examined. The faradic electrochemical impedance spectroscopy was used for determination of the hybridization of the DNA probe with a methylated DNA sequence. The fabricated biosensor shows promising analytical features with a wide detection of the linear range.

Keywords: Reduced graphene oxide, silver nanoparticles, nanomaterials, methylated DNA, electrochemical biosensor

1. INTRODUCTION

Epigenetics deals with modifications that inherently affect gene expression without altering the primary genetic information, especially the nucleotide sequence in the DNA. These modifications include DNA methylation, histone modification, chromatin remodelling and posttranscriptional gene regulation by miRNA. DNA methylation plays a crucial role in the regulation of several biological processes. Moreover, methylated DNA is associated with several diseases including cancer [1]. Early treatment of cancer requires preventive and accurate diagnosis, which is very expensive, time-consuming, requires specialized staff and complicated instrumentation and only can be performed in the hospitals [2]. Every year in the Czech Republic, nearly 100,000 of the world population suffer from malignant carcinoma, and last year nearly 30,000 people died because of cancer. However, due to the lack of rapid diagnostics, these numbers are rapidly increasing. The number of newly diagnosed patients is expected to increase rapidly in the coming years. Recent studies [3, 4, 5] show that methylated DNA is a new generation of biomarkers, which are a promising alternative for use in both clinical diagnostics and therapy [6]. Nevertheless, classical methods are expensive, time-consuming and require skilled staff. On the other hand, electrochemical biosensors are promising for clinical diagnosis and can be used as analytical tools in the point-of-care application.

The proposed work was aimed to create a simple and universal biosensor for the detection of DNA methylation and *M. SssI* activity assay. To achieve the better sensitivity and selectivity was used the nanocomposite

consisted from reduced graphene oxide with silver nanoparticles (rGO-AgNPs), because of the good chemical stability and ability to facilitate electron transfer between electrodes and biomolecules [7].

2. MATERIAL AND METHODS

2.1. Reagents

In this study were used synthetics oligonucleotides DNA_1: HS-$(CH_2)_6$-5'-CCT CGT GCG GGA TCA TTG TTA TTA GGCA-3' and DNA_2: 3'-GGA GCA CGC CCT AGT AAC AAT AAT CCGT-5', enzymes: CpG MTase *M.SssI* is supplied with 10× NEBuffer 2, 200× S-adenosylmethionine (SAM, 32 mM) and restriction endonuclease *HpaII* with 10× CutSmart buffer (Thermo Fisher), methylene blue (MB) chemicals for buffer preparation: sodium hydroxide (NaOH), potassium sulfate (K_2SO_4), 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). Reagents for the synthesis of GO and rGO-AgNPs: silver nitrate solution ($AgNO_3$), 37% HCl, potassium permanganate ($KMnO_4$), sodium borohydride ($NaBH_4$) and H_2O_2. All used chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated. High purity deionized water (Milli-Q Millipore 18.2 MΩ·cm^{-1}, Bedford, MA, USA) was used throughout the study.

2.2. Synthesis of GO and rGO-AgNPs

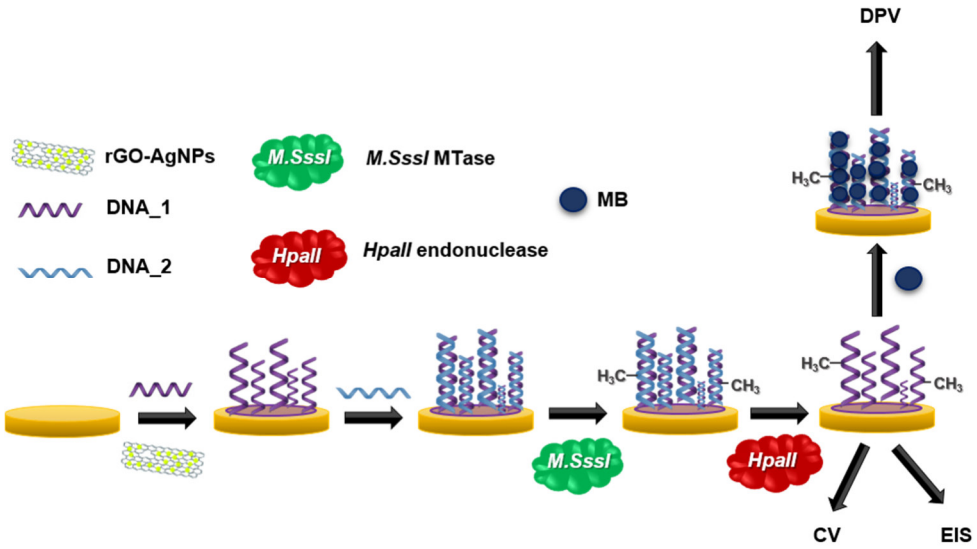
GO was prepared by chemical oxidation of 5 g graphite flakes (100 mesh, ≥75 % min) in a mixture of concentrated H_2SO_4 (670 ml) and 30 g $KMnO_4$ according to the simplified Hummer's method [8]. The reaction mixture was stirred vigorously. After 4 days, the oxidation of graphite was terminated by the addition of H_2O_2 solution (250 ml, 30 wt%). Formed GO was washed 3 times with 1 M HCl (37 wt%) and several times with Milli-Q water (total volume used 10 l) until constant pH value (3-4) was achieved. Afterwards, the 1 ml of GO solution (5 g·l^{-1}) was added dropwise into the 50 ml of $AgNO_3$ (10 mM) under vigorous stirring. After that 40 mg of sodium borohydride ($NaBH_4$) was added slowly to the reaction mixture and the resulting mixture was stirred intensively for 24 h to allow reduction. The final composite was washed with Milli-Q water several times.

2.3. The procedure of electrode surface preparation

Firstly, the bare gold electrode (GE) with a 2 mm radius (Ch Instruments Inc., Austin, USA) was mechanically and electrochemically polished. The three bare GEs were firstly 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 and an 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.

2.4. Biosensor development

The proposed biosensor was fabricated step by step as it described in Scheme 1. Firstly, the 5 µl of rGO-AgNPs was drop-casted onto the surface and let it dry. Then the 1 µM DNA_1 was immobilised onto rGO-AgNPs and let it incubate overnight in the fridge (4 °C). After incubation, the DNA_1 hybridized with 100 nM DNA_2. While the hybridization was successful, the electrode was treated by enzyme *M. SssI* methyltransferase, which added the methyl groups (–CH_3) to the fifth carbon of cytosine. At least the restriction *HpaII*, which blocked the cleaving at a specific site. To evaluate the sensitivity of developed biosensor, the *M. SssI* activity was tested. The modified GE was treated by a different concentrations of *M. SssI* (5; 25; 50; 100; 200; 300; 400 and 500 U·ml^{-1}) and measured in 20 µM MB solution.



Scheme 1 Step by step of the biosensor fabrication

2.5. Electrochemical measurements

Synthesised rGO-AgNPs was evaluated via scanning electrochemical microscopy (SEM). Process of biosensor development was characterised via electrochemical impedance spectroscopy (EIS) in Faradaic mode, with a frequency range from 100 kHz to 100 MHz. All the electrochemical measurements were performed in a standard three-electrode system. Sensitivity and reliability were characterised by differential pulse voltammetry (DPV). EIS and DPV were performed in 5 mM $Fe(CN)_6^{4-}$/ $Fe(CN)_6^{3-}$ diluted in 100 mM phosphate buffer (PB, pH 7.0). Parameters of DPV were following: potential range from 0.0 V to −0.6 V, amplitude 0.02 V and scan-rate 0.015 $V \cdot s^{-1}$.

3. RESULTS AND DISCUSSION

3.1. Synthesis of GO and rGO-AgNPs

AgNPs were stabilized on the GO surface by using $NaBH_4$ as a reducing agent. The resulting material is rGO-Ag nanocomposite with uniformly distributed AgNPs onto rGO. The morphology of nanocomposite rGO-Ag was observed using SEM (**Figure 1**).

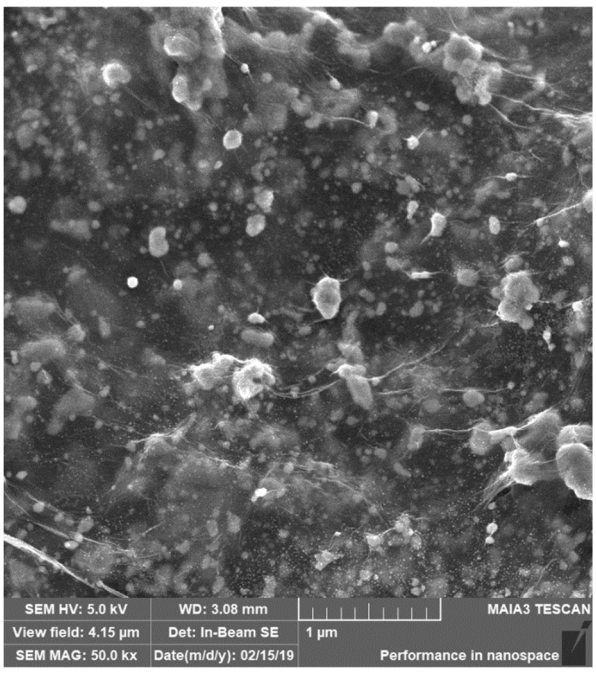


Figure 1 SEM picture of the rGO-AgNPs nanocomposite

3.2. Electrochemical performance of the developed biosensor

The modified surface of GE by rGO-AgNPs nanocomposite was characterized by Electrochemical Impedance Spectroscopy (EIS). Recorded Nyquist plots (**Figure 3**) show increasing charge transfer value due to the change between redox potential and the GE surface. Moreover, the sensitivity of the proposed biosensor was obtained by differential pulse voltammetry (DPV). The linear equation was obtained from calibration curve $I(\mu A) = -0.0005c$ (U/ml) − 0.1003; (R^2 = 0.9922) and has shown a broad linear range from 5 U/ml to 500 U/ml of *M. SssI*. The error bars were calculated as a standard deviation from measurement (n = 3).

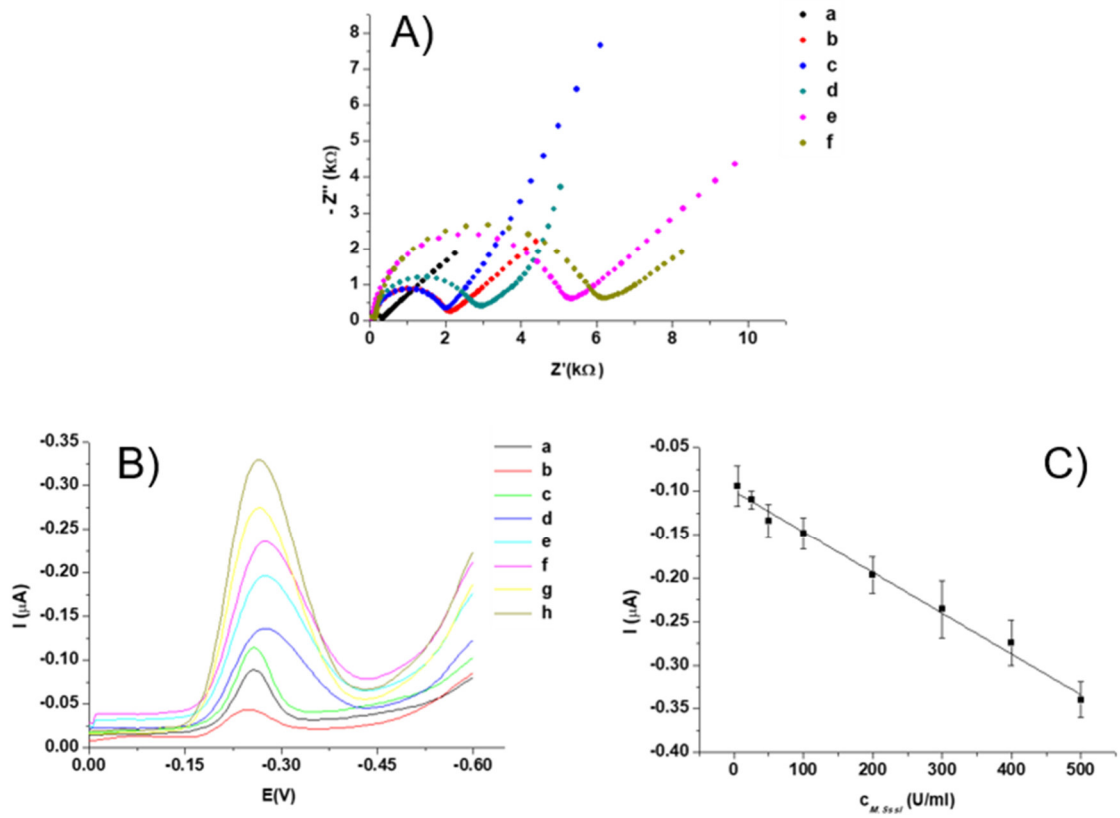


Figure 2 A) Nyquist plots obtained from EIS measurement in 5 mM $Fe(CN)_6^{4-}$/ $Fe(CN)_6^{3-}$ in 100 mM PB. (a) The bare GE, (b) GE/rGO-AgNPs, (c) GE/rGO-AgNPs/DNA_1, (d) GE/rGO-AgNPs/probe DNA_1/ DNA_2, (e) GE/rGO-AgNPs/DNA_1/ DNA_2/ *M. SssI* MTase (500 U/ml)/ *HpaII* (20 U·ml^{-1}) and (f) GE/rGO-AuNPs/ DNA_1/ DNA_2/ *M. SssI* MTase (500 U·ml^{-1}). B) DPV records obtained from various concentrations measurement. The DNA was methylated by 5; 25; 50; 100; 200; 300; 400 and 500 U/ml M. SssI. C) Calibration curve responds to the accumulated MB in the helix structure. Error bars were calculated in dependence on standard deviations from the measurement.

4. CONCLUSION

Herein, a simple and sensitive biosensor for specific detection of DNA methylation and *M. SssI* MTase was fabricated. The biosensor modified with rGO-AgNPs nanocomposite has shown the determination of *M. SssI* with a broad linear range from 5 U·ml^{-1} to 500 U·ml^{-1}. Our biosensor provides an easy measurement of DNA methylation. The main advantage of this is, that it does not require expensive instruments, PCR amplification and multiple-step procedures. From the preliminary data is obvious, that our biosensor can be easily transferred to lab-on-a-chip and be part of smart device, which will be created in the future.

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