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Simple and label-free electrochemical impedance Amelogenin gene hybridization biosensing based on reduced graphene oxide

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ABSTRACT

The increasing desire for sensitive, easy, low-cost, and label free methods for the detection of DNA sequences has become a vital matter in biomedical research. For the first time a novel label-free biosensor for sensitive detection of Amelogenin gene (AMEL) using reduced graphene oxide modified glassy carbon electrode (GCE/RGO) has been developed. In this work, detection of DNA hybridization of the target and probe DNA was investigated by electrochemical impedance spectroscopy (EIS) and cyclic voltammetry (CV). The optimum conditions were found for the immobilization of probe on RGO surface and its hybridization with the target DNA. CV and EIS carried out in an aqueous solution containing $[Fe(CN)_6]^{3-/4-}$ redox pair have been used for the biosensor characterization. The biosensor has a wide linear range from 1.0×10^{-20} to 1.0×10^{-14} M with the lower detection limit of 3.2×10^{-21} M. Moreover, the present electrochemical detection offers some unique advantages such as ultrahigh sensitivity, simplicity, and feasibility for apparatus miniaturization in analytical tests. The excellent performance of the biosensor is attributed to large surface-to-volume ratio and high conductivity of RGO, which enhances the probe absorption and promotes direct electron transfer between probe and the electrode surface. This electrochemical DNA sensor could be used for the detection of specific ssDNA sequence in real biological samples.

1. Introduction

Nucleic acid hybridization has become a fundamental technique in biology for the detection and analysis of specific DNA sequences. The detection of specific nucleic acid sequences has become increasingly important in the diagnosis and treatment of genetic disease, detection of infectious agents, drug screening, food safety, and environmental monitoring (Chen et al., 2009; Kalogianni et al., 2006). Many attempts have been made to generate inexpensive, easy-to-use, fast-response devices to address the challenges connected to the development of DNA biosensors. Electrochemical-based sensors have attracted more attention than other methods because they are highly sensitive, easy, inexpensive, and compatible with micro fabrication technologies (White and Plaxco, 2010). DNA electrochemical biosensors based on demonstrating the variations between single-stranded DNA (ss-DNA) probes and hybridized DNA (ds-DNA) have attracted great interest (Jin et al., 2007).

Graphene has been introduced as the building block of graphite for more than 60 years, but its experimental isolation was first attained in 2004. In spite of its short history, graphene has attracted much notice because of its excellent mechanical, thermal and electronic properties (Novoselov et al., 2004). This unique nanostructure holds vast promise for potential applications in many technological fields such as Nano electronics (Xinhuang, et al., 2009), biosensors (Xiao et al., 2013), Nano composites (Hu et al., 2011a, 2011b), batteries, super capacitors (Vivekchand et al., 2008), gas sensors (Leenaerts et al., 2008), pH sensor (Ang et al., 2008) and hydrogen storage (Geim and Novoselov, 2007). Graphene is composed of planar sheets of sp^2 bonded carbon atoms. The most common approach to graphite exfoliation is the use of a strong oxidant for obtaining graphene oxide (GO) as nonconductive hydrophilic carbon material. As recently proved, the solution-based route involves chemical oxidation of graphite to hydrophilic graphite oxide, which can be readily exfoliated as GO sheets by ultra-sonication in water. Graphene oxide can be reverted back to conducting graphene by chemical reduction, for example, using hydrazine (Stankovich et al., 2006). This unique nanostructure

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material has high surface area, excellent electrical conductivity and electron mobility at room temperature, robust mechanical properties, and flexibility. These special properties of graphene may provide to fabricate novel biosensors for virtual applications. The high surface area is helpful in increasing the surface loading of the probe DNA. The excellent conductivity and small band gap are favorable for conducting electrons from the biomolecules (Stankovich et al., 2006). Graphene-based chemical sensors can also have a much higher sensitivity because of the low electronic noise from thermal effect (Ao et al., 2008; Pozo et al., 2005). Furthermore, compared with CNTs, graphene can be obtained easily by chemical change of the inexpensive graphite (Xu et al., 2008).

Sex determination using genomic DNA from several origins is often an important tool in forensic science or in routine genotyping. AMEL is one of the major matrix proteins secreted by the tooth enamel (Faerman et al., 1995). The AMEL gene, coding for a highly conserved protein, is located on the X and the Y chromosomes in humans. The two alleles are similar for the exonic sequences but differ in the intronic sequences (Slavkin, 1997). Thus the females (XX) have two identical AMEL genes but the males (XY) have two non-identical genes. AMEL is a very useful marker for sex determination especially in forensic applications, where the determination of gender in biological samples has a primary importance for evidentiary and investigative purposes.

EIS has increasingly become method of choice for electrochemical measurements of molecular interactions and is widely used in a variety of biosensing applications (Lisdat and Schafer, 2008). There are a growing number of publications on the use of EIS based on immobilized DNA probes, to detect complementary ssDNA target through hybridization. The method is very sensitive and can be used for detection of a wide range of molecular recognition events happening on the electrode surface without any label. The method provides unique advantages compared to other electrochemical methods, such as high sensitivity and ability to separate the surface binding events from the solution impedance. EIS is a method of measuring the impedance value of the electrode surface during the process of frequency variation. EIS is able to provide various properties of interface of the electrode and solution, including the electrode impedance, capacitance of the electric double layer, and the surface electron transfer resistance (R_{ct}). This technique can be used to characterize a DNA hybridization sensor to realize sensitive indicator-free detection of the gene sequences. Hybridization reaction of DNA on the electrode surface results in change of the R_{ct} value upon formation of duplex between probe and target DNA. The quantity of the negative charge on the electrode surface increases greatly due to DNA duplex formation and thereby further impeding the electron transfer.

In this article, we report a new, simple, ultra-sensitive, highly selective and label-free electrochemical AMEL gene hybridization biosensor based on reduced graphene oxide as the platform. All the processes including modification of GCE with RGO, ss-DNA immobilization and complementary sequence hybridization were studied using both CV and EIS techniques. DNA immobilization, hybridization on the platform caused changes in the interfacial and conformation, which could be readily monitored by impedance measurement. Under optimal conditions the device can achieve high sensitivity to detect 3.2×10^{-21} M and high selectivity in real samples of target DNA. Because of this extremely low detection limit we believe that our explorations may present a basis for further research and advancement in graphene-based impedimetric biosensing. These results confirm that the AMEL gene can be used for rapid sex determination with a high efficiency and accuracy. In our previous paper, carbon paste electrode (CPE) was modified with gold nanoparticles (AuNPs) for immobilization of thiolated bioreceptors (Mazloum-Ardakani et al., 2013). Although elegant it had disadvantages of (1) low reproducibility due to use of carbon paste electrode; (2) long assay time because of the time required for hybridization reactions and self-assembly of ssDNA in the presence of methylene blue; and (3) high detection limit. To eliminate of these disadvantages, we propose to the best of our knowledge for the first time the determination of AMEL gene by label-free electrochemical impedance method on reduced graphene oxide modified GCE. The newly developed sensor had an extremely high sensitivity, a low limit of detection, a wide dynamic range, an excellent selectivity and a short assay time. The biosensor successfully detected the AMEL gene in real samples.

2. Experimental

2.1. Materials

Specific primers and probes were designed based on AMEL gene obtained from the Gen Bank database. The oligonucleotides were designed by primer design software (Premier 5.0, Premier Biosoft, Canada), and their secondary structure were examined with Gene Runner (version 3.05, Hastings Software, USA). Deoxyribonucleotide triphosphate (dNTP) and Taq DNA polymerase were purchased from Sina Clon Company, Iran. All oligonucleotides were synthesized by Macrogen (Korea). Chemical reagents including absolute ethanol, sodium dihydrogen phosphate, disodium hydrogen phosphate, 1-ethyl-3-(3-dimethyl amino propyl) carbo-diimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), 2-(N-morpholino) ethane sulfonic acid (MES), Tween 20 (Polyoxyethylenesorbitan monolaurate), potassium ferrocyanide, potassium ferricyanide, graphite powders, $KMnO_4$, hydrazine monohydrate (N_2H_4, 98%) and sulfuric acid were purchased from commercial sources (Merck or Sigma) in analytical grade and used without further purification. All solutions were prepared with deionized water (DI: 18 MΩ cm^{-1} resistivity).

The sequences are as follows:

Probe sequence:
AMGX: 3′-TATCCCAGATGTTTCTC-NH_2-5′
Complementary sequence:
AMGX: 3′-GAGAAACATCTGGGATA-5′
Noncomplementary sequence (random sequence):
AMGY: 5′-CACTTTATTTGGGATG-3′

The stock solutions of the oligonucleotides (100.0 μM) were prepared in 0.1 M pH 7.4 phosphate buffer saline (PBS) and kept frozen at −20 °C. The washing solution was 0.05 M phosphate buffer (pH=7.4) containing 0.3 M of NaCl. The pH was adjusted with either NaOH or HCl solutions. The double distilled water and buffers were sterilized using autoclave.

2.2. Instrumentation

The electrochemical measurements were performed with an Autolab potentiostat/galvanostat model PGSTAT 30 (Eco Chemic, Utrecht, Netherlands) and a NOVA 1.7 software at laboratory temperature (25 ± 1 °C). The utilized three-electrode system was composed of a glassy carbon working electrode (surface area of 7.07 mm^2), an Ag/AgCl (1.0 M KCl) reference electrode, and a platinum auxiliary electrode. All the potentials in the text are reported with respect to the reference electrode. The pH measurements were done with a Metrohm model 691 pH/mV meters. CV was recorded at a scan rate of 100 mV s^{-1} and EIS measurements were recorded between 100 kHz and 0.01 Hz at 10 mV, AC amplitude. All experiments were carried out in 0.5 mM [Fe