Analytical Biochemistry 443 (2013) 132–138

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

Sex determination based on amelogenin DNA by modified electrode with gold nanoparticle

Mohammad Mazloum-Ardakani [a,*], Nooshin Rajabzadeh [a], Ali Benvidi [a], Mohammad Mehdi Heidari [b]

[a] Department of Chemistry, Faculty of Science, Yazd University, Yazd 89195-741, Iran
[b] Department of Biology, Faculty of Science, Yazd University, Yazd 89195-741, Iran

ARTICLE INFO

Article history:
Received 11 June 2013
Received in revised form 27 August 2013
Accepted 29 August 2013
Available online 6 September 2013

Keywords:
DNA biosensor
DNA synthesis
Amelogenin gene
Gold nanoparticles
Electrochemical impedance spectroscopy

ABSTRACT

We have developed a simple and renewable electrochemical biosensor based on carbon paste electrode (CPE) for the detection of DNA synthesis and hybridization. CPE was modified with gold nanoparticles (AuNPs), which are helpful for immobilization of thiolated bioreceptors. AuNPs were characterized by scanning electron microscopy (SEM). Self-assembled monolayers (SAMs) of thiolated single-stranded DNA (SH–ssDNA) of the amelogenin gene was formed on CPE. The immobilization of the probe and its hybridization with the target DNA was optimized using different experimental conditions. The modified electrode was characterized by electrochemical impedance spectroscopy (EIS) and cyclic voltammetry (CV). The electrochemical response of ssDNA hybridization and DNA synthesis was measured using differential pulse voltammetry (DPV) with methylene blue (MB) as an electroactive indicator. The new biosensor can distinguish between complementary and non-complementary strands of amelogenin ssDNA. Genomic DNA was extracted from blood and was detected based on changes in the MB reduction signal. These results demonstrated that the new biosensor could be used for sex determination. The proposed biosensor in this study could be used for detection and discrimination of polymerase chain reaction (PCR) products of amelogenin DNA.

DNA is the largest known biologically active macromolecule. Therefore, it is vital to measure the interaction of DNA with diverse molecules and advance new strategies for sequence-specific detection of DNA in samples [1]. The expansion of DNA sensors has recently attracted attention in connection with research efforts directed at gene analysis, detection of genetic tissue match, and forensic applications [2,3]. DNA biosensors hold enormous potential for disease diagnosis, detection of infectious agents, drug screening, and forensic applications [4]. Many efforts have been made to produce devices to meet the challenges connected with the development of DNA biosensors. Electrochemical sensors have attracted the most attention when compared with the other methods because of their high sensitivity, ease of use, affordability, portability, and compatibility with the micro fabrication technologies [5].

Different kinds of solid electrodes such as metal and carbon electrodes have been employed as the transducers for electrochemical DNA biosensors [6,7]. Among these solid electrodes, carbon paste electrodes (CPEs)[1] have some specific advantages that include a wide potential window, low background current, and ease of fabrication [8]. Most important, CPEs can be easily endowed with specific properties by introduction of functional materials. Moreover, the surface of the electrode is polishable and renewable [9,10]. Many protocols have been proposed for electrochemical monitoring of DNA hybridization. For example, metal coordination complexes [11] and intercalating organic compounds [12] are usually employed as electroactive indicators for DNA hybridization. The electrochemical response of these labels or indicators changes on DNA hybridization.

Methylene blue (MB) is an organic dye belonging to the phenothiazine family and is a well-known DNA hybridization indicator as it associates with the free guanine bases of single-stranded DNA (ssDNA) [13]. MB has a high affinity for ssDNA, and this association generates a marked electrochemical signal [12]. Thus, MB was used

[1] *Abbreviations used:* CPE, carbon paste electrode; MB, methylene blue; ssDNA, single-stranded DNA; AuNP, gold nanoparticle; NP, nanoparticle; PCR, polymerase chain reaction; SAM, self-assembled monolayer; CV, cyclic voltammetry; EIS, electrochemical impedance spectroscopy; DPV, differential pulse voltammetry; dNTP, deoxyribonucleotide triphosphate; MCH, 6-mercapto-1-hexanol; SCE, saturated calomel electrode; SEM, scanning electron microscopy; PBS, phosphate-buffered solution; dsDNA, double-stranded DNA.

* Corresponding author. Fax: +98 3518210644.
E-mail address: mazloum@yazd.ac.ir (M. Mazloum-Ardakani).

0003-2697/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.ab.2013.08.028

as an indicator in this study. The quantity of immobilized DNA and the accessibility of probe DNA are extremely important for biosensors detecting DNA hybridization. By increasing the immobilized DNA amount and controlling the molecular orientation of probe oligonucleotides, a marked increase in the detection capability of DNA biosensor can be achieved [14,15]. To raise the immobilization amount of probe DNA and improve its DNA hybridization efficiency, different kinds of nanomaterials have been applied to modify the electrode surface. These modifications can increase the surface area and also result in better orientation of the immobilized DNA, thereby enhancing detection [1,16,17]. It has been reported that modification with gold nanoparticles (AuNPs) could increase the surface area of the electrode and enhance the immobilization amount and accessibility of probe DNA [17]. Different strategies have been developed to construct AuNP-modified electrodes. These include electroless deposition [18], sol–gel process [19], hydrothermal precipitation [20], and assembly by bifunctional chemical linkers [21]. Apart from the above-described methods, a unique process termed electrodeposition produces nanoparticles (NPs) with controlled characteristics, size, morphology, and composition. It is simple, fast, inexpensive, and among the most familiar binder-free techniques employed for the preparation of NPs. The advantage of this technique is that the NPs get directly attached to the substrate and, in comparison with other techniques, the particle size, crystallographic orientation, mass, thickness, and morphology of the nanomaterials can be controlled by adjusting the operating conditions. Thiol–metal (SH–Au) linkages are commonly used to covalently attach biomolecules to the surface of AuNPs because of the strong affinity between sulfur and gold atoms.

Nucleic acid hybridization of single-stranded probes with targets is routinely used in various analytical strategies for the determination of genetic markers associated with disease and identification of organisms. Nucleic acid hybridization has become a fundamental technique in molecular biology for the detection and analysis of specific DNA sequences. Such analysis plays a significant role in many areas, including clinical diagnosis, forensic and environmental analysis, and monitoring of food quality. Conventional methods of DNA sequence determination (e.g., polymerase chain reaction [PCR], gel electrophoresis, Southern blotting) have been recently enriched by new instruments such as DNA biosensors enabling DNA sequence detection by hybridization. So far, however, DNA detection with the use of biosensors is usually preceded by PCR amplification, and this makes such assays very sensitive as a few copies of DNA can be detected in this way [22]. Many methods of detection of hybridization have been proposed. The most popular approaches include techniques rooted in electrochemical, plasmon resonance, acoustic wave, electrostatic, interferometric, and luminescent processes [23].

The amelogenin gene presents on the human X (AMEL X), and the human Y (AMEL Y) chromosomes [24] showed size differences between these two chromosomes; therefore, this gene has been used to differentiate males from females [25,26]. The amelogenin gene can be used in sex determination of samples from unknown human origin through PCR. In the self-assembled monolayer (SAM) technique, the recognition interface is fabricated using an alkane thiol ssDNA. The electrochemical methods of cyclic voltammetry (CV), electrochemical impedance spectroscopy (EIS), and differential pulse voltammetry (DPV) have been used to study DNA synthesis and the hybridization process. We devised a simple electrodeposition method to achieve AuNPs modification on the CPE surface. In this research, for the first time, DNA synthesis has been detected because the incorporation of a nucleotide into the DNA molecule causes an increase in the probe length, leading to a significantly enhanced voltammetric signal. We report, for the first time, a new electrochemical biosensor for sex determination based on the detection of amelogenin DNA in genomic DNA sample.

Materials and methods

Materials

Specific primers and probes were designed based on the amelogenin gene using the GenBank database. The oligonucleotides were designed by primer design software (Premier 5.0, Premier Biosoft, Canada), and their secondary structure was examined with Gene Runner (version 3.05, Hastings Software, USA). All oligonucleotides were synthesized by Macrogen (Korea). Deoxyribonucleotide triphosphate (dNTP) and Klenow enzyme (the large fragment of *Escherichia coli* DNA polymerase I) were purchased from SinaClon (Iran). 6-Mercapto-1-hexanol (MCH) was purchased from Aldrich. Other chemical reagents, including $HAuCl_4$, MB, and absolute ethanol, were purchased from Merck. All solutions were prepared with double-distilled water. Tris–HCl buffer (20.0 mM Tris + 20.0 mM NaCl, pH 7.4) was used for the dilution of samples. All of the chemicals were used as received without further purification. The sequences of the probe and complementary were as follows:

Probe sequence: AMGX, 5′-(SH)-TATCCCAGATGTTTCTC-3′
Complementary sequence: AMGX, 5′-GAGAAACATCTGGGATA-3′.

Instruments

EIS and electrochemical measurements were performed using an Autolab potentiostat/galvanostat (PGSTAT 302N, Eco Chemie, The Netherlands) and a potentiostat/galvanostat (SAMA 500 electroanalyzer system, Iran), respectively. The three-electrode system used was composed of a CPE (3 mm diameter) as the working electrode, a saturated calomel electrode (SCE) as the reference electrode, and a platinum electrode as the auxiliary electrode.

All of the values for the potential in the text are reported with respect to this reference electrode. The pH measurements were performed using a Metrohm model 691 pH/mV meter.

Electrode preparation and modification

The CPE was prepared by mixing 0.50 g of graphite powder and 0.7 ml of paraffin oil in a mortar and pestle until a uniform paste was obtained. The amounts of the materials used were obtained experimentally after the optimization process. This paste was then packed into the end of a glass tube (~10 cm long). A copper wire was inserted into the carbon paste and provided an electrical contact. When necessary, the surface of the carbon paste was polished with smooth paper to obtain a shiny appearance. The AuNPs/CPE was obtained by CV scanning from a solution containing 0.01 M Na_2SO_4, 0.01 M H_2SO_4, and 1.0 mM HAu(III) $Cl_4{\cdot}3H_2O$ at a scan rate of 60 mV/s for five scans. $NaNO_3$ was employed as a supporting electrolyte instead of KCl because KCl has been reported to have a negative effect on Au electrodeposition by favoring the coalescence phenomenon, leading to the formation of less numerous and large-sized NPs [27].

Immobilization and electroactivation of probe DNA at AuNPs/CPE

The AuNPs/CPE was washed with double-distilled water. The SAM formation was conducted by droplet coating of the electrode surface. For this purpose, 5 μl of the DNA probe solution (1 μM) was pipetted on the surface and allowed to dry overnight in a wet chamber. This electrode was named as ssDNA/AuNPs/CPE, where ssDNA is a segment of the amelogenin gene. Following SAM formation, the electrode was dipped into the MCH solution (1.0 mM) at room temperature for 60 min. MCH treatment is very important for the removal of nonspecific probe adsorbed on the

surface and also to fill in the bare areas of the CPE surface that have not been covered by DNA molecules. The electrode was rinsed repeatedly with buffer after each stage. Thereafter, the electrode was soaked in the Tris–HCl buffer (pH 7.4) containing 20.0 μM MB for 10 min and then washed with copious amounts of water and Tris–HCl buffer (pH 7.4). This electrode was named as MB/ssDNA/AuNPs/CPE.

Hybridization

The probe-modified electrode was hybridized with the sample DNA using three different procedures. In the first method, the ssDNA/AuNPs/CPE was placed upside down in a humidity chamber and 5 droplets of complementary DNA solution was added to the electrode surface. The chamber was sealed to prevent water evaporation and kept overnight at room temperature for hybridization. In the second method, the hybridization reaction was carried out by immersing the ssDNA/AuNPs/CPE in a Tris–HCl buffer solution (pH 7.4) containing a certain concentration of complementary DNA at 42 °C for 1 h. After that, the electrode was washed with copious amounts of water and Tris–HCl buffer to remove the unhybridized ssDNA. The third procedure was similar to the second method except that the beaker was placed on a heater and the solution was slowly heated to approximately 93 °C for 5 min and then gradually cooled to room temperature. These three procedures were named as the droplet, solution, and solution preheating hybridization methods, respectively. The results showed that the solution preheating hybridization method was more effective than the other two methods in the conditions used for the experiments; consequently, this method was used for future experiments.

The strategy used in the current research is shown in Scheme 1.

Electrochemical detection

DPV of the accumulated MB was obtained in Tris–HCl buffer (pH 7.4) containing 2.0×10^{-5} M MB (DPV parameters: pulse amplitude, 5 mV; pulse width, 50 ms). CV measurements of 1.0 mM $[Fe(CN)_6]^{3-/4-}$ were performed with the supporting electrolyte of 0.1 M KCl. The scan rate was 100 mV/s. EIS was carried out in 1.0 mM $[Fe(CN)_6]^{3-/4-}$ solution containing 0.1 M KCl. The AC voltage amplitude was 50 mV, and the voltage frequencies ranged from 10^4 to 0.1 Hz. The result for each electrode is reported as the mean value of three parallel measurements.

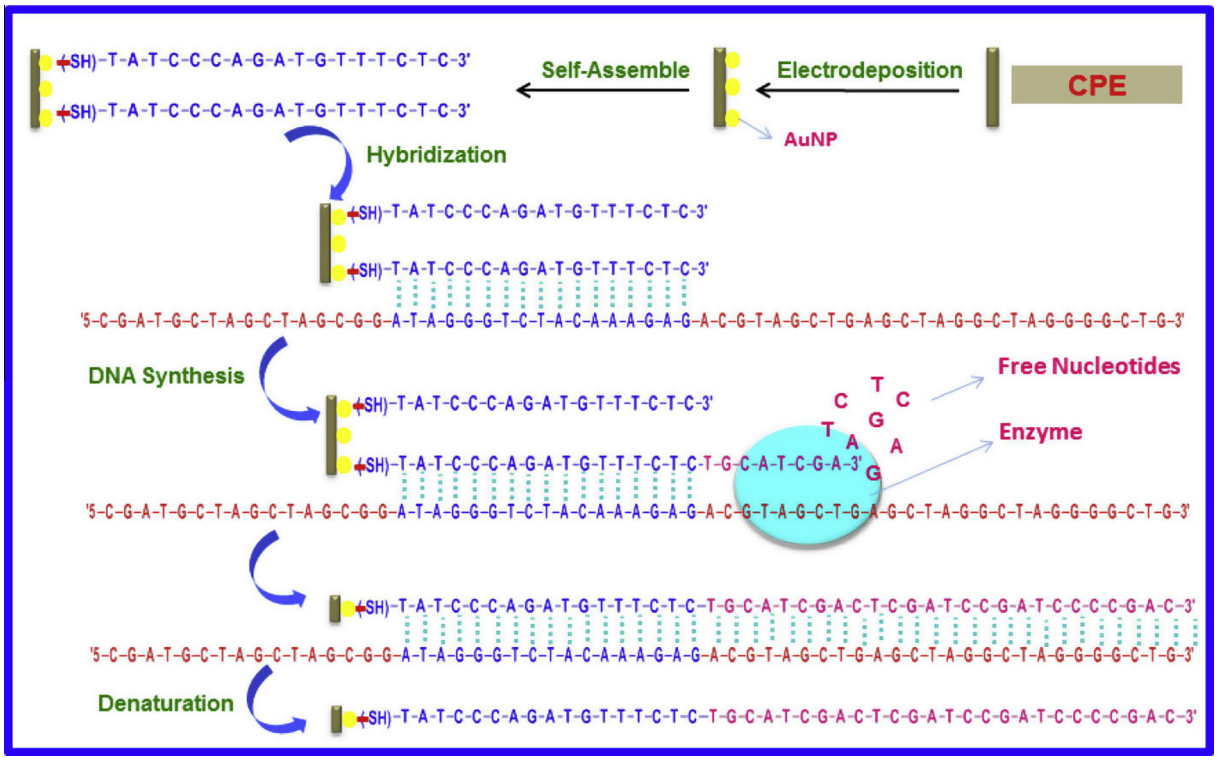
Genome DNA extraction

DNA was isolated from peripheral blood samples using a DNA extraction kit (DNAfast Kit, Genfanavaran, Iran).

Results and discussion

Synthesis and characteristics of AuNPs electrodeposited on CPE

A 0.01-M Na_2SO_4 aqueous solution containing 0.01 M H_2SO_4 and 1.0 mM $HAuCl_4{\cdot}3H_2O$ was used for electrodeposition of gold particles on CPE. AuNPs were electrodeposited on CPE by potential sweeping by cycling the potential of working electrode between +1.20 and −0.20 V for five cycles. The cyclic voltammograms of this process are shown in Fig. 1. These parameters were obtained by optimization. The forward scan exhibits the reduction of Au(III) to Au(0) with a cathode peak at 0.38 V, including the deposition of AuNPs onto the electrode surface. The shape of the first voltammogram is consistent with previous reports using similar conditions [28]. The reduction waves of Au(III) occurred at more negative potentials in the first cycle and then shifted to more positive potentials in the subsequent four cycles. This is consistent with thermodynamic behavior that predicts easier growth of previously formed AuNPs than the process of new AuNP nucleation on CPE. This occurs because the deposition of gold requires less energy on gold than on CPE [28,29]. In the second voltammogram, the reduction peak of Au(III) shifted from 0.38 to 0.68 V, indicating that



Scheme 1. Schematic diagram of the DNA biosensor fabrication.

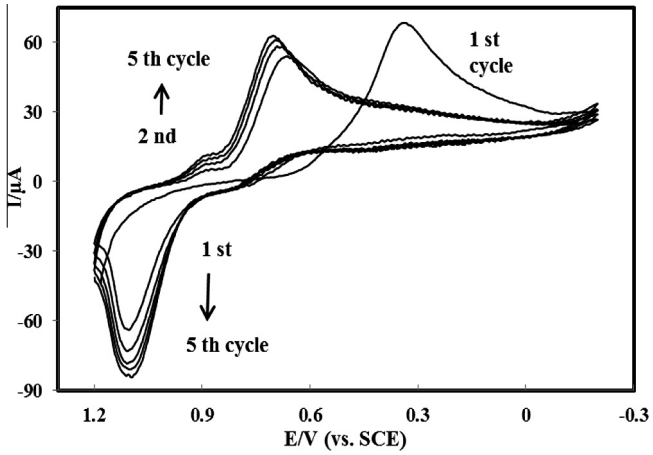


Fig.1. Staircase cyclic voltammograms recorded at a CPE immersed in the 0.01-M Na_2SO_4 solution containing 0.01 M H_2SO_4 and 1 mM $HAuCl_4{\cdot}3H_2O$. Scan rate: 60 mV/s. Numbers of potential cycles are indicated.

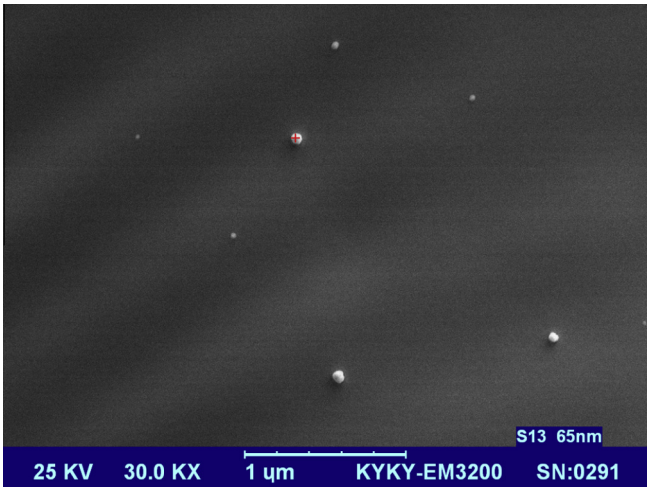


Fig.2. SEM image of the surface AuNPs/CPE.

Au deposition occurred preferentially on the NPs created during the first cycle.

AuNPs were characterized by scanning electron microscopy (SEM), as shown in Fig. 2. SEM was used to evaluate the physical appearance and surface characteristics of AuNPs on the electrode surface at a higher magnification. As can be seen, many spherical AuNPs are electrodeposited on the surface CPE.

Electrochemical characterization of modified electrodes

CV of $[Fe(CN)_6]^{3-}/[Fe(CN)_6]^{4-}$ at modified electrodes

CV is an effective and convenient method for proving the features of modified electrode surfaces. The changes in peak current and the separation of peak potentials in cyclic voltammograms at different electrode surfaces are theoretically related to the electron transfer rate constant. Each step of CPE surface modification and ssDNA immobilization was monitored by CV using an $[Fe(CN)_6]^{3-/4-}$ mixture in a phosphate-buffered solution (PBS, pH 6.0) containing 0.1 M KCl as a redox probe. In each cyclic voltammogram, only the second cycle was considered because no major changes were seen in the subsequent cycles. $[Fe(CN)_6]^{3-/4-}$ undergoes a reversible electrochemical reaction at different electrodes and is widely used as an electrochemical probe to investigate the characteristics of films on electrode surfaces [28]. Fig. 3 shows the cyclic voltammograms of different electrodes in 1.0 mM $[Fe(CN)_6]^{3-/4-}$ in PBS containing 0.1 M KCl at a scan rate of 100 mV/s. For uncoated CPE, a quasi-reversible electrochemical response for $Fe(CN)_6^{3-/4-}$, with a peak potential separation (ΔE_p = 0.375 V), was observed (Fig. 3, curve a). When AuNPs electrodeposited on the CPE, there was a drastic decrease in the peak potential separation (ΔE_p = 0.085 V) and the cathode and anode peak currents increased in comparison with the bare CPE (curve b). The peak currents at the AuNPs/CPE (curve b) were larger than those at the bare CPE (curve a), implying that AuNPs modified on the bare CPE increased the electroactive surface of the electrode and also increased the conductivity. The peak potential separation, which is inversely proportional to the electron transfer rate [28], is used for electrochemical evaluation of the electrode conductivity. Obviously, the AuNPs electrodeposited on the CPE surface and increased the conductivity of the electrode. Curve c was obtained at ssDNA/AuNPs/CPE with ΔE_p = 0.151 V. The peak current at ssDNA/AuNPs/CPE (curve c) was decreased dramatically compared with that at AuNPs/CPE, which could be attributed to two facts: (i) the probe molecules blocking the electrode surface and preventing $[Fe(CN)_6]^{3-/4-}$ ions from reaching the electrode surface and (ii) the electrostatic repulsion between negatively charged phosphate skeletons of immobilized DNA on the AuNPs/CPE and the anionic redox couple. Following hybridization of the DNA probe with the target complementary DNA, the peak-to-peak separation was increased (ΔE_p = 0.211 V) and the redox peak currents were decreased significantly (curve d). These observations represent less electron transfer following DNA hybridization attributed to the two mentioned facts: more electrode surface blocking because of hybridization and more repulsion between negatively charged hybridized DNA and $[Fe(CN)_6]^{3-/4-}$ anions.

EIS of modified electrodes

EIS is a technique of choice for electrochemical measurements of molecular interactions and is widely used in a variety of biosensing applications [27]. There are a growing number of publications on the use of EIS based on immobilized DNA probes that detect complementary ssDNA target molecules through hybridization. The method is very sensitive and can be used for a "label-free" detection of a wide range of molecular recognition events happening at the electrode surface. The method provides unique advantages compared with other electrochemical methods. These include

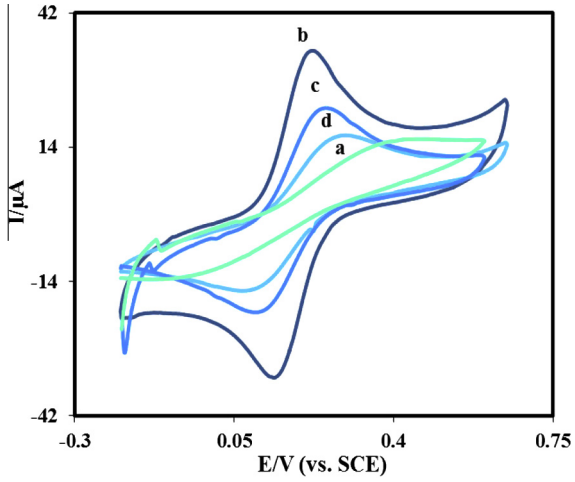


Fig.3. Cyclic voltammograms of 1.0 mM $[Fe(CN)_6]^{3-/4-}$ in 0.1 M KCl at bare CPE (a), AuNPs/CPE (b), ssDNA/AuNPs/CPE (c), and dsDNA/AuNPs/CPE (d). Scan rate: 100 mV/s.

high sensitivity, ease of signal quantification, and the ability to separate the surface binding events from the solution impedance. EIS measures the impedance value of the electrode surface during the process of frequency variation. EIS is able to offer various properties of interface of the electrode and solution, including the electrode impedance, the capacity of the electric double layer, and the surface electron transfer resistance (R_{ct}). This technique was used to characterize a DNA hybridization sensor to realize sensitive, indicator-free detection of the gene sequences.

EIS enables the analysis of the complex electrical resistance of a system. This analytical method is very sensitive to changes on the electrode surface as well as in the bulk solution; therefore, it is an appropriate application tool for use in DNA detection. The EIS technique is an inherently label-free detection method, which is especially beneficial because there is no need to also modify the biomolecules of interest with markers such as fluorescent dyes, enzymes, or other redox labels. The electrochemical complex impedance $Z_{(w)}$ can be represented as the sum of the real $Z'_{(w)}$ and imaginary $Z''_{(w)}$ components, where w is angular frequency:

$$Z_{(w)} = Z'_{(w)} + jZ''_{(w)}, \quad \text{where} \quad j = \sqrt{-1}. \tag{1}$$

$$Z''_{(w)} = -1/Wc. \tag{2}$$

Hybridization reaction of DNA at the electrode surface results in a change of R_{ct} value due to the formation of a duplex between the probe and the target DNA. The quantity of negative charge on the surface of the electrode increases largely due to hybridization and, thus, further impedes the electron transfer. The results obtained from EIS are consistent with those of CV and are in agreement with the fact that more uniform AuNPs were present on the surface of CPE and a large surface area of CPE was covered with AuNPs.

DPV of MB at modified electrodes

MB is an organic dye belonging to the phenothiazine family, and it binds specifically to the guanine (G) bases in DNA [13]. Fig. 4 shows the DPV signals of 2.0×10^{-5} M MB at bare CPE (curve a), AuNPs/CPE (curve b), and ssDNA/AuNPs/CPE (curve c) as well as signals of MB at the ssDNA probe immobilized on the electrode after hybridization with the complementary DNA sequence (curve d) and non-complementary sequence (curve e). Although the existence of AuNPs film greatly enlarged the surface area of the electrode, the signal obtained at AuNPs/CPE was increased when compared with that at bare CPE. The highest MB reduction signal was obtained at ssDNA/AuNPs/CPE because MB has a strong affinity to the free guanine bases; hence, the greatest amount of MB accumulation occurred at this surface. A significant decrease of the peak current value of MB was observed after the ssDNA probe was hybridized with the complementary target sequence because the interaction of MB and guanine residues on the probe was prevented by duplex formation on the electrode surface [13]. The decrease of the peak current value of MB was not obvious after the ssDNA probe was hybridized with the non-complementary sequence, indicating that hybridization reaction did not occur.

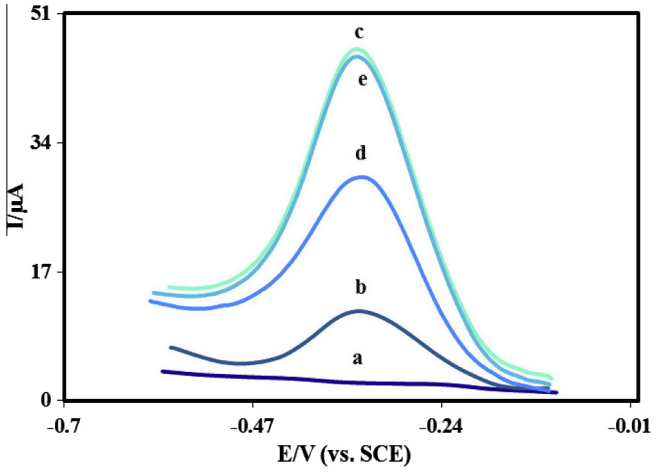


Fig.4. Differential pulse voltammograms using 2.0×10^{-5} M MB as the redox indicator at the bare CPE (a), AuNPs/CPE (b), and ssDNA/AuNPs/CPE (c) as well as ssDNA/AuNPs/CPE hybridized with complementary DNA sequence (d) and non-complementary DNA sequence (e). DPV parameters: pulse amplitude, 5 mV; pulse width, 50 ms.

Optimization of experimental conditions

Optimization of the hybridization process

To obtain the best experimental conditions, three DNA hybridization methods were tested and compared. In the first method, called the droplet hybridization method, the hybridization process was performed by incubation of 5 μl of the complementary solution on the ssDNA/AuNPs/CPE overnight at room temperature (25 ± 1 °C) in a high-humidity container to prevent evaporation. Then, the electrode was rinsed thoroughly in a washing solution and MB was accumulated on the electrode surface. Curve a of Fig. 5 shows the DPV of the accumulated MB on the electrode surface in Tris–HCl buffer solution. In the second method, hybridization reaction was carried out by immersing the ssDNA/AuNPs/CPE in a Tris–HCl buffer solution (pH 7.4) containing a certain concentration of complementary DNA at 42 °C for 1 h. The DPV of the accumulated MB is presented in curve b of Fig. 5. Finally, in the third method, called the preheating solution hybridization method, the ssDNA/AuNPs/CPE was immersed in the hybridization solution at 93 °C for 5 min. Then, the solution was gradually cooled. Thereafter, the electrode was rinsed with a washing solution and MB was accumulated on the electrode surface. The DPV of the accumulated MB in the preheating solution hybridization method is presented in curve c of Fig. 5.

As illustrated, the lowest current response was obtained in the third method. This may be due to complete denaturation of the double-stranded DNA (dsDNA) at 93 °C and the favorable orientation of the target DNA, facilitating the hybridization process. Based on these observations, the preheating solution hybridization method was chosen as the optimal procedure for the hybridization process.

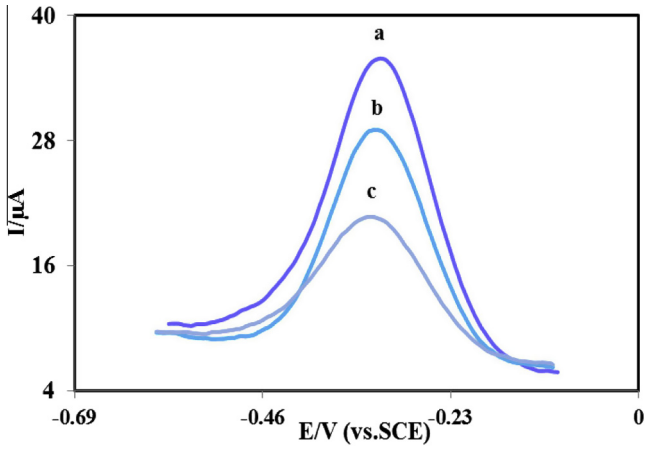


Fig.5. Differential pulse voltammograms of the MB accumulated on the surface of dsDNA/AuNPs/CPE that was prepared with different hybridization methods of droplet hybridization (a), solution hybridization (b), and preheating solution hybridization (c).

Effect of MB concentration

The concentration of MB had a pronounced effect on the hybridization response of the oligonucleotides and directly affected the amount of MB accumulated on ssDNA probe and dsDNA, thereby having a significant effect on the sensitivity of the biosensor. To investigate the effect of MB concentration, the ssDNA/AuNPs/CPE was immersed in Tris–HCl buffer (pH 7.4) containing different concentrations of MB for 10 min. The response of MB increased sharply with the increasing concentrations from 5.0×10^{-6} to 2.0×10^{-5} M. The signal plateau appeared at 2.0×10^{-5} M or above. Therefore, 2.0×10^{-5} M MB was used as the optimal concentration for further experiments.

Effect of MB accumulation time

The time required for the accumulation of MB was also investigated. The ssDNA-modified electrode was incubated in Tris buffer (pH 7.4) containing 2.0×10^{-5} M MB. The voltammetric signal obtained from the ssDNA/AuNPs/CPE increased linearly with the accumulation of MB up to 10 min and then leveled off. Therefore, the optimal accumulation time was chosen as 10 min.

Effect of MCH incubation time

To remove the nonspecifically adsorbed probe molecules from the electrode surface, the modified electrode was stirred in the solution of MCH for a determined time. Some DNA molecules might not orient in the right direction at electrode surface during SAM formation. This could be attributed to nonvertical attachment of DNA molecules on the electrode during SAM formation. These molecules adsorb nonspecifically on the electrode surface. MCH not only removes these DNA molecules from the electrode surface but also binds to the nonspecific sites on the DNA monolayer. The ssDNA probe-modified electrode was incubated in 1 M MCH solution. The peak current of MB increased with the prolongation of the accumulation time. The response signal stabilized after 60 min. Therefore, the optimal time of 60 min was considered for the incubation of MCH.

DNA syntheses of amelogenin gene by DNA polymerase

As shown in Scheme 1, oligonucleotides (the "probe") are immobilized on the surface of CPE. When a complementary oligonucleotide (PCR product) is introduced (the "target"), it hybridizes onto its corresponding probe. Polymerase enzyme (Klenow fragment) is subsequently added along with the required free nucleotides (dNTP) needed for polymerization. For the detection method discussed in this article, the probe and the target oligonucleotide (amelogenin gene fragment) must not be the same length because we can detect the incorporation of nucleotides into the double-stranded sequence during polymerization. Curve a of Fig. 6 shows the DPV signals of 2.0×10^{-5} M MB at the ssDNA/AuNPs/CPE. As shown in this figure, when the planar AuNPs/CPE surface was immobilized by ssDNA probe, the strong association of MB with the immobilized ssDNA probe led to a significantly enhanced voltammetric signal. Curve b of Fig. 6 shows the DPV signal of ssDNA immobilized electrode after hybridization with the PCR product. The PCR product is longer than ssDNA and has a part of complementary sequence that can hybridize with the ssDNA probe. A decrease of signal may be attributed to less MB accumulation on the dsDNA caused by guanine bases becoming inaccessible to MB after hybridization. This result indicates the hybridization of the PCR product with the probe-modified CPE. Then, the dsDNA/AuNPs/CPE was rinsed in a solution containing 2 μl of buffer, 2 μl of Klenow enzyme (5 U/μl), 2 μl of dNTP (5 mM/μl), and 14 μl of water at 37 °C for 1 h until strain complementary DNA was fabricated according to the model. Curve c of Fig. 6 shows the DPV signal of electrode after syntheses. For denaturation of the dsDNA, the electrode was immersed in water at 93 °C for 5 min. The dsDNA was denatured and the target DNA was eliminated. Only the extended probes were maintained on the CPE. Finally, MB reacted with the extended probes and the electrochemical signal of indicator was measured. This signal was higher than that of the CPE modified only by original probe. As shown in curve d of Fig. 6, the DPV signal increased, indicating the presence of free guanine residues on the probe DNA.

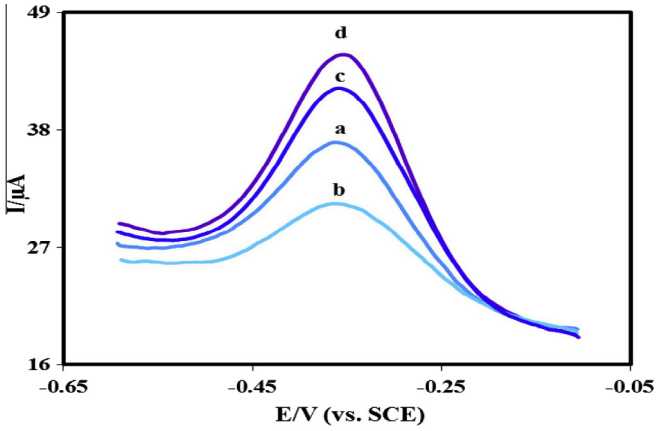


Fig.6. Differential pulse voltammograms of the MB accumulated on the surface of ssDNA/AuNPs/CPE (a), ssDNA/AuNPs/CPE hybridized with PCR product (b), dsDNA/AuNPs/CPE after synthesis (c), and dsDNA/AuNPs/CPE after denaturation (d).

Detection of genomic DNA

Table 1 shows the detection of genomic samples on modified electrodes. Detection of genomic samples is important for the analysis of clinical samples and genetic organisms. One of the aims of our study was to establish a sensitive method to detect the genomic DNA sequence. In this experiment, five genomic DNA samples were extracted from blood using a DNA extraction kit and were employed as the target. Initially ssDNA probe was immobilized on the five modified electrodes. As shown in Table 1, the means of I_p values of DPV were recorded for five probe DNAs. Each measurement was repeated at least five times using different electrodes. Then, the modified electrodes were immersed in the various genomic DNA samples. In the DPV results, the I_p values of the dsDNA were decreased compared with those of the original probe. This result demonstrates the hybridization of genomic DNA sequence with the probes. Then, the electrodes were immersed in 94 °C buffer solution to dissociate the strands. After the dsDNA denatured, the target DNAs were eliminated and the I_p values increased to the initial value. Therefore, this method was successful in the detection of genomic DNA, and the regeneration of the biosensor was very good.

Table 1
Means of peak currents for five probe DNAs and their complementary strains in the genome sample by DPV.

Target DNA	I_p (μA)		
	Probe DNA	dsDNA	Denaturation
1	12.18 ± 0.14	9.94 ± 0.10	11.05 ± 0.02
2	10.41 ± 0.08	8.95 ± 0.09	10.02 ± 0.03
3	15.01 ± 0.10	12.06 ± 0.12	14.50 ± 0.15
4	12.80 ± 0.15	9.70 ± 0.05	11.08 ± 0.13
5	17.01 ± 0.11	14.03 ± 0.10	16.08 ± 0.08

Note: Each measurement was repeated at least five times using different electrodes.

Conclusions

In this study, we have reported the construction of a DNA electrochemical biosensor by immobilization of AuNPs on the surface of CPE. The AuNPs could enhance the DNA immobilization and hybridization. The biosensor fabrication process was thoroughly investigated with CV and EIS using $[Fe(CN)_6]^{3-/4-}$ as an electrochemical redox coupler. Based on the significant difference between the voltammetric signals of MB binding to ssDNA and dsDNA, the modified electrode was applied for the detection of target DNA with MB as a hybridization indicator. We developed a novel electrochemical biosensor for DNA synthesis and sex determination. The constructed DNA biosensor was established with robust stability. The method was successful in determination of sex and was successfully employed in the detection of genomic DNA samples.

Acknowledgment

The authors thank the Yazd University Research Council, IUT Research Council, and Excellence in Sensors for financial support of this research.

References

[1] M. Mazloum-Ardakani, M.M. Heidari, E. Salehpour, Digestion of restriction enzyme for the detection of single-base mismatch in DNA, Anal. Biochem. 421 (2012) 125–129.
[2] A.W. Peterson, L.K. Wolf, R.M. Georgiodis, Hybridization of mismatched or partially matched DNA at surfaces, J. Am. Chem. Soc. 124 (2002) 14601–14607.
[3] S. Reisberga, B. Piroa, V. Noela, M.C. Pham, Selectivity and sensitivity of a reagentless electrochemical DNA sensor studied by square wave voltammetry and fluorescence, Bioelectrochemistry 69 (2006) 172–179.
[4] G. Mandong, L. Yanqing, G. Hongxia, W. Xiaoqin, F. Lifang, Electrochemical detection of short sequences related to the hepatitis B virus using MB on chitosan modified CPE, Bioelectrochemistry 70 (2007) 245–249.
[5] R.J. White, K.W. Plaxco, Exploiting binding-induced changes in probe flexibility for the optimization of electrochemical biosensors, Anal. Chem. 82 (2010) 73–76.
[6] M.H. Pournaghi-Azar, M.S. Hejazi, E. Alipour, Developing an electrochemical deoxyribonucleic acid (DNA) biosensor on the basis of human interleukine-2 gene using an electroactive label, Anal. Chim. Acta 570 (2006) 144–150.
[7] C. Fan, H. Song, X. Hu, G. Li, J. Zhu, X. Xu, D. Zhu, Voltammetric response and determination of DNA with a silver electrode, Anal. Biochem. 271 (1999) 1–7.
[8] A.K. Ioannou, A.A. Pantazaki, S.T.H. Girousi, M.C. Millot, C. Vidal-Madjar, A.N. Voulgaropoulos, DNA biosensor based on carbon paste electrodes modified by polymer multilayer, Electroanalysis 18 (2006) 456–464.
[9] M. Mazloum-Ardakani, N. Rajabzadeh, A. Dehghani, A. Sheikh-Mohseni, A. Benvidi, Carbon nanoparticles and a new derivative of hydroquinone for modification of a carbon paste electrode for simultaneous determination of epinephrine and acetaminophen, Anal. Methods 4 (2012) 2127–2133.
[10] W. Yang, M. Ozasoz, D.B. Hibbert, J.J. Gooding, Evidence for the direct interaction between methylene blue and guanine bases using DNA-modified carbon paste electrodes, Electroanalysis 14 (2002) 1299–1302.
[11] M.E. Napier, H.H. Thorp, Modification of electrodes with dicarboxylate self-assembly monolayers for attachment and detection of nucleic acids, Langmuir 13 (1997) 6342–6344.
[12] H.C.M. Yau, H.L. Chan, M. Yang, Electrochemical properties of DNA-intercalating doxorubicin and methylene blue on hexadecyl mercaptan-doped 5-thiol-labeled DNA-modified gold electrodes, Biosens. Bioelectron. 18 (2003) 873–879.
[13] L.F. Zhang, S.H. Zuo, H.H. Yuan, M.B. Lan, A. Geoffrey, C. Lawrance, G. Wei, Electrochemical detection of DNA hybridization by using a zirconia modified renewable carbon paste electrode, Bioelectrochemistry 74 (2009) 223–226.
[14] C.F. Ding, F. Zhao, M.L. Zhang, S.S. Zhang, Hybridization biosensor using 2,9-dimethyl-1,10-phenantroline cobalt as electrochemical indicator for detection of hepatitis B virus DNA, Bioelectrochemistry 72 (2008) 28–33.
[15] V. Ostatná, N. Dolinnaya, S. Andreev, T. Oretskaya, J. Wang, T. Hianik, The detection of DNA deamination by electrocatalysis at DNA-modified electrodes, Bioelectrochemistry 67 (2005) 205–210.
[16] I. Willner, B. Willner, E. Katz, Biomolecule–nanoparticle hybrid systems for bioelectronic applications, Bioelectrochemistry 70 (2007) 2–11.
[17] M. Mazloum-Ardakani, M.M. Heidari, M. Naderi, Detection of amplified SRY gene by a novel electrochemical biosensor based on gold nanoparticles, Scientia Iranica 19 (2012) 341–966.
[18] S. Liu, J. Liu, L. Wang, F. Zhao, Development of electrochemical DNA biosensor based on gold nanoparticle modified electrode by electroless deposition, Bioelectrochemistry 79 (2010) 37–42.
[19] M. Hong, S. Lee, S. Kim, Use of KCl aqueous electrolyte for 2 V manganese oxide/activated carbon hybrid capacitor, Electrochem. Solid-State Lett. 5 (2002) A227–A230.
[20] L. Cote, A. Teja, A. Wilkinson, Z. Zhang, Continuous hydrothermal synthesis of $CoFe_2O_4$ nanoparticles, Fluid Phase Equilibr. 210 (2003) 307–317.
[21] D. Tian, C. Duan, W. Wang, N. Li, H. Zhang, H. Cui, Y. Lu, Sandwich-type electrochemiluminescence immune sensor based on *N*-(aminobutyl)-*N*-ethylisoluminol labeling and gold nanoparticle amplification, Talanta 78 (2009) 399–404.
[22] T.G. Drummond, M.G. Hill, J.K. Barton, Electrochemical DNA sensors, Nat. Biotechnol. 21 (2003) 1192–1199.
[23] N. Svanvik, G. Westman, D. Wang, M. Kubista, Light-up probes: Thiazole orange-conjugated peptide nucleic acid for detection of target nucleic acid in homogeneous solution, Anal. Biochem. 281 (2000) 26–35.
[24] D. Bailey, N. Affara, M. Ferguson-Smith, The X-Y homologous gene amelogenin maps to the short arms of both the X and the Y chromosomes and is highly conserved in primates, Genomics 14 (1992) 203–205.
[25] A. Mannucci, K. Sullivan, P. Ivanov, P. Gill, Forensic application of a rapid and quantitative DNA sex test by amplification of the X-Y homologous gene amelogenin, Int. J. Legal Med. 106 (1994) 190–193.
[26] H. Haas-Rochholz, G. Weiler, Additional primer sets for an amelogenin gene PCR-based DNA-sex test, Int. J. Legal Med. 110 (1997) 312–315.
[27] Y. Wang, J. Deng, J. Di, Y. Tu, Catalytic application of gold nanoparticles electrodeposited by fast scan cyclic voltammetry to glycerol electrooxidation in alkaline electrolyte, Electrochem. Commun. 11 (2009) 1034–1037.
[28] J.B. Raoof, R. Ojania, S.M. Golabib, E. Hamidi-Asla, M.S. Hejazi, Preparation of an electrochemical PNA biosensor for detection of target DNA sequence and single nucleotide mutation on p53 tumor suppressor gene corresponding oligonucleotide, Sens. Actuators B 157 (2011) 195–201.
[29] L. Komsiyska, G. Staikov, Gold electrodeposition in organic media, Electrochim. Acta 54 (2008) 168–172.