Bioelectrochemistry 109 (2016) 63–69

Contents lists available at ScienceDirect

Bioelectrochemistry

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

Electrochemical biosensor modified with dsDNA monolayer for restriction enzyme activity determination☆

Joanna Zajda, Łukasz Górski *, Elżbieta Malinowska

Faculty of Chemistry, Warsaw University of Technology, Noakowskiego 3, 00-664 Warsaw, Poland

ARTICLE INFO

Article history:
Received 7 September 2015
Received in revised form 28 January 2016
Accepted 31 January 2016
Available online 1 February 2016

Keywords:
Electrochemical sensor
DNA-based biosensor
Restriction endonuclease activity determination

ABSTRACT

A simple and cost effective method for the determination of restriction endonuclease activity is presented. dsDNA immobilized at a gold electrode surface is used as the enzymatic substrate, and an external cationic redox probe is employed in voltammetric measurements for analytical signal generation. The assessment of enzyme activity is based on a decrease of a current signal derived from reduction of methylene blue which is present in the sample solution. For this reason, the covalent attachment of the label molecule is not required which significantly reduces costs of the analysis and simplifies the entire determination procedure. The influence of buffer components on utilized dsDNA/MCH monolayer stability and integrity is also verified. Electrochemical impedance spectroscopy measurements reveal that due to pinhole formation during enzyme activity measurement the presence of any surfactants should be avoided. Additionally, it is shown that the sensitivity of the electrochemical biosensor can be tuned by changing the restriction site location along the DNA length. Under optimal conditions the proposed biosensor exhibits a linear response toward *Pvu*II activity within a range from 0.25 to 1.50 U/μL.

1. Introduction

Restriction endonucleases, also known as molecular scissors, play a key role in modern genetic engineering and molecular diagnosis [1]. Particularly, type II enzymes, that give unique and defined digestion products, are essential tools in sequencing of large DNA strands, genotyping, mapping, and recombinant technology [2–6]. In addition to that, dysfunction of XPG endonuclease has recently been linked to a high risk of skin cancer as well as skeletal, neurological and developmental abnormalities [7,8]. Considering the biological significance and extensive use of restriction enzymes, their activity assays are of great importance.

Conventional methods for restriction enzyme activity determination, such as gel electrophoresis [9], filter binding [10], high-performance liquid chromatography (HPLC) [11], and enzyme-linked immunosorbent assay (ELISA) [12] have been widely used. However, the protocols for these methods are discontinuous, time and DNA consuming, laborious, and usually require isotope labeling. Many of these limitations have been addressed by the development of alternative optical and electrochemical methods. Optical assays utilizing oligonucleotides labeled with spectrally paired fluorophores and quenchers (*e.g.* TAMRA and DABCYL) [13–15], hairpin DNAs [16], hairpin molecular beacon DNA probes [17,18], hairpin-type oligonucleotides labeled with a single fluorophore [19], and double stranded oligonucleotides that in a course of enzymatic reaction yields a G-quadruplex aptamer [20,21], and responsive systems with DNA-gold nanoparticles assembly [22,23] have been proposed. Additionally, electrochemical methods that, due to high sensitivity, ease of miniaturization and direct electronic readout, are currently one of the most popular for oligonucleotide detection and identification have been employed for this purpose. Restriction endonuclease activity has been determined using changes in either charge transfer (CT) chemistry [24], amount of CdSe QD remaining on the biosensor [25], interfacial electron transfer resistance [26,27] or G-quadruplex–hemin complex formation [28]. Both optical and electrochemical methods allow continuous monitoring of the enzymatic reaction, and determination of kinetic parameters in a short time period. However, modification of DNA with a label/fluorophore molecule to provide the analytical signal significantly increases the costs of the entire analysis. Large molecules that are used to label DNA substrates may interfere with recognition by the enzyme and influence the activity of certain restriction enzymes. In addition, some of the applied procedures are laborious and rather complicated. For these reasons, a simple and cost effective method for endonuclease activity determination is of great interest.

In this paper we present a simple, electrochemical biosensor that allows DNA cleavage monitoring for restriction endonuclease activity determination. Short double-stranded DNA fragments containing the restriction site of a chosen enzyme immobilized on a gold electrode surface are used as an enzymatic substrate. The changes within the dsDNA monolayer resulting from the enzymatic reaction are monitored using methylene blue (MB) that intrinsically associates noncovalently with the DNA [29,30] as a redox-active probe. The square wave voltammetry

☆ The authors declare no competing financial interest.

* Corresponding author.
E-mail address: lukegor@ch.pw.edu.pl (Ł Górski).

http://dx.doi.org/10.1016/j.bioelechem.2016.01.009
1567-5394/

(SWV) measurements of enzyme activity were used and were based on the current signal decrease derived from the redox active probe present in the test sample solution. By using MB, an expensive modification of deoxyoligonucleotide strand is not required and cost effective analysis can be performed.

2. Experimental section

2.1. Reagents

All the deoxyoligonucleotides used comprised of 20 bases and were purchased from Genomed Sp. z o.o. Poland. The base sequences were as follows: **T1**: 5′-SH–$(CH_2)_6$–GGGCG AGCAG CTGCG TCGGG-3′, **T2**: 5′-SH–$(CH_2)_6$–GGCAG CTGCG TCGCG CCGGG-3′, **C1**: 3′-CCCGC TCGTC GACGC AGCCC-5′, **C2**: 3′-CCGTC GACGC AGCGC GGCCC-5′. 6-Mercapto-1-hexanol (purum, ≥97% GC) (MCH), methylene blue (MB), hexaammineruthenium (II) chloride (98%), potassium ferricyanide, potassium ferrocyanide, tris(hydroxymethyl)aminomethane (Tris), magnesium chloride, sodium chloride, potassium chloride, potassium phosphate monobasic, TRITON X-100, glycerol, DL-dithiothreitol (≥99%) (DTT), and bovine serum albumin (BSA) were from Sigma-Aldrich (Germany) and were used as received. Sulfuric acid (≥95% pure P.A.), hydrochloric acid (35–58% pure P.A.), and hydrogen peroxide (30% pure P.A.) were acquired from POCh S.A., Poland. Restriction endonuclease *Pvu*II (from *Proteus vulgaris*, 10 U/μL) with recommended buffer G were purchased from Thermo Scientific and used without further purification. Oligonucleotide stock solutions were prepared with deionized water and stored in a −20 °C freezer before use. All solutions were prepared with Milli-Q water (18.2 MΩ, Millipore Corp.)

2.2. Electrodes pretreatment and DNA immobilization

Before any electrochemical experiments, the gold disk electrodes (2 mm diameter) were polished on microcloth with alumina powder of grain size 0.05 μm (CH Instruments). The electrodes were then washed with water and sonicated for 5 min in deionized water at 40 °C to remove any remaining particles. Next, piranha solution (sulfuric acid and hydrogen peroxide in ratio 7:3) was dropped on the working gold disk electrodes and left for 5 min. After removing the solution, electrodes were rinsed thoroughly with deionized water. Subsequently, electrodes were electrochemically cleaned in 10 mM Tris buffer pH 7.0 by scanning the potential from −600 mV to 600 mV *versus* an Ag/AgCl reference electrode, until the CV characteristic for a clean gold was obtained.

The ssDNA monolayer was prepared as described previously [31]. Briefly, after electrode cleaning, 25 μL of 4 μM solution of thiolated deoxyoligonucleotide (**T1** or **T2**) in 1 M KH_2PO_4 (pH 4.5) was dropped on the gold working disk electrode. The ssDNA immobilization was carried out for 120 min. Next, the solution was removed and the electrodes were washed with deionized water. Subsequently, electrodes were incubated in 4 μM solution of MCH in immobilization buffer (1 M KH_2PO_4, pH 4.5) for 60 min. After that time, electrodes were rinsed thoroughly with water and 25 μL of 4 μM solution of complementary oligonucleotide (**C1** or **C2**) in 10 mM Tris–HCl buffer containing 1 M NaCl, pH 7.0 was dropped on the ssDNA/MCH modified surface. Hybridization was performed for 60 min. Finally, the solution was removed, electrodes were washed with hybridization buffer solution (10 mM Tris–HCl, 1 M NaCl, pH 7.0), and the electrochemical measurements were conducted.

2.3. Electrochemical measurements

The DNA surface density and hybridization efficiency were determined based on chronocoulometric method previously reported by Steel et al. [32]. The DNA functionalized electrodes were first immersed in 10 mM Tris–HCl buffer pH 7.4, potential was stepped from 100 to −400 mV *vs* Ag/AgCl for 500 ms and the resulting charge flow measured. Then, the electrodes were transferred to 100 μM hexaammineruthenium(II) chloride ($Ru(NH_3)_6^{3+}$) in 10 mM Tris–HCl buffer, pH 7.4, and the measurements were repeated. Prior to chronocoulometric measurements both solutions were purged with nitrogen for 10 min and blanketed with N_2 during the experiment. Before hybridization with complementary oligonucleotide, electrodes were incubated in 10 mM Tris–HCl, 1 M NaCl, pH 7.0 for 10 min to remove $Ru(NH_3)_6^{3+}$.

The influence of buffer components *i.e.*, DTT, BSA, TRITON X-100, and glycerol on self-assembled dsDNA monolayer (dsDNA/MCH-SAM) stability was evaluated by electrochemical impedance spectroscopy (EIS) carried out at DC potential of 230 mV and 5 mV AC amplitude. Frequency was in the range from 0.1 Hz to 100 kHz. The EIS measurements were performed in 50 mM Tris–HCl solution containing 100 mM KCl and 5 mM concentration of $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ at pH 7.5. The obtained results were analyzed based on an equivalent circuit comprising of a resistor connected in series to a capacitor. The impedance of dsDNA/MCH modified electrodes was measured before and after 20 min incubation with the chosen compound.

Square wave voltammetric measurements were performed at a pulse amplitude of 25 mV, increment of 4 mV, and a frequency of 15 Hz. The potential was changed within the range of −500 mV to 300 mV *vs* Ag/AgCl reference electrode. Measurements were conducted in 50 μM MB, 10 mM Tris–HCl, 10 mM $MgCl_2$, 50 mM NaCl at pH 7.5 after 10 min of equilibration in the solution. Before the incubation in enzyme solution, MB was removed by sensor immersion in 10 mM Tris–HCl, 10 mM $MgCl_2$, 50 mM NaCl, pH 7.5 for 10 min.

All electrochemical measurements were performed with a CHI Electrochemical Workstation Model 660D (CH Instruments Inc., Austin, TX, USA) connected to PC, and carried out in a conventional three-electrode system consisting of a gold disk electrode (CH Instruments, Austin, TX, USA), a gold wire auxiliary electrode, and a Ag/AgCl/1 M KCl reference electrode at ambient temperature (24 ± 2 °C).

3. Results and discussion

3.1. Quantitation of DNA density immobilized on gold and hybridization efficiency

The gold electrode with immobilized dsDNA was used as substrate for the enzymatic reaction. For this reason it was crucial to investigate the quantity of the recognition sites potentially accessible for enzymatic cleavage, as well as the reproducibility of the DNA immobilization method. The surface density of ssDNA and hybridization efficiency with a complementary strand was determined using widely utilized chronocoulometry [32].

A self-assembled monolayer was formed by the chemisorption of thiolated ssDNA at the gold electrode surface, forming Au–SR bonds. By assuming that $Ru(NH_3)_6^{3+}$ binds stoichiometrically to the anionic sugar-phosphate backbone of DNA, probe DNA coverage can be calculated from the number of electrostatically associated cationic redox molecules [33]. In low ionic strength buffer containing a multivalent redox cation, $Ru(NH_3)_6^{3+}$ exchanges preferentially with the native monovalent DNA counterions and becomes electrostatically trapped at the interface. The counterions are essentially completely replaced in the ratio 1:3. Therefore, in a chronocoulometric experiment the charge Q as a function of time *t* is the sum of the charge resulting respectively, from the redox reaction of the molecules diffusing from the solution, the double layer charge, and the charge derived from reaction of species adsorbed on the electrode surface. The total charge is given by the integrated Cottrell equation [33]:

$$Q = \frac{2nFAD_0^{1/2}C_0^*}{\pi^{1/2}} t^{1/2} + Q_{dl} + nFA\Gamma_0 \quad (1)$$

where *n* is the number of electrons per molecule for reduction, *F* is the Faraday constant ($C \cdot mol^{-1}$), *A* is the electrode area (cm^2), D_0 is the

diffusion coefficient ($cm^2 \cdot s^{-1}$), C_0^* is the bulk concentration of redox species ($mol \cdot cm^{-3}$), Q_{dl} is the capacitive charge (C), and $nFA\Gamma_0$ is the charge from the reduction of Γ_0 ($mol \cdot cm^{-2}$) of adsorbed $Ru(NH_3)_6^{3+}$ (Q_{ads}). The Anson plot of a chronocoulometric data (Q *vs* $t^{1/2}$) yields an intercept at $t = 0$ that corresponds to the sum of Q_{dl} and $nFA\Gamma_0$. Assuming that the double layer capacitance is equal in the presence of the cationic redox molecules and in the blank solution, the surface excess $nFA\Gamma_0$ can be calculated from the difference in the intercepts.

The conversion of measured surface excess to DNA surface density can be undertaken only when redox marker concentrations provide saturation conditions *i.e.* DNA charge is completely compensated by $Ru(NH_3)_6^{3+}$. The surface density is then determined from the surface excess of redox marker as:

$$\Gamma_{DNA} = \Gamma_0 ({}^{z}/_{m}) N_A \quad (2)$$

where Γ_{DNA} is the probe surface density ($molecules \cdot cm^{-2}$), z is the charge of the redox molecule, m is the number of bases in the probe DNA, and N_A is the Avogadro number.

In Fig. 1 the Anson's plots obtained for an electrode modified with thiolated ssDNA, and for the same electrode after hybridization with a complementary sequence are presented. To ensure diffusion of the electroactive specie so that the Cottrell equation is valid,100 μM $Ru(NH_3)_6^{3+}$ in 10 mM Tris–HCl buffer solution was used in all experiments. For an electrode placed in 10 mM Tris–HCl buffer solution, a straight line with a positive intercept related to double-layer charging was observed, while in the presence of $Ru(NH_3)_6^{3+}$, a typical plot for electroactive species adsorption was recorded. Following the potential step, electroactive molecules adsorbed at the electrode surface are instantaneously electrolyzed. This reaction gives rise to current and to a large value of charge at the initial point. Subsequently, plots are linear, since the charge arising from diffusion requires time for the electroactive component to reach the electrode surface and to react. In the case of the dsDNA/MCH modified electrode, more $Ru(NH_3)_6^{3+}$ molecules were adsorbed at the surface, therefore a larger value of charge at the initial point was observed than before hybridization with the complementary strand (either **C1** or **C2**).

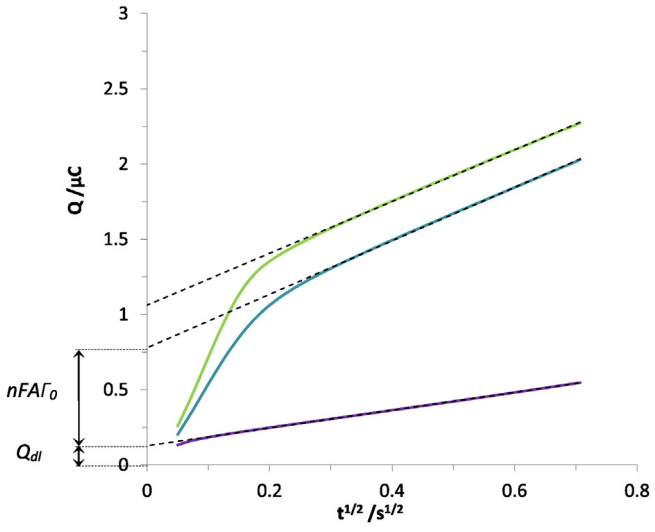


Fig. 1. Anson's plots of representative chronocoulometric data obtained for electrode modified with thiolated ssDNA and MCH in 10 mM Tris–HCl buffer solution at pH 7.0 (purple line), for ssDNA/MCH modified electrode in 100 μM $Ru(NH_3)_6^{3+}$, 10 mM Tris–HCl buffer, pH 7.0 (blue line), and after hybridization with complementary sequence (dsDNA) in 100 μM $Ru(NH_3)_6^{3+}$, 10 mM Tris–HCl buffer, pH 7.0 (green line). Dotted lines represent least squares fit to linear region. 6-mercaptohexanol (MCH) was used to prevent nonspecific adsorption. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

DNA densities were calculated using the geometric area of the disk gold electrodes. When measuring the density of hybridized target in the low ionic strength Tris–HCl buffer, in the absence of $Ru(NH_3)_6^{3+}$, a partial loss of the duplex can occur. However, in the presence of the electroactive probe ($Ru(NH_3)_6^{3+}$), due to stabilization of the duplex by the trivalent redox molecule, no loss of target has been reported previously [34]. In addition, the capacitive charge (Q_{dl}) before and after hybridization, due to an approximately equal capacitance, was shown to vary by less than 1% [34]. Therefore, the Q_{dl} value measured for ssDNA in 10 mM Tris–HCl buffer at pH 7.4 was used in all calculations. The surface density and hybridization efficiency obtained for the applied DNA immobilization and hybridization method were 5.1 ± 0.4 × 10^{12} $molecules \cdot cm^{-2}$, and 39.52 ± 3.74%, respectively (SD was calculated for 9 sensors). It should be noted that the calculated values are in good agreement with the data previously reported in the literature for gold electrodes modified with DNA [32,34].

3.2. Examination of dsDNA/MCH SAM stability

To maintain their catalytic activity commercially available restriction endonucleases are supplied in appropriate storage buffer solutions. These Tris–HCl or phosphate buffer solutions typically contain EDTA (1 mM), DTT (1 mM), BSA (0.2 mg/mL), and glycerol at concentration of 50% (*v*/*v*) [35]. Moreover, in some cases detergents like nonionic TRITON X-100 are added to reduce surface tension, and enable enzymes to function properly [36]. Nonetheless, some of these compounds can potentially affect the integrity of dsDNA/MCH SAMs created on a gold electrode. To verify the possibility of an unwanted interaction between the storage buffer components and mixed self-assembled monolayer utilized, electrochemical impedance spectroscopy measurements were performed. The influence of BSA, DTT, glycerol, and TRITON X-100 on SAM stability and organization was examined. All impedance measurements were carried out in the presence of the redox couple ferro/ferricyanide with a concentration of 5 mM each. Nyquist plots, derived from the impedance data, before and after 20 min incubation with a chosen compound are shown in Fig. 2. In the presence of the $FeCN_6^{4-/3-}$ couple, a characteristic shape of Nyquist plots for DNA modified electrodes was observed. Within the high frequency range, a semicircle was observed, which is associated with a resistance (R_{ct}) at the electrode/solution interface. For the lower frequency range, a linear dependence of the impedance components (real Z', and imaginary Z''), typical for a diffusion controlled electrode process ("Warburg" impedance), was recorded.

In a solution at pH 7.5, due to its sugar-phosphate backbone nature, the dsDNA/MCH monolayer is negatively charged [37]. Therefore, a repulsion of the $Fe(CN)_6^{3-/4-}$ redox couple can be observed which is demonstrated by the relatively high R_{ct} value (Fig. 2, blue lines). Once the dsDNA/MCH modified electrode was incubated in 10 mM Tris–HCl, 10 mM $MgCl_2$, 50 mM NaCl buffer solution at pH 7.5 containing either BSA, DTT or glycerol (Fig. 2A, B and C, respectively), no significant change in charge transfer resistance was observed. On the other hand, the R_{ct} value remarkably decreased after the incubation of dsDNA/MCH modified gold electrode in 0.02% TRITON X-100 solution (Fig. 2D). This undesirable drop in the resistance is most likely caused by the interaction of surfactant with the alkanethiol monolayer. Surfactant molecules can adsorb on the surface or at the interface, and to some extent change the surface free energy. Surfactant molecules can also, certainly, intercalate into a previously formed thiol monolayer. Therefore, the integrity of the organized SAM is disrupted, and pinholes are formed, which facilitates a direct redox reaction at the electrode surface resulting in a reduced R_{ct} value. The influence of surfactant on the electrochemical properties of a gold electrode modified with a thiol monolayer was previously described by Yong et al. [38]. These authors investigated the interaction of the cationic surfactant hexadecyltrimethylammonium bromide with a 1-dodecanethiol self-assembled monolayer. They observed a decrease in charge transfer

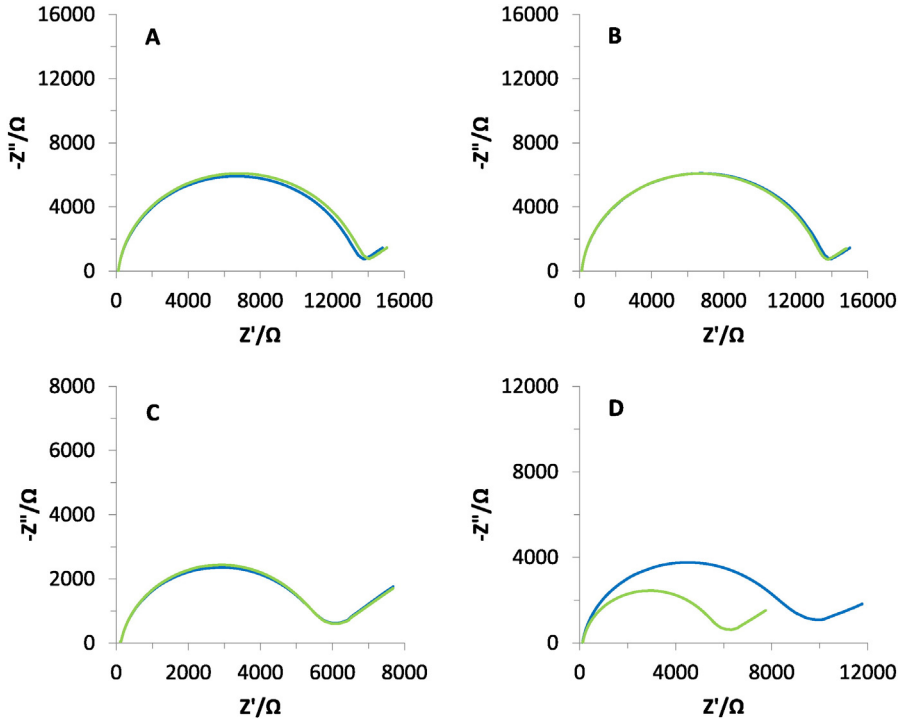


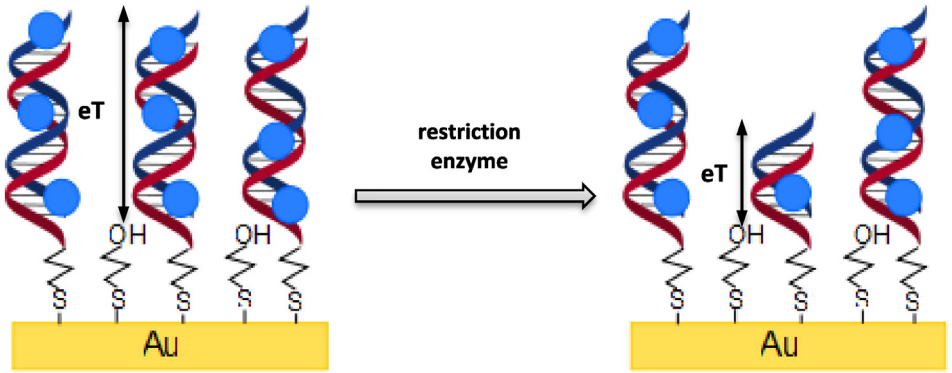
Fig. 2. Nyquist plots obtained for dsDNA/MCH modified electrodes before (blue line) and after 20 min incubation in: (A) 0.1 mg/mL BSA, (B) 0.1 mM DTT, (C) 5% glycerol, (D) 0.02% TRITON X-100 in 10 mM Tris–HCl, 10 mM $MgCl_2$, and 50 mM NaCl (green line). Analyses were performed after 5 min incubation in 50 mM Tris–HCl buffer solution containing 5 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ and 100 mM KCl at pH 7.5. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

resistance (R_{ct}) with increasing incubation time in surfactant solution. Although nonionic surfactant was tested in our work, the results presented in Fig. 2D show the same trend as for cationic surfactant. For this reason, to eliminate errors associated with changes in the electrochemical behavior of the dsDNA/MCH monolayer, the presence of any surfactant during the measurement of enzyme activity should be avoided. Since *Pvu*II restriction endonuclease does not require surfactant to maintain catalytic activity, it was chosen as a model enzyme.

3.3. Restriction endonuclease activity determination

As for each type II restriction endonuclease, *Pvu*II enzyme recognizes short, specific sequences of nucleotide bases and cleaves DNA at distinct sites within the recognition site. *Pvu*II restriction endonuclease recognizes the 5′ –CAGCTG– 3′ sequence, and cuts it in the middle, thus shorter oligonucleotide strands with blunt ends are formed upon enzymatic cleavage. In this work, two oligonucleotide sequences containing the *Pvu*II restriction site at different positions, 10 and 5 base pairs from the 5′-end, were used. dsDNA immobilized on the electrode surface served as the enzyme substrate, and was monitored for loss due to enzymatic cleavage using methylene blue (MB) as a redox-active probe (Scheme 1).

Thiolated ssDNA with a 6-carbon spacer at the 5′-terminus (**T1** or **T2**) was first immobilized on gold electrode through a thiol-Au covalent bond. Before hybridization with a complementary strand (**C1** or **C2**), 6-mercapto-1-hexanol (MCH) was added. This short bifunctional molecule is essential for a well-controlled DNA immobilization. MCH not only serves as a spacer that finely modulates the surface density of the DNA probes, but also effectively prevents nonspecific DNA–gold interactions [39,40]. A highly directional electron transfer (eT) through the DNA duplex was ensured using oligonucleotides with a length of 20 base pairs. The redox-active probe (MB) used here is known to interact electrostatically with both ssDNA and dsDNA. However, due to the increased local negative charge on dsDNA, and the intercalative mode of MB binding, “enhanced” interactions of MB with dsDNA can be anticipated. Also for longer DNA strands, providing higher negative charges as a result of a larger number of phosphate groups, substantially more MB molecules are expected to bind compared to shorter sequences.



Scheme 1. Representation of enzymatic cleavage of the substrate immobilized at the electrode surface catalyzed by *Pvu*II restriction endonuclease. Blue dots represent redox-active probe, methylene blue, and eT is the electron transfer. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

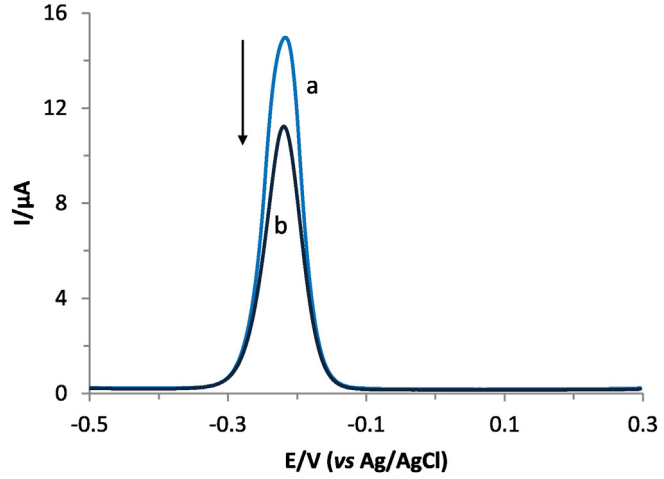


Fig. 3. SWV current response of dsDNA/MCH modified electrode: a) before and b) after enzymatic reaction. SWVs were measured after 10 min interaction with 50 μM MB in 10 mM Tris–HCl containing 10 mM $MgCl_2$ and 50 mM NaCl at pH 7.5.

Thus, as can be seen in Fig. 3, upon enzymatic cleavage the decreased current signal resulting from the MB reduction was observed.

As a result of applied cleaning method (*i.e.*, manual polishing with aluminum oxide slurry), each electrode has a slightly different surface area. Moreover, within the time in flexible dsDNA/MCH monolayer a dehybridization of complementary strand, or some unspecific interactions can occur. Thus, to take into account those variables, the biosensor response Δ was defined as a change of MB current signal, recorded in SWV measurements, using the following equation:

$$\Delta = \left(\frac{I_0 - I_{enz}}{I_0} - \frac{I_0' - I_{blank}}{I_0'}\right) \times 100\% \quad (3)$$

where I_0 and I_0' refer to current before the subjection of the sensor to sample solution, I_{enz} is current after incubation with an enzyme solution, and I_{blank} attributes to current after incubation in a blank solution (*i.e.*, 10 mM Tris–HCl, 10 mM $MgCl_2$, 50 mM NaCl, 0.1 mg/mL BSA at pH 7.5).

The kinetics of the enzymatic reaction catalyzed by the *Pvu*II endonuclease with the proposed biosensors was evaluated by plotting the Δ values against incubation times. In Fig. 4A and B the changes of the Δ values over time for 20-mer deoxyoligonucleotide with a restriction site along half of its length (**T1**/**C1**), and along a quarter of its length from the 5′-end (**T2**/**C2**) are presented. As a result of enzymatic cleavage, shorter strands with a length of 10 and 5 deoxyoligonucleotides, respectively, were formed at the electrode surface. As can be seen in Fig. 4, the analytical signal increases over incubation time for dsDNA/MCH modified gold electrodes with restriction endonuclease standard solution of activity 1 U/μL. For both probe sequences, the initial responses are linear and a slight flattening of the kinetic curve is observed after longer incubation times. However, in the case of the deoxyoligonucleotide with the restriction site closer to the electrode surface (**T2**/**C2**), the linear range is wider compared to the probe with the restriction site along half its length. For the probe containing the restriction site 5 base pairs from the electrode surface (**T2**/**C2**), after enzymatic cleavage less DNA can interact with the redox probe. Therefore, a bigger drop in analytical signal should be observed. Nonetheless, as the kinetic measurements revealed, a greater signal change was observed for the **T1**/**C1** dsDNA sequence with a restriction site along half its length (Fig. 4A). A greater than 16% signal change after 30 min of incubation with enzyme was registered for the **T1**/**C1** sequence compared to a 13% signal change for the **T2**/**C2** sequence. It should be noted that the large error bars represent pooled standard deviations calculated for two groups of at least 3 sensors. In the case of the **T2**/**C2** DNA sequence, where the restriction site is closer to the electrode surface, the smaller response is most likely related to hindered access of enzyme to the substrate. One can suggest, that the greater signal change could be observed if the restriction site was to be closer to the 3′-end of the thiolated strand, and more accessible to the enzyme. However, in that case shorter DNA fragments would be removed from the surface, thus, the decrease of MB reduction current, at high current background, would be less remarkable. In fact, the reduction current change resulting from the enzymatic reaction could be very small, almost unnoticeable, and overlooked.

Since greater changes in the reduction peak current were observed for a dsDNA sequence with a restriction site at half-length, electrodes modified with a **T1**/**C1** monolayer were used to construct the calibration curve. The electrodes modified with dsDNA/MCH monolayer were incubated for 15 min in *Pvu*II enzyme standard solutions, then MB reduction currents were recorded and Δ values plotted against enzyme activity. As shown in Fig. 5, a linear response was obtained in a range from 0.25 to 1.50 U/μL of enzyme activity. However, instead of a characteristic signal plateau for complete substrate cleavage, further decrease of Δ values was observed for higher enzyme activities. This drop was most likely associated with the increased glycerol concentrations present in the test sample solutions. Although glycerol does not change the electrochemical behavior of the dsDNA/MCH monolayer, as demonstrated by the EIS study (see Fig. 2C), it has been reported that glycerol can inhibit enzyme activity [41], and may even alter its specificity [42]. In our study, standard solutions of enzyme activities above 1.5 U/μL contained more than 8% (*v*/*v*) of glycerol. This glycerol concentration is generally considered to inhibit digestion by any restriction endonucleases [43]. For this reason, only data obtained for enzyme solutions of activity up to

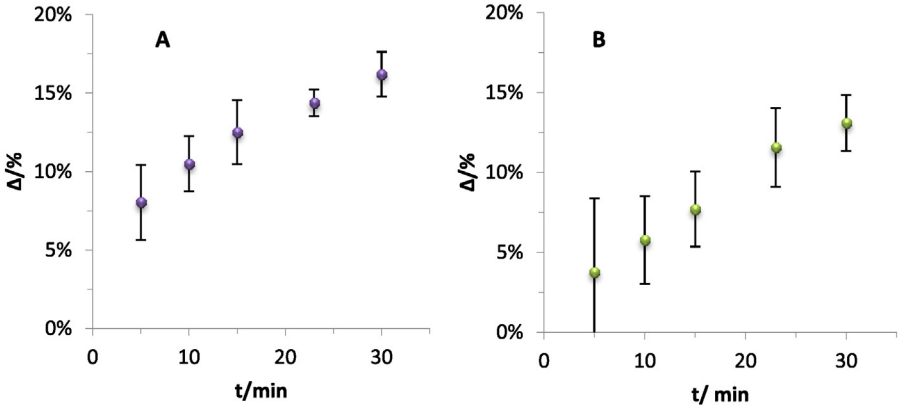


Fig. 4. Kinetics of enzymatic reaction obtained using dsDNA/MCH modified electrodes with *Pvu*II restriction site (**A**) at 10 base pairs (**T1**/**C1**) and (**B**) at 5 base pairs (**T2**/**C2**) from 5′ end of thiolated oligonucleotide strand. *Pvu*II restriction endonuclease activity was 1 U/μL. Error bars represent pooled SD (n = 3).

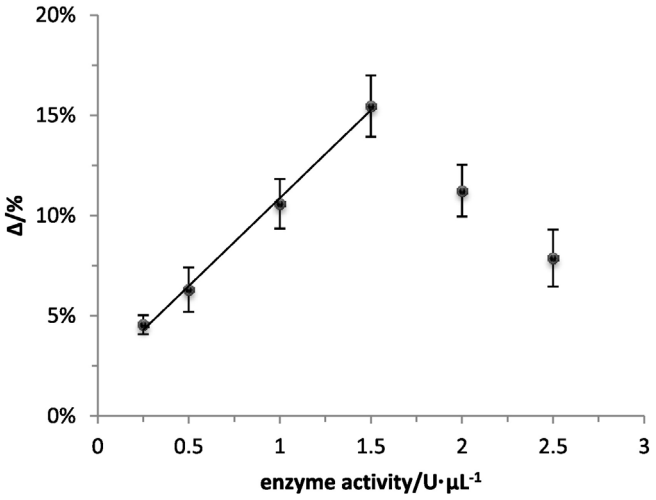


Fig. 5. Calibration curve of electrodes modified with the T1/C1 dsDNA sequence containing restriction site along half of its length toward *Pvu*II endonuclease activity. Electrodes were incubated with an enzyme standard solution of a chosen activity for 15 min. A linear regression line for the presented data has an equation of $y = 0.0877\times + 0.021$ with an $R^2 = 0.9968$. Error bars represent pooled SD (n = 6).

1.50 U/μL, which corresponds to 7.5% glycerol content, were used to plot the calibration curve presented in Fig. 5.

4. Conclusions

We have demonstrated that gold electrodes modified with dsDNA/MCH SAM containing restriction site of a chosen enzyme can be successfully used to monitor enzymatic DNA cleavage by restriction endonuclease. An external redox marker, methylene blue, was employed for voltammetric measurements which significantly reduces the costs of the entire analysis. Due to a decreased amount of dsDNA at the electrode surface resulting from the enzymatic reaction the current signal decreases proportionally to the reaction time, as well as to the enzyme activity. The influence of the buffer solution components (*i.e.*, DTT, BSA, TRITON-X, and glycerol) on DNA mixed self-assembled monolayer stability and integrity was examined. Electrochemical impedance spectroscopy measurements revealed that surfactant should be avoided due to its impact on the electrochemical behavior of dsDNA/MCH modified electrodes. The effect of the restriction site location on biosensor sensitivity was also investigated. It was shown that better sensitivity could be achieved for deoxyoligonucleotide sequences containing the restriction site along half of its length compared to sequence with a restriction site closer to the electrode surface. Diminish response in the case of the latter DNA probe was most likely related to the hindered access of the enzyme to the substrate. It was demonstrated that using the developed biosensor, *Pvu*II restriction endonuclease activity could be successfully determined in a range from 0.25 to 1.50 U/μL within 15 min. Moreover, the activity of the different restriction enzymes could be detected with the use of the proposed biosensor by simply changing the DNA sequence immobilized at the electrode surface. Considering the simplicity of proposed method and the low cost of the analysis it seems feasible to apply the developed biosensor for routine restriction endonuclease activity determination, as well as for screening tests based on XPG endonuclease activity, for skin cancer detection.

Acknowledgments

This work was financially supported by the European Union in the framework of the Regional Development through the Joint UW and WUT International PhD Program of Foundation for Polish Science (Grant MPD/2010/4), and by the Polish National Science Centre (NCN) grant OPUS 2013/11/B/ST4/00766.

References

[1] S. Chan, B.L. Stoddard, S. Xu, Natural and engineered nicking endonucleases—from cleavage mechanism to engineering of strand-specificity, Nucleic Acids Res. 39 (2011) 1–18.
[2] L.A. Allison, Fundamental molecular biology, Chapter 8: Recombinant DNA Technology and Molecular Cloning, Blackwell Publishing 2007, pp. 180–231.
[3] R.J. Roberts, How restriction enzymes became the workhorses of molecular biology, PNAS 102 (2005) 5905–5908.
[4] A.S. Bhagwat, Restriction enzymes: prosperities and use, Methods Enzymol. 216 (1992) 199–2244.
[5] A. Pingoud, A. Jeltsch, Structure and function of type II restriction endonucleases, Nucleic Acids Res. 29 (2001) 3705–3727.
[6] P.B. Gray, K.G. Anderson, Fatherhood: Evolution and Human Paternal Behavior, Harvard University Press, 2010.
[7] D. Schärer, XPG: its products and biological roles, Adv. Exp. Med. Biol. 637 (2008) 905–921.
[8] A. O'Donovan, A.A. Davies, J.G. Moggs, S.C. West, R.D. Wood, XPG endonuclease makes the 3′ incision in human DNA nucleotide excision repair, Nature 371 (1994) 432–435.
[9] A.P. Sharp, B. Sugden, J. Sambrook, Detection of two restriction endonuclease activities in *Haemophilus parainfluenzae* using analytical agarose-ethidium bromide electrophoresis, Biochemistry 12 (1973) 3055–3063.
[10] T.G. Fanning, H.-J. Kreutzfeldt, R.W. Davies, P.H. Shreier, Detection of restriction endonucleases with a lac repressor-lac operator filter binding assay, FEBS Lett. 61 (1976) 237–239.
[11] L.W. McLaughlin, F. Benseler, E. Graeser, N. Piel, S. Scholtissek, Effect of functional group changes in the EcoRI recognition site on the cleavage reaction, catalyzed by the endonuclease, Biochemistry 26 (1987) 7238–7245.
[12] A. Jeltsch, A. Fritz, J. Alves, H. Wolfes, A. Pingoud, A fast and accurate enzyme-linked immunosorbent assay for determination of the DNA cleavage activity of restriction endonucleases, Anal. Biochem. 213 (1993) 234–240.
[13] K. Eisenschmidt, T. Lanio, A. Jeltsh, A. Pingoud, A fluorimetric assay for on-line detection of DNA cleavage by restriction endonucleases, J. Biotechnol. 96 (2002) 185–191.
[14] S.P. Lee, M.L. Censullo, M.G. Kim, J.R. Knutson, M.K. Han, Characterization of endonucleolytic activity of HIV-1 integrase using a fluorogenic substrate, Anal. Biochem. 227 (1995) 295–301.
[15] S.P. Lee, D. Porter, J.G. Chirikjian, J.R. Kunston, M.K. Han, A fluorometric assay for DNA cleavage reactions characterized with BamHI restriction endonuclease, Anal. Biochem. 220 (1994) 377–383.
[16] J. Zhang, Z. Shi, Y. Jin, Enzyme-free and label-free signal amplification for monitoring endonuclease activity and inhibition via hybridization chain reaction, Analyst 140 (2015) 3500–3506.
[17] J.J. Li, R. Geyer, W. Tan, Using molecular beacons as a sensitive fluorescence assay for enzymatic cleavage as single-stranded DNA, Nucleic Acids Res. 28 (2000) ES2.
[18] C. Ma, Z. Tang, K. Wang, W. Tan, X. Yang, W. Li, Z. Li, X. Lv, Real-time monitoring of restriction endonuclease activity using molecular beacon, Anal. Biochem. 363 (2007) 294–296.
[19] T.T. Nikiforov, Fluorogenic polymerase, endonuclease, and ligase assays based on DNA substrates labeled with a single fluorophore, Anal. Biochem. 412 (2011) 229–236.
[20] Z. Zhou, Y. Du, L. Zhang, S. Dong, A label-free, G-quadruplex DNAzyme-based fluorescent probe for signal-amplified DNA detection and turn-on assay of endonuclease, Biosens. Bioelectron. 34 (2012) 100–105.
[21] L. Lu, D.S.-H. Chan, D.W.J. Kwong, H.-Z. He, C.-H. Leung, D.-L. Ma, Detection of nicking endonuclease activity using a G-quadruplex-selective luminescent switch-on probe, Chem. Sci. 5 (2014) 4561–4568.
[22] X.Y. Xu, M.S. Han, C.A. Mirkin, A gold-nanoparticle based real-time colorimetric screening method for endonuclease activity and inhibition, Angew. Chem. Int. Ed. Engl. 46 (2007) 3468–3470.
[23] G. Song, C. Chen, J. Ren, X. Qu, A simple, universal colorimetric assay for endonucleases/methylotransferase activity and inhibition based on an enzyme-responsive nanoparticle system, ACS Nano 3 (2009) 1183–1189.
[24] E.M. Boon, J.E. Salas, J.K. Barton, An electrical probe of protein-DNA interactions on DNA-modified surfaces, Nat. Biotechnol. 20 (2002) 282–287.
[25] C. Zhang, J. Lou, W. Tu, J. Bao, Z. Dai, Ultrasensitive electrochemical biosensing for DNA using quantum dots combined with restriction endonuclease, Analyst 140 (2015) 506–511.
[26] E. Katz, I. Willner, Immunosensors and DNA sensors based on impedance spectroscopy, in: V. Mirsky, O. Wolfbeis (Eds.), Ultrathin Electrochemical Chemo- and Biosensors. Technology and Performance, Springer Series on Chemical Sensors and Biosensors. Methods and ApplicationsSpringer-Verlag, Berlin 2004, pp. 67–106 Chapter 4.
[27] E. Katz, Y. Weizmann, I. Willner, Magnetoswitchable reactions of DNA monolayers on electrodes: gating the processes by hydrophobic magnetic nanoparticles, J. Am. Chem. Soc. 127 (2005) 9191–9200.
[28] N. Yang, Y. Cao, P. Han, X. Zhu, L. Sun, G. Li, Tools for investigation of the RNA endonuclease activity of mammalian Argonaute2 protein, Anal. Chem. 84 (2012) 2492–2497.
[29] S.O. Kelly, J.K. Barton, Electrochemistry of methylene blue bound to a DNA-modified electrode, Bioconjug. Chem. 8 (1997) 31–37.

[30] E. Farjami, L. Clima, K.V. Gothelf, E.E. Ferapontova, DNA interactions with a methylene blue redox indicator depend on the DNA length and are sequence specific, Analyst 135 (2010) 1443–1448.
[31] M. Jarczewska, R. Ziółkowski, Ł. Górski, E. Malinowska, Electrochemical uranyl cation biosensor with DNA oligonucleotides as receptor layer, Bioelectrochemistry 96 (2014) 1–6.
[32] A.B. Steel, T.M. Herne, M.J. Tarlov, Electrochemical quantitation of DNA Immobilized on gold, Anal. Chem. 70 (1998) 4670–4677.
[33] A.J. Bard, L.R. Faulkner, Electrochemical Methods Fundamentals and Applications, second ed. John Wiley & Sons, New York, 2001.
[34] S.D. Keighley, P. Li, P. Estrela, P. Migliorato, Optimization of DNA immobilization on gold electrodes for label-free detection by electrochemical impedance spectroscopy, Biosens. Bioelectron. 23 (2008) 1291–1297.
[35] http://www.thermoscientificbio.com/restriction-and-modifying-enzymes/restriction-enzymes/conventional (accessed Aug 14, 2015)
[36] http://www.methodbook.net/dna/restrdig.html (accessed Aug 14, 2015)
[37] J. Wang, G. Rivas, J.R. Fernandes, J.L.L. Paz, M. Jiang, R. Waymire, Indicator-free electrochemical DNA hybridization biosensor, Anal. Chim. Acta 375 (1998) 197–203.
[38] M. Yong, W. Jian-Guo, H. Fei, H. Jun-Jie, Z. Shu-Liang, Influence of surfactant on electrochemical behavior of gold electrode modified with thiol monolayer, Acta Chim. Sin. 64 (2006) 1309–1313.
[39] T.M. Herne, M.J. Tarlov, Characterization of DNA probes immobilized on gold surfaces, J. Am. Chem. Soc. 119 (1997) 8916–8920.
[40] R. Levicky, T.M. Herne, M.J. Tarlov, S.K. Satija, Using self-assembly to control the structure of DNA monolayers on gold: a neutron reflectivity study, J. Am. Chem. Soc. 120 (1998) 9787–9792.
[41] http://www.imbb.forth.gr/groups/minotech-new/pdf/restriction_endonuclease_troubleshooting_guide.pdf (accessed Aug 14, 2015)
[42] S.J. Karcher, Molecular Biology: A Project Approach, Academic Press Inc, San Diego, 1995.
[43] http://www.ibibiobase.com/projects/reb/FAQs.htm (accessed Aug 14, 2015)