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Detection of *bar* gene encoding phosphinothricin herbicide resistance in plants by electrochemical biosensor

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ABSTRACT

An electrochemical biosensor for the detection of *bar* gene coding phosphinothricin herbicide resistance is presented. The detection was based on hybridization reaction between the specific to *bar* gene 19-mer probe immobilized on the electrode surface and complementary DNA in a sample. Single-stranded DNA probe specific to *bar* gene was covalently attached by 5′-phosphate end to the surface of carbon paste electrode. Outer layer of a conventional CPE was provided with carboxyl groups of stearic acid. ssDNA was coupled to the electrode through ethylenediamine with the use of water-soluble 1-ethyl-3(3′-dimethylaminopropyl)-carbodiimide and *N*-hydroxy-sulfosuccinimide as activating reagents. Hybridization reaction at the electrode surface was detected via $Co(bpy)_3^{3+}$, which posses a much higher affinity to the resulting DNA duplex compared to ssDNA probe. Detection limit of the sensor was 0.1 μM of target DNA fragments and its response was linear from 5 to 20 μM. Hybridization event was also detected by measuring guanine peak but this approach presented distinctly higher detection limit (1 μM) and lower reproducibility. Complete time of one measurement with the use of the biosensor including covalent attachment of ethylenediamine (linker) and ssDNA probe to the electrode, hybridization with target and interaction with electroactive indicator was about 70 min.

1. Introduction

Nucleic acid hybridization has become one of the most important techniques in molecular biology for the detection and analysis of specific DNA sequences. Determination of nucleic acid sequence plays an increasingly significant role in many areas, ranging from clinical diagnosis, forensic and environmental analyses to food safety monitoring. Conventional methods are often laborious and expensive, therefore new DNA biosensors selectively detecting DNA sequences by hybridization appear very promising analytical tool [1–6]. DNA hybridization biosensor consists of a biological recognition element associated with a transducer translating recognition event into a physically measurable value [7]. Methods of signal transduction include mostly electrochemical, piezoelectric or optical systems [2,8]. Electrochemical transducers are relatively simple devices, rather cheap, appropriately sensitive and selective, and generally eligible for routine tests, therefore they seem to be particularly attractive for the detection of specific DNA sequences [2,3,5,6,9,10]. In an electrochemical DNA hybridization sensor usually a short single-stranded DNA (a probe) is immobilized on working electrode to create recognition element. Among variety of working electrodes carbon paste electrodes (CPE) have been widely used to assemble DNA biosensor [2,11]. They can be modified by different methods for example by simple mixing appropriate compounds with the carbon paste [6,12,13]. The probe immobilized on the electrode surface is able to form specific double-stranded hybrid with its complementary nucleic acid (target) in the presence of a mixture of many other nucleic acid fragments [4,6,11,14]. The hybridization event is commonly detected with the use of electroactive redox indicator that enables discrimination between single-stranded and newly formed on the electrode surface double-stranded DNA [1,2,5,6,10,15]. In this analytical approach bipyridyl or phenanthroline metal chelates (Ru, Os, Co) are frequently used.

The efficiency of DNA hybridization sensor is highly influenced by the method of probe immobilization and the hybridization procedure [6,8]. The probe attachment is the key factor in the overall performance of electrochemical DNA biosensor and wide range of immobilization methods was described [2,5,6,11]. One of the most commonly used approaches is controlled-potential adsorption on the CPE surface [16–21]. This immobilization method does not require any reagents or nucleic acid chemical modifications. However, multiple sites of ssDNA binding result often in poor hybridization efficiency and a probe is not prevented from desorption [2,6,11]. More suitable approach could be one point attachment of the ssDNA molecule by 5′ or 3′ end providing higher hybridization efficiency. Covalent immobilization of ssDNA enables proper orientation of coupled probes and furnishes a stable layer under experimental conditions [2,11,14].

A method of covalent immobilization of ssDNA fragments on the modified carbon paste electrode was described by Millan et al. [12]. The

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probe deposition on a modified carbon paste was performed in the presence of water-soluble carbodiimide 1-(3-dimethylaminopropyl)-3-ethyl-carbodiimide (EDC) and *N*-hydroxy-sulfosuccinimide (NHS). Carbodiimide derivatives are widely used for forming either amide or phosphoamidate bonds between the DNA and carboxyl or amino groups of the modified carbon or gold electrodes surface [2,6,14].

In this work rapid and sensitive electrochemical DNA biosensor for the identification and quantitative determination of *bar* gene is presented. The majority of genetically modified plants have been transformed with constructs conferring herbicide resistance, commonly phosphinothricin tolerance genes (*bar*, *pat*). Therefore the detection of these target sequences is a way of GM food analysis [22,23]. DNA sequence determination was performed electrochemically using the stearic acid modified carbon paste electrode with covalently attached probe specific for the *bar* gene. The complementary nucleic acids fragments were detected via hybridization with the use of electroactive indicator or also without indicator on the base of guanine oxidation peak [1,2,6,10,15]. An optimization of biosensor performance procedure was established and this is discussed in the paper. One of the important factors influencing biosensor performance is a hybridization efficiency related very much to the electrode surface density of a probe [8,9,24,25]. This is also considered in the paper.

2. Materials and methods

2.1. Reagents and electrochemical measurements

All solutions were prepared using deionized water. Oligonucleotides (19-mer probe, 19-mer complementary target and 15-mer noncomplementary DNA fragment) were purchased in Tib Molbiol (Poznan, Poland) and had the following sequences:

probe 5′ GTCAACTTCCGTACCGAGC
target (*bar* gene) 5′ GCTCGGTACGGAAGTTGAC
noncomplementary DNA fragment 5′ TGTCTCAAACTGTTT
15 mer oligoG and 15 mer oligoC

Ethylenediamine was from Fluka. 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide (EDC), *N*-hydroxy-sulfosuccinimide (NHS), stearic acid, 2,2′-bipyridine cobalt(II) chloride, perchloric acid, graphite powder, mineral oil were from Sigma. All other chemicals were analytical grade from Polskie Odczynniki Chemiczne, Poland. Tris(2,2′-bipyridyl)cobalt (III) perchlorate ($Co(bpy)_3(ClO_4)_3$) was prepared according to described procedures and recrystallized twice from water prior to use [26–28].

Electrochemical measurements were performed with a potentiostat PGSTAT12 with GPES, version 4.9 software (Eco Chemie, Utrecht, The Netherlands). The experimental conditions for electrochemical analysis were: the three electrode system consisted of a carbon paste working electrode, Ag/AgCl reference electrode and a platinum wire counter electrode. The carbon paste was prepared by mixing graphite powder with mineral oil with the ratio 70:30. The resulting paste was packed into Teflon tube of 0.1 cm internal diameter. Electrical connection was supplied with a copper wire. Only the outer layer of electrode was prepared from modified carbon paste enriched with stearic acid in the concentration of 5% (w/w). The surface of the working electrode was always renewed before use by removing outer layer of carbon paste and polishing to a smoothed finish on a weighing paper.

Experiments were carried out in a 1 ml cylindrical cell. All potentials were referred to Ag/AgCl electrode. All measurements were done in room temperature.

Experimental conditions for square wave voltammetry were: frequency of 100 Hz, amplitude of 0.04 V and step potential of 0.015 V.

For the presentation of all experimental voltammograms Origin, version 6.0 (Microcal Software) was used.

Calculations were executed on the ground average from five measurements.

2.2. Immobilization of ssDNA probe on the modified CPE

Sequence specific DNA sensor was developed by the covalent attachment of single-stranded nucleic acid fragments to stearic acid modified carbon paste electrode according to our procedure described previously [29].

Before immobilization the carbon paste electrode was electrochemically pretreated in 0.05 M phosphate buffer, pH 7.0 for 60 s at +1.7 V. After pretreatment the electrode was rinsed for 15 min in 0.05 M phosphate buffer, pH 7.0 containing 5 mM EDC, 8 mM NHS and 75 mM ethylenediamine. Then the electrode was washed for 2 min in a buffer and immersed for 15 min at +0.5 V in the phosphate buffer containing 5 mM EDC, 8 mM NHS and DNA probe at the concentration of 10 μM. After immobilization DNA modified electrode was rinsed for 15 min and used to hybridization with target DNA fragments.

2.3. Electrochemical detection of bar gene by hybridization

For hybridization a probe modified electrode was immersed in 0.05 M phosphate buffer, pH 7.4 containing 0.5 M NaCl and a 19-mer complementary DNA fragments (target) at concentrations ranging from 0.05 to 40 μM. Then the electrode was washed for 15 min in a buffer and rinsed for 5 min at +0.5 V to phosphate buffer containing 38.5 μM $Co(bpy)_3^{3+}$. This step was avoided when hybridization was detected by guanine signal. After that the electrode was washed and immersed to blank phosphate buffer where finally a square wave voltammetric scan was recorded. The same reaction was performed with the use of noncomplementary DNA fragments at the concentration of 5 μM.

3. Results and discussion

Detection of complementary DNA fragments specific to *bar* gene was performed by electrochemical biosensor. A recognition layer was the surface of a carbon paste electrode with covalently immobilized 19-mer oligonucleotide as a probe. Detection of target DNA was accomplished after hybridization by measuring electrochemical signal of two electroactive agents: bipyridyl cobalt(III) complex interacting with DNA and guanine, electroactive component of DNA. Electrochemical signal (SWV) of $Co(bpy)_3^{3+}$ was expected to be higher after hybridization because it binds more strongly to DNA duplex than to single-stranded DNA leading to an increase of indicator surface concentration [3,5,6].

Electrode surface recognition layer of a biosensor was prepared by immobilization of DNA probe via ethylenediamine to stearic acid modified carbon paste electrode as previously described [29]. Optimal stearic acid concentration in the outer layer of CPE was established for 5% (w/w). Carboxyl groups of stearic acid were activated by water-soluble carbodiimide 1-(3-dimethylaminopropyl)-3-ethyl-carbodiimide (EDC) and *N*-hydroxy-sulfosuccinimide (NHS) and then substituted in nucleophilic substitution reaction with the amine groups of ethylenediamine to form finally an amide linkage. According to our previously reported protocol this step lasted only 5 min and the concentration of ethylenediamine was 10 mM [29]. However, further investigation brought us about revision of some earlier findings. Increasing ethylenediamine concentration and also extending the time of ethylenediamine reaction with carboxyl groups on carbon paste electrode positively influenced the efficiency of DNA probe immobilization. The relationship was examined by the modification of CPE according to procedure described in Materials and methods, whereat ethylenediamine concentration ranged from 10 to 80 mM and reaction time of ethylenediamine with an electrode varied from 5 to 70 min. In these experiments a simple 15-mer oligonucleotide (oligoG) was immobilized on modified carbon paste electrodes and its immobilization efficiency was estimated by measuring guanine signal (Figs. 1 and 2).

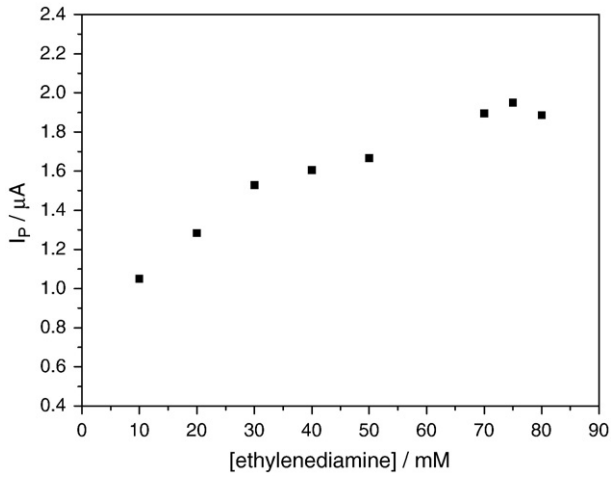

Fig. 1. Effect of ethylenediamine concentration on 5 µM 15-mer oligoG immobilization efficiency expressed as guanine peak height. Time of ethylenediamine and oligoG binding was 15 min. As activators 5 mM EDC and 8 mM NHS were used. SWV conditions: frequency of 100 Hz, amplitude of 0.04 V, step potential of 0.015 V in 0.05 M phosphate buffer (pH 7.0).

Guanine signal of an electrode was steadily raised when ethylenediamine concentration increased up to 75 mM and then remained rather stable (Fig. 1). It was concluded from these experiments that 75 mM concentration of ethylenediamine for carbon paste activation provided the highest DNA immobilization efficiency and this concentration was used in further experiments. Similarly, effect of ethylenediamine reaction time was examined and it was found that 15 min time resulted in the highest oligonucleotide immobilization efficiency (Fig. 2).

In conclusion, the amount of oligonucleotide immobilized on the carbon paste electrode increased by 30% when ethylenediamine concentration rose from 10 to 75 mM and reaction time was extended from 5 to 15 min. Applying these new parameters optimal oligonucleotide concentration for DNA layer construction was established. Results presented on Fig. 3 show that the highest response of the DNA modified electrode expressed as guanine signal was found in case of 10–12 µM oligonucleotide concentration used for immobilization.

Carbon paste electrode modified according to presented procedure and supplied with appropriate DNA probe can be used as a hybridization biosensor for detection of target DNA. Experimental conditions for hybridization described in the literature are very

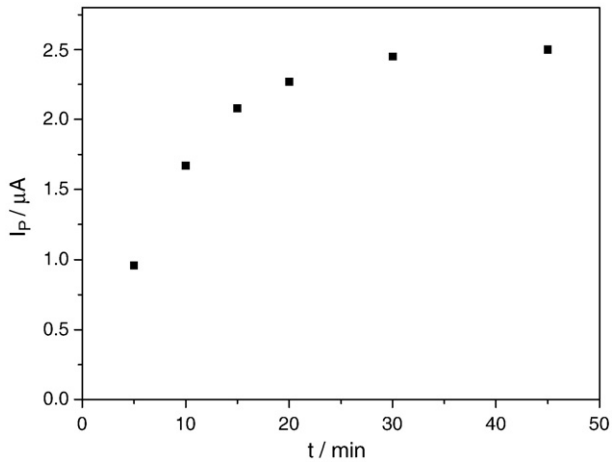

Fig. 2. Effect of 75 mM ethylenediamine reaction time on 5 µM 15-mer oligoG immobilization efficiency expressed as guanine peak height. Time of oligoG bonding was 15 min. As activators 5 mM EDC and 8 mM NHS were used. SWV conditions as on Fig. 1.

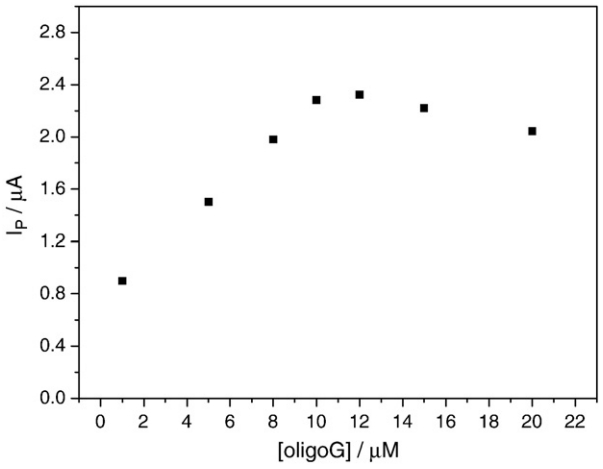

Fig. 3. Effect of 15-mer oligoG concentration on its relative immobilization efficiency expressed as guanine peak height. Time of oligoG bonding was 15 min. As activators 5 mM EDC and 8 mM NHS were used. SWV conditions as on Fig. 1.

differentiated and its duration generally varied from 2 min to 1 h [5,11,25]. Millan et al. [12] suggests that reaction of complementary fragments association in the high salt concentration runs at maximum rate in the first 10 min only. Peterson et al. [25] reported that at low probe density hybridization reaction reached maximum already after 15 min. In the series of experiments using again oligonucleotide oligoC as a probe and oligoG as a target an optimal hybridization time was established (Fig. 4). Process of hybridization should last long enough to provide low detection limit. On the other hand, too long reaction can bring about non specific hybridization.

Results presented on Fig. 4 show that guanine signal rose dynamically up to 15 min of hybridization reaction and after that growth rate was significantly lower. Therefore hybridization time 15 min was assumed as optimal and it was used in further experiments.

Detection of *bar* gene encoding phophinothricin herbicide was performed using developed electrochemical biosensor with a carbon paste electrode modified with stearic acid and ethylenediamine as described above. The recognition element was ssDNA probe attached covalently to ethylenediamine. The probe was 19-mer oligonucleotide complementary to *bar* gene. Hybridization was detected by bipirydyl cobalt (III) complex. Reproducibility of voltammetric signals was very much influenced by a time of electrode washing after interaction with $Co(bpy)_3^{3+}$ (Fig. 5).

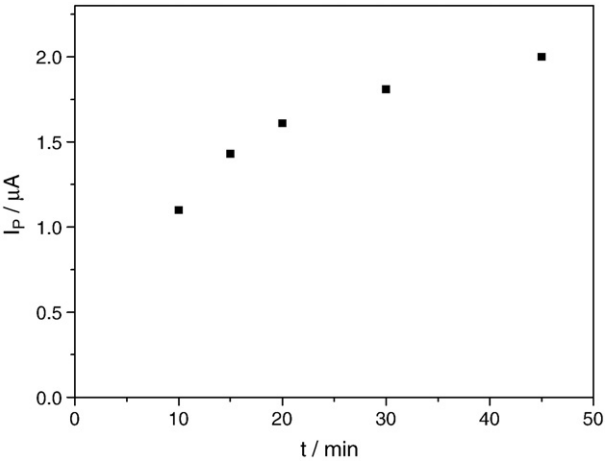

Fig. 4. Effect of hybridization time on the guanine peak height after hybridization performed between 15-mer oligoC immobilized on the electrode and 15-mer oligoG in solution. Oligonucleotides concentration was 10 µM. SWV conditions as on Fig. 1.

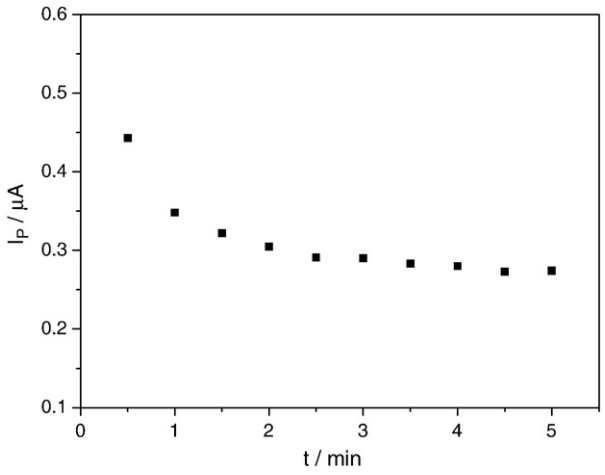

Fig. 5. Relationship between SWV signals of 38.5 µM $Co(bpy)_3^{3+}$ and washing time of the DNA modified CPE after hybridization between DNA probe specific for *bar* gene immobilized on the electrode and complementary DNA fragment in solution at the concentration of 3 µM. SWV conditions as on Fig. 1.

In this work several different electrode washing times in stirred buffer solution were used, i.e. from 30 s to 5 min. Finally, 3 min time of washing was established and this time ensured satisfactory reproducibility although obtained voltammetric signals were slightly lower.

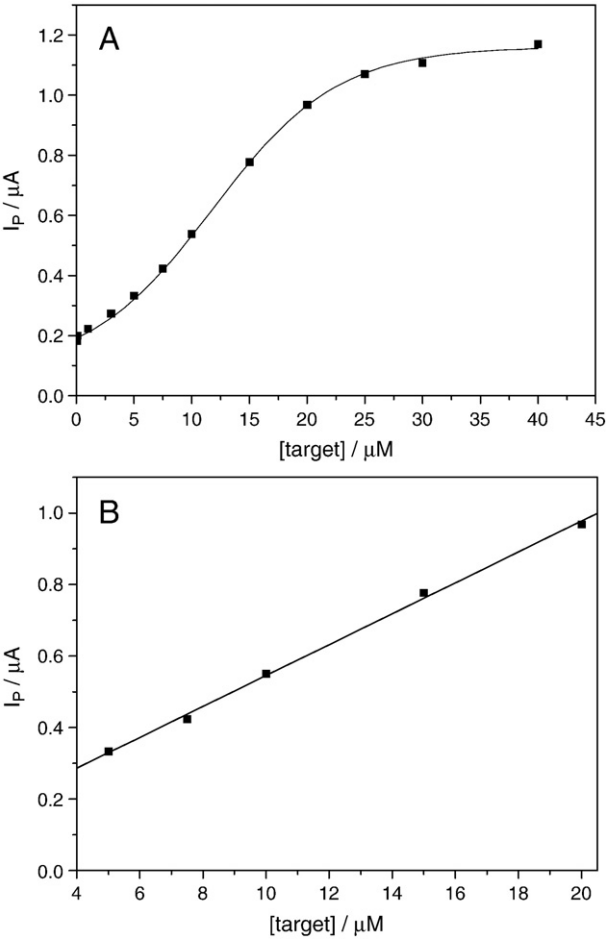

Fig. 6. SWV peak current of 38.5 µM $Co(bpy)_3^{3+}$ (Ep=0.039 V) as a function of different target DNA concentration during hybridization (A). A linear relationship was found in the range from 5 to 20 µM of target concentration (B). SWV conditions as on Fig. 1.

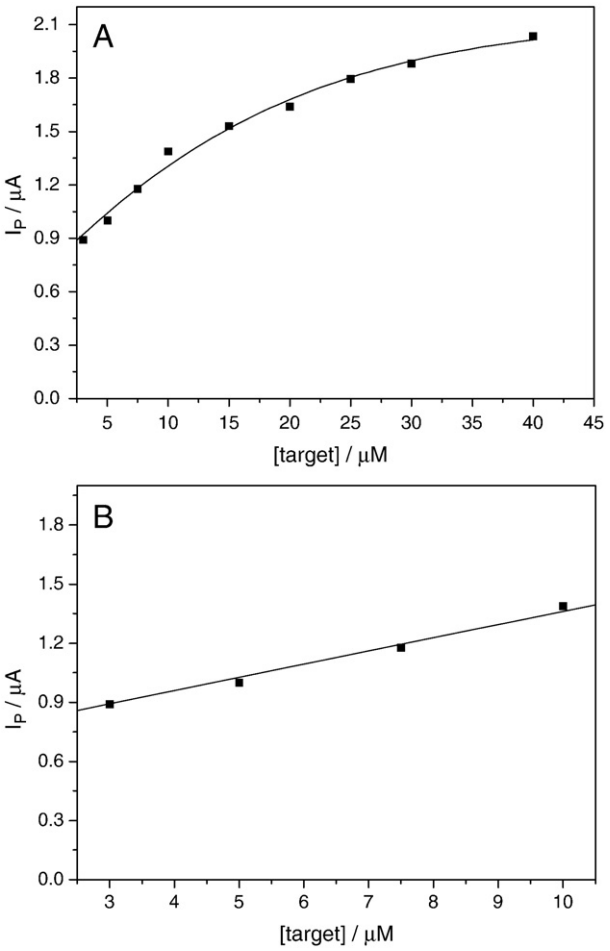

Fig. 7. Relationship between SWV guanine peak current (Ep=1.026 V) and the concentration of target DNA during hybridization (A). A linear relationship was found in the range from 3 to 10 µM of target concentration (B). SWV conditions as on Fig. 1.

Elaborated analytical procedure was used to find a relationship between concentration of DNA fragments specific for *bar* gene and electrochemical signal of $Co(bpy)_3^{3+}$ (Fig. 6A). This relationship presents linearity in the range from 5 to 20 µM concentration of target DNA (Fig. 6B).

$Co(bpy)_3^{3+}$ binds not only to dsDNA but also to single-stranded DNA therefore detection of target at very low concentration was quite limited. Detection limit for target DNA fragments achieved in these experiments was 0.05 µM. Hybridization event could be also examined by the observation of guanine peak present in SW voltammogram. Relationship between a concentration of DNA fragments specific for *bar* gene and guanine peak current was presented on Fig. 7A.

Detection limit obtained with the use of bipyridyl complex enabled to determine 0.05 µM concentrations of target fragments, whereas voltammetric signals of guanine oxidation achieved for target

Table 1
Peak current and relative standard deviation of $Co(bpy)_3^{3+}$ and guanine signals after hybridization of the probe with target DNA (5 and 20 µM)

	$Co(bpy)_3^{3+}$		Guanine	
Target DNA	Peak current	RSD	Peak current	RSD
(µM)	(µA)	(%)	(µA)	(%)
5	0.336	4	1.005	21
20	0.969	6	1.636	20

Average values of 5 repeated measurements.

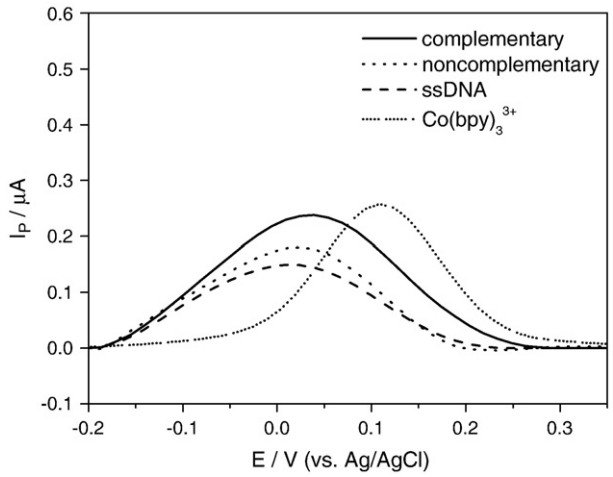

Fig. 8. SW voltammograms of $Co(bpy)_3^{3+}$ (38.5 μM) on bare CPE and after interaction with ssDNA probe, after hybridization with complementary (0.1 μM) and noncomplementary (5 μM) DNA fragments. SWV conditions as on Fig. 1.

concentrations below 1 μM were hardly discriminated. Linear relationship between guanine oxidation peak and target DNA concentrations was found in the range from 3 to 10 μM (Fig. 7B). Data attained for guanine signals were slightly reproducible, values of relative standard deviations exceeded 20% when RSD for $Co(bpy)_3^{3+}$ signals amounted only 4–8%. Some exemplary data of RSD values obtained in repeated measurements are presented in Table 1.

Control experiments were performed with the use of noncomplementary DNA fragments in the hybridization mixture (Fig. 8).

Obtained data showed that in the presence of noncomplementary DNA fragments in a hybridization mixture resulted $Co(bpy)_3^{3+}$ signals were slightly higher than those obtained for ssDNA probe immobilized on an electrode. This was also observed by some other authors. Ozkan et al. [30] applied methylene blue as a hybridization indicator and they also obtained some small differences between signals of noncomplementary DNA fragments and ssDNA probes. Also Wang and co-workers [31] attained similar results. Some increase of a signal probably results from nonspecific interaction between a probe and noncomplementary DNA fragment, however, it is difficult to elucidate this phenomenon. In our experiments in a hybridization solution the concentration of noncomplementary fragments was 5 μM and obtained signals were significantly smaller than for complementary fragments at the concentration of 0.1 μM (Fig. 8). And this concentration was assumed as a detection limit of target DNA fragments in analyzed sample with the use of described procedure.

It was also observed (Fig. 8) that a peak potential of $Co(bpy)_3^{3+}$ was shifted negatively from about +100 mV to +39 mV for dsDNA (obtained after hybridization with target) and to +24 mV for ssDNA modified CPE (interaction with probe and also hybridization performed with noncomplementary DNA fragments). The peak potential of $Co(bpy)_3^{3+}$ was shifted negatively after interaction with DNA and this result evidenced an electrostatic interaction of bipyridine complex with DNA via anionic phosphate residues [32]. The difference between a shift of $Co(bpy)_3^{3+}$ SWV peak potential after interaction with ssDNA (both a probe and also noncomplementary DNA fragments) and DNA duplex obtained after hybridization with complementary target could probably indicate slightly different mechanism of interaction of this complex with dsDNA than with ssDNA.

4. Conclusions

The aim of this work was to construct a hybridization biosensor for the detection of DNA fragments specific for *bar* gene coding phosphinothricin resistance. Sensor presented in this work enables rapid detection of specific DNA fragments. Complete time of one analysis including covalent attachment of ethylenediamine (linker) and ssDNA probe to the electrode, hybridization with target and interaction with electroactive indicator was about 70 min.

Carbon paste electrode modified by stearic acid with covalently immobilized ssDNA probe specific for *bar* gene was used for hybridization with complementary fragments. Short DNA immobilization time resulted in active layers with low density DNA probes enabling an easy access of complementary fragments to a sensor. Relationship between SWV signals of $Co(bpy)_3^{3+}$ used as hybridization indicator and a concentration of complementary DNA fragments was found. Hybridization lasting only 15 min led to the detection limit of 0.1 μM targets in the presence of noncomplementary DNA fragments.

Obtained biosensor presented good reproducibility and an easy way of regeneration. Established procedure provides the electrochemical biosensor for simple, rapid, sensitive and reliable detection of specific DNA fragments. It will be used for the determination of real samples isolated from genetically modified plants.

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