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Electrochemical detection of foodborne pathogen *Aeromonas hydrophila* by DNA hybridization biosensor

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ABSTRACT

The paper describes an electrochemical DNA biosensor used for the detection of *Aeromonas hydrophila*. This opportunistic pathogen is recognized as an emerging foodborne hazard and is associated with a variety of virulence factors including production of cytotoxic enterotoxin aerolysin. The genosensor recognition layer was prepared using mixed self-assembled monolayer (SAM) consisting of thiolated single-stranded DNA probe (ssDNA) and diluent molecules – mercaptoalcohol: mercaptoethanol (MCE) or mercaptohexanol (MCH) or mercaptononanol (MCN). The voltammetric examination of double-layer capacitance of biosensor recognition interface supported by chronocoulometric quantitation of DNA present on the electrode surface showed that mixed ssDNA and MCH monolayer revealed the lowest defectiveness. Its double-layer capacitance equaled 4.0 $\mu F\,cm^{-2}$ and ssDNA probe surface coverage reached 8.5×10^{11} molecules cm^{-2} of gold electrode surface. Chronocoulometric quantitation of DNA and square wave voltammetry (SWV) measurements of electroactive indicator, methylene blue (MB) were performed to investigate the influence of hybridization reaction time, concentration of target DNA fragments, and presence of non-complementary DNA on the electrochemical response of genosensor recognition interface. The biosensor enabled distinction between the DNA samples isolated from *A. hydrophila* (present at the concentration of 2.5 $\mu g\,cm^{-3}$) and other microbial DNA.

1. Introduction

The nucleic acid hybridization has become an important approach in molecular diagnosis for the detection and analysis of specific DNA sequences. Its determination plays a significant role in clinical diagnosis, forensic and environmental analyses, and food safety monitoring (de-los-Santos-Alvarez et al., 2004; Odenthal and Gooding, 2007). Following this increasing demand, the DNA hybridization biosensor (also called genosensor) has been subjected to considerable development (Bakker and Qin, 2006). In a typical configuration, the biosensor consists of single-stranded DNA fragments (ssDNA probes) associated with a transducer, which translates the recognition event into a physically measurable signal. Highly selective base-pairing interaction between the probe and target DNA leads to the formation of specific double-stranded hybrid (dsDNA) in the genosensor detection layer (Palecek and Fojta, 2005). The hybridization reaction is then recognized usually by electrochemical, piezoelectric or optical transduction system (Odenthal and Gooding, 2007; Lucarelli et al., 2008). Taking into consideration its high sensitivity, small dimensions, low cost and suitability to miniaturization, the electrochemical approach is one of the most interesting solutions in evolving biosensing techniques (Bakker and Qin, 2006). The hybridization event is detected via direct measurements of electrical response of DNA strands or application of electroactive redox indicator that provides discrimination between ssDNA probes and newly formed dsDNA (de-los-Santos-Alvarez et al., 2004; Dharuman and Hahn, 2008; Lucarelli et al., 2008).

One of the goals important for genosensor development is its application in food safety monitoring (Deisingh and Thompson, 2004). Due to numerous disadvantages of conventional approaches, there is a permanent demand for rapid and reliable alternative methods of foodborne pathogens detection (Brettar and Hofle, 2008). Changes in food processing technologies, patterns of distribution, and eating habits have led to the increase in microbiological hazard associated with the group of so-called "emerging pathogens" (Daskalov, 2006). Among the microorganisms classified in this group there is the gram-negative rod *Aeromonas hydrophila* (EPA, 2006). The significance of this opportunistic pathogen comes mainly from its wide distribution in aquatic environment and foods, psychrophilic nature and ability to produce a broad range of virulence factors (Janda and Abbott, 2010). One of the virulent toxins secreted by the bacterium *A. hydrophila* is aerolysin (Abrami et al., 2000; Singh et al., 2010). Approaches alternative to laborious

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classical methods of effective control of the pathogen in food still require development (EPA, 2006). The procedures based on the use of immunoassays or polymerase chain reaction (PCR) was already proposed (Kingombe et al., 2004; Arora et al., 2006). The nucleic acid-based detection of *A. hydrophila* a DNA piezoelectric biosensor was also presented (Tombelli et al., 2000). Although the detection procedure was simple, the preparation of biosensor detection interface was quite time- and labour-consuming. Biotinylated ssDNA probe was attached on the streptavidin coated gold disk, which was previously modified with thiol/carboxylated dextran. The quartz crystal microbalance (QCM) technique enabled efficient detection of specific DNA fragments but was not able to monitor the mechanism of target DNA adsorption onto the interface. Other electrochemical hybridization biosensors were developed for the detection of nucleic acid fragments specific to pathogen *Cryptosporidium* (Wang et al., 1997) and for identification of a specific DNA sequence from potentially aflatoxigenic *Aspergillus* species (Snevajsova et al., 2010). Both sensors are based on non-covalent immobilization of ssDNA probes in the detection interface, which could be a drawback of presented solutions.

It has been attempted in this work to develop an electrochemical DNA sensor for the detection of *A. hydrophila* in food via identification of nucleic acid fragments typical of the aerolysin gene. The crucial aspect of such biosensor is the construction of the DNA recognition interface (Wong et al., 2005; Odenthal and Gooding, 2007). The main goal is to obtain a recognition layer that will ensure high hybridization efficiency and decrease non-specific adsorption of single-stranded DNA (Dharuman and Hahn, 2008). The effective biosensor detection layer could be obtained by the application of thiolated DNA probes immobilized on the gold electrode surface followed by the adsorption of alkanethiol diluent (Wong et al., 2005). The formed mixed monolayer modifying the electrode surface provides interfacial properties demanded in an efficient DNA hybridization process. Incorporation of mercaptoalcohol molecules (possessing a hydroxyl group at the distal end) repeals non-specific DNA–gold binding and ensures a less defective biosensor detection layer (Steel et al., 1998; Wong et al., 2005).

Equally important to the construction of biosensor detection layer is the choice of the discrimination system for the signal arisen from the DNA hybridization. (de-los-Santos-Alvarez et al., 2004; Bakker and Qin, 2006). Due to their simplicity and sensitivity, various redox molecules are incorporated in the detection of DNA duplex formation. Presented procedures included metallic complexes (Ru, Os, Co or Cd) or organic dyes (Odenthal and Gooding, 2007; Dharuman and Hahn, 2008). Both kinds of the molecules display significantly different electrochemical signals in the presence of ssDNA or dsDNA modified electrode. Methylene blue (MB), an organic dye classified into the phenolthiazine family, is an example of such indicator (Yang et al., 2002). It was used as a reporting element in several electrochemical genosensors already presented in the literature (Tichoniuk et al., 2008; Nasef et al., 2010).

In this study, a DNA hybridization biosensor capable of detecting nucleic acid fragments specific for the foodborne pathogen *A. hydrophila* is reported. The detection layer of the biosensing device based on mixed self-assembled monolayer consisted of thiolated single-stranded DNA probes and mercaptoalcohol molecules. The selection of an optimal diluent from the group of mercaptoethanol, mercaptohexanol and mercaptononanol was performed on the basis of voltammetric examination of double-layer capacitance and chronocoulometric (CC) quantitation of DNA present on the electrode surface. Chronocoulometric procedure using hexaammineruthenium(III) chloride and square wave voltammetry (SWV) measurements of electroactive indicator methylene blue (MB) were applied to investigate the influence of hybridization reaction time, concentration of target DNA fragments and presence of non-complementary DNA on the electrochemical response of genosensor recognition interface. The biosensor was used for the discrimination between ss- and dsDNA, and the voltammetric detection of target nucleic acid fragments.

2. Materials and methods

2.1. Reagents and solutions

2-Mercapto-1-ethanol (MCE; 99% purity), 6-mercapto-1-hexanol (MCH; 97% purity), 9-mercapto-1-nonanol (MCN; 96% purity), methylene blue trihydrate (MB; 98.5% purity), hexaammineruthenium(III) chloride (RuHex; 98% purity) were purchased from Sigma–Aldrich (Poznan, Poland). Reagent grade NaCl, KCl, KH_2PO_4, Na_2HPO_4, H_2SO_4, Tris–HCl, were obtained from Polskie Odczynniki Chemiczne (Gliwice, Poland).

All solutions were prepared using deionized water. The following several buffers and solutions were employed: 50 mM phosphate buffer, pH 7.6 (ssDNA immobilization buffer); 10 mM Tris–HCl buffer, pH 7.4; 10 mM Tris–HCl with 1 M NaCl, pH 7.4 (hybridization buffer); 1 mM KH_2PO_4 (electrolyte solution for double-layer capacitance measurement); 50 mM phosphate buffer with 5 mM KCl, pH 7.6 (electrochemical buffer for square wave voltammetry measurements). Additionally, 12.5 μM MB solution was made in electrochemical buffer for SWV measurement and 2 mM RuHex stock solution was prepared in 10 mM Tris–HCl buffer (pH 7.4).

Oligonucleotides (20-mer probe, 20-mer target DNA, 21-mer non-complementary DNA fragment, and PCR reaction primers) were purchased in Genomed (Warsaw, Poland) and had the following sequences:

thiolated ssDNA probe: 5′ GTGGTGGGCTGGGCGATCAA-*p*-$(CH_2)_3$-SH
target DNA (fragment of *A. hydrophila* aerolisyne gene): 5′ TTGATCGCCCAGCCCACCAC
non-complementary DNA fragment: 5′ GCATGACGTTATTTATGAGAT

Primers for the amplification PCR reaction of *A. hydrophila* aerolysin gene fragment:

forward: 5′CTGCGAGGGTTATCGTTGTG
backward: 5′ GTGTCGCTGTCGTTGATCG

The sequences of ssDNA probe, a target nucleic acid fragment, and both primers were chosen by exploring the National Center for Biotechnology Information (NCBI) data base. The oligonucleotides had been positively verified in respect of high selectivity and specificity to *A. hydrophila* aerolysin gene via Basic Local Alignment Search Tool (BLAST) program. Additionally, all nucleic acid fragments used in the research show no tendency to form a "hairpin" secondary structure and the dimers do not couple with themselves (examined using DNA calculator: http://www.sigma-genosys.com/calc/DNACalc.asp).

All oligonucleotide stock solutions were prepared in sterile water, divided into analytical portions and kept frozen. They were diluted in appropriate buffer before use.

Nucleic acid from *A. hydrophila* and reference bacterial cultures were isolated and purified using Genomic Maxi DNA isolation kit (A&A Biotechnology, Gdynia, Poland) via the ion exchange column. The purity of isolated genomic DNA was examined by measuring absorbance of the diluted samples (at the analytical wavelengths 230, 260 and 280 nm). Stock solutions of isolated bacterial DNA were stored in a freezer. Before interaction with the biosensor detection layer, DNA solutions were diluted in hybridization buffer and denatured in 96 °C for 3 min. After heating, the sample was

rapidly cooled in ice bath for 2 min directly before the hybridization reaction.

2.2. Instrumentation and measurements

All electrochemical measurements were carried out using a potentiostat PGSTAT12 with GPES 4.9 software package (Eco Chemie, Utrecht, The Netherlands). Cyclic voltammetry (CV), square wave voltammetry (SWV) and chronocoulometry (CC) experiments were performed with the three-electrode system consisting of a bare or modified gold working electrode, Ag/AgCl (3 M KCl) reference electrode and a platinum wire counter electrode. Surface of the working electrode (1.2 mm diameter) before modification and/or electrochemical measurements was polished with 1 μm alumina slurry, followed by 0.05 μm alumina, on microcloth pads (Buehler, Lake Bluff, USA). After removal of the polishing slurry from the gold surface and cleaning in ultrasonic bath, the electrode was immersed in an aqueous solution of 0.05 M H_2SO_4 where it was subjected to electrochemical etching by the cycling potential between −0.3 and +1.5 V until reproducible cyclic voltammograms were reached. The electrochemical area of the working electrode was estimated from the reduction of gold oxide on the basis of conversion factor 390 μC cm^{-2} (Trasatti and Petrii, 1991) and equaled 0.064 cm^2 (n = 8, RSD 5%). All calculations involving the electrode surface area were referred to the estimated value.

CV experiments (associated with the double-layer capacitance measurement) were performed in the potential range between 0.0 and +0.7 V, at a sweep rate of 0.1 V s^{-1} and a step potential of 2.4 mV. The electric capacity was obtained by dividing the measured CV charging current by the scan rate and referring it to the electrochemical area of the gold electrode (Finklea, 1996). SWV voltammograms of MB were measured between −0.4 and +0.05 V, at a pulse amplitude of 40 mV, with a potential step of 1 mV and a frequency of 100 Hz. Chronocoulometric measurements were carried out with a pulse period of 500 ms, a pulse width of 0.5 V, and a potential step between +0.1 and −0.4 V.

All potentials were referred to the Ag/AgCl electrode at room temperature. Solutions were degassed with argon for ca. 10 min prior to performing the electrochemical measurements in a 1 ml cylindrical cell.

Results of the voltammetric and chronocoulometric experiments were presented using Origin software, version 8.0 (OriginLab Corp., Northampton, USA). Calculations were made using an average from at least five measurements.

2.3. Procedures

2.3.1. Preparation of the DNA recognition interface

Modification of the working electrode surface relied on the formation of self-assembled monolayer consisting of the thiolated ssDNA probe and/or mercaptoalcohol. The procedure was based on the immersion of the bare gold electrode in immobilization buffer solution containing 1 μM ssDNA probe for 60 min, followed by rinsing with phosphate buffer. An electrode modified with the monolayer of thiolated ssDNA probes (ssDNA/AuE) was obtained. Formation of mixed SAM on the transducer surface resulted from further immersion of the ssDNA/AuE in 10 mM diluent (MCE, MCH or MCN) solution in 10 mM Tris–HCl buffer (pH 7.4) for 30 min, followed by rinsing with the buffer. The procedure resulted in the formation of a mixed monolayer containing both thiolated probe and diluent molecules in the biosensor recognition interface (ssDNA/MCE/AuE, ssDNA/MCH/AuE or ssDNA/MCN/AuE, respectively). Omitting the probe immobilization step, the gold electrode modified with SAM of particular mercaptoalcohol was obtained (MCE/AuE, MCH/AuE and MCN/AuE, respectively). The quality of monolayers formed on the gold surface was examined via CV measurement of double-layer capacitance of interface between the electrode and the electrolyte solution (1 M KH_2PO_4).

2.3.2. Quantitation of DNA

The nucleic acid coverage on the gold electrode surface was calculated from the number of RuHex molecules electrostatically associated with DNA immobilized into the biosensor detection layer. The chronocoulometric technique enabled quantitation of DNA present on gold, which was outlined in the literature (Steel et al., 1998; Wong et al., 2005). In this work, the chronocoulomograms of the DNA immobilized on the electrode surface were measured in 10 mM Tris–HCl buffer (pH 7.4) prior to the exposure of RuHex molecules. In the next step measurements were taken of cationic redox molecules accumulated (during 120 s) on the transducer surface immersed in the Tris–HCl buffer solution (pH 7.4) enriched with RuHex in the concentration range from 0.05 to 2 mM. The charge associated with the amount of RuHex accumulated onto DNA molecules was calculated as the difference in the chronocoulometric intercepts from the Cottrell equation (Steel et al., 1998) in the absence or presence of the redox molecules in the Tris–HCl buffer solution. For further calculation of DNA quantitation only the CC measurements obtained under the complete charge compensation of DNA by RuHex were taken. Estimated DNA surface coverage (Γ_{DNA}) was finally obtained from the relationship

$$\Gamma_{DNA} = \Gamma \left(\frac{z}{m}\right) N_A$$

where Γ was the surface coverage of RuHex, z was the charge of the redox molecule, m was the number of bases in probe DNA, and N_A was Avogadro's number (Steel et al., 1998).

2.3.3. Hybridization and detection of immobilized DNA

The hybridization reaction was performed by immersion of a probe modified gold electrode in hybridization buffer containing denatured target DNA for a defined period of time in room temperature with stirring (200 rpm). The electrode was further transferred into the 50 mM phosphate buffer solution (pH 7.6) to remove the non-bonded nucleic acid.

The results of hybridization reaction were examined via estimation of DNA surface coverage on the electrode surface using chronocoulometric measurements – only in case of oligonucleotide interactions. The hybridization event was detected by SWV measurement of electrochemical response of methylene blue accumulated on the electrode surface. The electrode was immersed for 3 min with stirring (200 rpm) in the 50 mM phosphate buffer (pH 7.6) solution containing 12.5 μM MB and 5 mM KCl. Consequently, the electrode was placed for 1 min in the stirred indicator-free phosphate buffer to remove unbound MB compounds. The electrochemical response of accumulated methylene blue was SWV measured after transferring the working electrode to a cell with the 50 mM phosphate buffer (pH 7.6) solution containing 5 mM KCl.

3. Results and discussion

3.1. Preparation of DNA recognition interface

Electrochemical detection of specific nucleic acid fragments was based on hybridization reaction with ssDNA probe immobilized in the biosensor recognition interface. Efficiency of the hybridization event strongly depends on the preparation of a detection layer. For the purpose of this study, the 20-mer ssDNA probe was modified with a 3′-mercaptopropyl linker group at its 3′-end. The introduction of thiol group to single-stranded oligonucleotides enabled their immobilization on the electrode surface via single-point

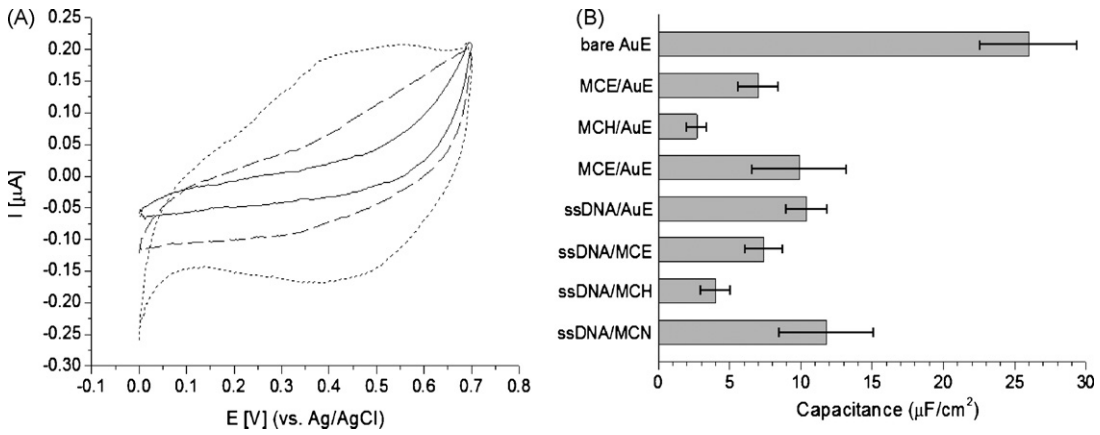

Fig. 1. CV voltammograms of double-layer capacitance measurement for bare gold electrode (···), electrodes modified with ssDNA probe (– – –) and ssDNA/MCH (— — —). (A) Electrochemical capacitance values (horizontal columns) and its standard deviation (RSD, horizontal lines) measured for bare and modified gold electrodes. (B) CV conditions: sweep rate of 0.1 V s^{-1}, step potential of 2.4 mV.

attachment associated with gold and sulfur self-assembling chemistry. Steel et al. (1998) and also Wong et al. (2005) investigated the process of gold electrode modification with the thiol-derivatized ssDNA probes. Their studies showed that introduction of alkanethiol diluent into the DNA recognition interface significantly enhanced the quality of SAM structure and positively influenced effectiveness of the hybridization event. In order to verify the statement, three different diluents – mercaptoethanol (MCE), mercaptohexanol (MCH), and mercaptononanol (MCN) – were used in the modification of gold electrode surface. The ability to form a high quality, self-assembled monolayer on the gold electrode surface was examined both for one-component and two-component mixed SAMs. Interfaces consisting of ssDNA probes (ssDNA/AuE), one of the examined mercaptoalcohols (MCE/AuE, MCH/AuE or MCN/AuE, respectively), or a combination of probe and diluent molecules (ssDNA/MCE, ssDNA/MCH, ssDNA/MCN) were prepared according to the procedure described in Sections 2.3 and 2.3.1. The capacity to construct a SAM monolayer of the thiolated molecules was examined via electrochemical double-layer capacitance measurement in an electrolyte solution (1 M KH_2PO_4) (Fig. 1A and B). The procedure of CV measurements was described in Section 2.2.

Thiolated molecules immobilized on the gold surface led to a significant decrease of capacity currents as a result of the changes in dielectric constant values of particular layers between the electrode and the electrolyte solution (Fig. 1A). Consequently, the measured capacitance for the ssDNA recognition interface (10.4 μF cm^{-2}, n = 6, RSD 14%) was more than two times lower than the value obtained for the bare gold electrode (26.0 μF cm^{-2}, n = 15, RSD 13%). Introduction of the MCH diluent into the ssDNA monolayer led to a further decrease of observed double-layer capacitance to the value of 2.7 μF cm^{-2} (n = 6, RSD 27%).

On the basis of capacitance measurements it could be estimated to what degree the monolayer was penetrated by water or ions. Capacitance of the bare gold electrode lies usually in the range of 20–50 μF cm^{-2}, while the electrode modified with a well-organized alkanethiol monolayer has the electric capacity from 1 to 5 μF cm^{-2} (Finklea, 1996). It could be concluded that the higher the capacitance the more defective the monolayer. Comparing the results of CV measurements obtained for MCE/AuE, MCH/AuE and MCN/AuE, the SAM structure containing mercaptohexanol had the highest quality regarding its defectiveness (Fig. 1B). A similar conclusion resulted from the comparison of double-layer capacitance values for gold electrodes modified with mixed monolayers. The combination of ssDNA probe and mercaptohexanol diluent provided an interface with the lowest capacitance value (4.0 μF cm^2, n = 5, RSD 27%) and therefore the lowest defectiveness in its structure. Similar electric capacity values were also obtained for the well-organized mixed probe-DNA/MCH monolayer in the study of electrochemical quantitation of DNA immobilized on gold (Steel et al., 1998).

In order to check the accessibility of ssDNA probes in the biosensor recognition layer, DNA quantitation via chronocoulometric measurements for electrodes modified with ssDNA/MCE, ssDNA/MCH and ssDNA/MCN was carried out. The procedure for CC experiments was described in Sections 2.2 and 2.3.2. Obtained binding isotherms for hexaaminеruthenium(III) chloride accumulated on gold electrodes modified with ssDNA/MCE and ssDNA/MCN monolayers could not be used for the DNA quantitation. Both interfaces showed defectiveness precluding estimation of adsorbed RuHex concentration and calculation of ssDNA probe coverage on the transducer surface. In the case of a mixed self-assembled monolayer containing mercaptohexanol diluent the saturation of DNA recognition interface with adsorbed hexaaminеruthenium(III) chloride was reached with indicator concentration of 1 mM in 10 mM Tris–HCl buffer (pH 7.4). The charge from the reduction of accumulated RuHex equaled 0.059 μC (n = 10, RSD 41%). DNA probe coverage on the gold electrode modified with ssDNA/MCH reached the value of 8.5×10^{11} molecules cm^{-2} of the gold electrode surface (n = 10, RSD 41%). For further experiments, the recognition layer consisting of the thiolated ssDNA probe and mercapothexanol diluent was chosen.

3.2. Optimization of hybridization time on DNA duplex formation

The biosensor recognition array was applied in experiments on hybridization reaction between the probe and the target DNA fragment. Examined interaction ranged from 5 to 60 min (Table 1). The goal of this experiment was to determine optimal hybridization time ensuring specific binding of the target nucleic acid fragments with the ssDNA probe and minimizing non-specific interaction. The procedures of hybridization reaction and determination of its results (both CC and SWV measurements) were described in Sections 2.3.2 and 2.3.3. Applied in the experiments was the 10 μM target DNA (tDNA) solution in hybridization buffer.

Chronocoulometric measurements of the charge from the reduction RuHex accumulated on the ssDNA/MCH modified electrode surface before and after the interaction with 10 μM target DNA fragments showed that the amount of the indicator in the biosensor detection layer was generally stable already after 15 min of the examined reaction (Table 1A). This time was sufficient to produce a nearly doubled response from the accumulated indicator. Presented chronocoulometric results were collected at the indicator concentration of 1 mM (for 5 min interaction) and 1.5 mM (for

Table 1
Results of CC experiments (A) and SWV measurements (B) for ssDNA/MCH modified electrodes before and after interaction with target tDNA fragment for 5, 15, 30 or 60 min in hybridization buffer solution.

	Time of interaction ssDNA probe and tDNA				
	No interaction	5 min interaction	15 min interaction	30 min interaction	60 min interaction
(A) CC measurements					
The charge from the reduction of RuHex [μC]	0.059	0.093	0.109	0.106	0.111
(Number of measurements /RSD [%])	(10/41)	(8/41)	(8/27)	(8/26)	(10/46)
(B) SWV measurements					
The peak signal from the redox reaction of MB [μA]	1.12	0.86	0.83	0.86	0.97
(Number of measurements /RSD [%])	(5/14)	(9/43)	(9/20)	(5/35)	(5/28)

longer interaction times). Variability of obtained results showed that both too short (for example 5 min) and prolonged (1 h) time of hybridization led to lower repeatability of RuHex accumulation outcomes. The 15 min interaction between the probe and tDNA fragments produced the signal of indicator reduction equaled 0.109 μC. Hence, the calculated accumulation rate of DNA molecules was 15.9×10^{11} on the electrode surface ($n = 8$, RSD 27%).

Application of the voltammetric measurement of MB accumulated in the biosensor detection layer before and after the interaction of the probe with target DNA indicated that the 15-min reaction time provided the highest efficiency of DNA duplex formation (Table 1B). Methylene blue exhibited preferential binding features to free guanine bases in a nucleic acid strand and provided a higher accumulation rate (and therefore a more intensive redox signal) on the surface of ssDNA modified electrodes than in the presence of dsDNA, 1.12 μA ($n = 5$, RSD 14%) and 0.83 μA ($n = 9$, RSD 20%), respectively. The repeatability of MB voltammetric response was also satisfying in this case. The results of both SWV measurements and chronocoulometric experiments predestined the 15-min hybridization procedure to further DNA biosensor applications.

3.3. DNA recognition interface

The biosensor recognition interface was examined in relation to the changes in the target DNA concentration and the presence of non-complementary nucleic acid fragments in hybridization solution. The procedure of the interaction between the probe and oligonucleotides (1, 5 and 10 μM complementary tDNA, and 10 μM non-complementary ntDNA), and determination of this reaction results (both via CC and SWV measurements) were described in Sections 2.3.2 and 2.3.3. A convenient interpretation of some SWV results was provided by the conversion of the signal of MB accumulated on the electrode surface after hybridization procedure into a percentage reduction rate of results measured for the ssDNA/MCH modified electrode (Fig. 2A).

Accumulation rate of methylene blue in the biosensor detection interface (revealed in SWV measurements of indicator redox signal) showed strong correlation between the concentration of target tDNA in hybridization solution (in range from 1 to 10 μM) and the decrease of MB signal after formation of DNA duplex on the electrode surface (Fig. 2A). The linear correlation coefficient for this relationship reached the value of 0.99. The voltammetric MB signal was decreased the most significantly for the tDNA concentration of 10 μM (reduction by 28%) and was equal to 0.83 μA ($n = 5$, RSD 20%). The results of chronocoulometric measurements also confirmed this tendency. The amount of DNA accumulated on the gold electrode after the hybridization reaction increased proportionally to the concentration of tDNA in hybridization buffer. For example, the estimated DNA concentration was 11.7×10^{11} molecules cm^{-2} of gold electrode surface ($n = 10$, RSD 32%) for the 5 μM tDNA solution, and equaled 15.9×10^{11} molecules cm^{-2} ($n = 8$, RSD 27%) in case of 10 μM tDNA concentration. The referred piezoelectric biosensor (Tombelli et al., 2000) had the lowest detected concentration of target DNA fragments, which was slightly lower than the presented biosensing assay. On the other hand, the application of electrochemical tools (chronocoulometric DNA quantitation and SWV voltammetry of MB) enabled precise monitoring of the form of DNA accumulated in the detection interface, which was limited with the QCM technique. Additionally, experiments performed with the hybridization solution containing 10 μM non-complementary ntDNA fragments showed that the biosensor recognition system revealed nearly the same voltammetric signal of accumulated MB – 1.17 μA ($n = 5$, RSD 28%) in comparison with 1.12 μA ($n = 5$, RSD 14%) for the ssDNA/MCH modified gold electrode (Fig. 2B). Chronocoulometric examination of DNA sur-

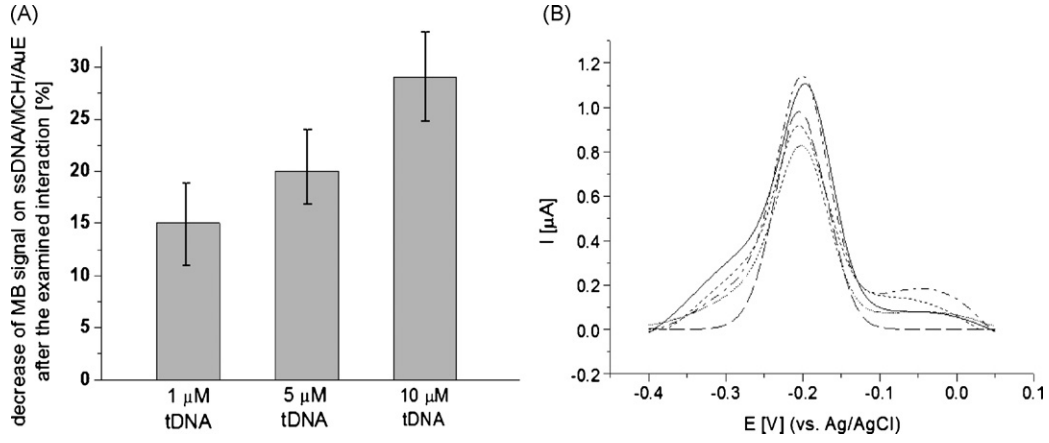

Fig. 2. Results of SWV measurements of methylene blue (bars) and its RSD (lines) obtained on ssDNA/MCH modified gold electrode after hybridization with target tDNA fragments (in the concentration of 1, 5 or 10 μM) presented as a percentage reduction of MB signal obtained for ssDNA/MCH modified electrode (A). SW voltammograms of MB (12.5 μM) on ssDNA/MCH modified electrode (— — —), and after hybridization with target tDNA (1 μM, 5 μM or 10 μM) (– – –, - - - and ···, respectively), or interaction with 10 μM non-complementary ntDNA (– – – – – –) (B). SWV conditions: pulse amplitude of 40 mV, potential step of 1 mV, frequency of 100 Hz.

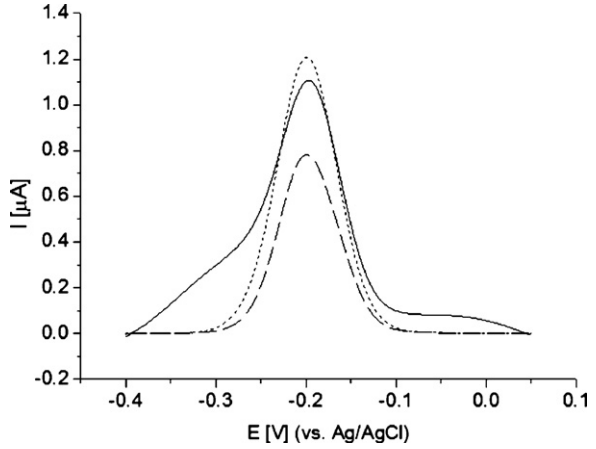

Fig. 3. SW voltammograms of MB (12.5 μM) on ssDNA/MCH modified electrode (———), after hybridization with DNA isolated from *A. hydrophila* (2.5 μg cm^{-3}) (– – –) or interaction with reference bacterial DNA sample (4 μg cm^{-3}) (···). SWV conditions as in Fig. 2B.

face concentration after the interaction with 10 μM ntDNA gave the result value of 11.5×10^7 molecules cm^{-2}. This increase was associated with non-specific accumulation of non-complementary oligonucleotides on the electrode surface and, considering the results of SW voltammetric experiments for ntDNA, it could be concluded that the biosensor detection interface was not significantly susceptible to the influence of non-complementary nucleic acid fragments.

3.4. Detection of A. hydrophila

DNA hybridization biosensor was applied for the detection of nucleic acid fragments specific for the *A. hydrophila* aerolysin gene (Fig. 3). The hybridization reaction was performed according to the procedure described in Section 2.3.3. The hybridization solution contained nucleic acid fragments in the concentration of 2.5 μg cm^{-3} isolated from *A. hydrophila* bacteria and amplified via the polymerase chain reaction (PCR). In a reference experiment, DNA isolated from other bacterial microorganism (*Propionibacterium jenseni*) was applied (in the concentration of 4.0 μg cm^{-3}). In both cases the results of interaction with ssDNA probe were assessed using SW voltammetric measurement of MB indicator according to the procedure described in Section 2.3.

The voltammetric measurements of redox signal of methylene blue accumulated in the biosensor detection layer enabled discrimination between the analytical response obtained in the presence and absence of target nucleic acid fragments in examined DNA samples. The MB peak height measured on the electrode immersed in a solution containing nucleic acid isolated from *A. hydrophila* was significantly reduced to the value of 0.78 μA ($n = 5$, RSD 8%) in comparison to the results obtained on the ssDNA/MCH modified electrode (1.12 μA, $n = 5$, RSD 14%). Newly formed DNA duplexes decreased the rate of indicator accumulation in the biosensor detection interface. The reference experiment showed that MB redox signal was slightly higher than the results obtained for the ssDNA/MCH modified electrode and reached the value of 1.25 μA ($n = 5$, RSD 21%). Nucleic acid isolated from other than *A. hydrophila* bacteria neither underwent the hybridization reaction nor attached non-specifically to the detection layer. Comparing the biosensing protocols for identification of PCR products specific to pathogenic microorganisms, the presented detection interface was a much less complicated preparation approach than the referred piezoelectric device (Tombelli et al., 2000), and a more controllable construction than the sensors designed for the pathogenic *Aspergillus* species (Snevajsova et al., 2010) or *Cryptosporidium* bacteria (Wang et al., 1997).

4. Conclusions

The aim of this study was to construct a hybridization biosensor for the detection of *A. hydrophila*. The presented genosensor enabled discrimination between the DNA specific one for the aerolysin gene of *A. hydrophila* and the DNA samples isolated from other bacterial microorganisms. The self-assembled monolayer of thiolated ssDNA probes and diluent molecules provided stable biosensing interface characterized by low defectiveness and good accessibility of immobilized ssDNA probes. It was found that mercaptohexanol was the diluent compound (in comparison with other tested mercaptoalcohols) most suitable to chemical modification of the gold electrode surface. Detection of specific nucleic acid fragments was performed with a simple and reliable hybridization monitoring system based on the electroactive indicator of methylene blue. The optimal hybridization reaction time of 15 min was determined by the identification of the DNA duplex on the recognition interface. The biosensor analytical response was adequate to the concentration of target DNA fragments in the hybridization solution and was not interfered with by the presence of non-complementary nucleic acid fragments. Establishing the procedure provided promising features towards the development of a DNA hybridization biosensor for the detection of foodborne pathogens.

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