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Electrochemical DNA biosensor for the detection of pathogenic bacteria *Aeromonas hydrophila*

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ABSTRACT

Two electrochemical biosensors for the detection of bacteria *Aeromonas hydrophila* are presented. *A. hydrophila* is a foodborne human pathogen of emerging importance, very often isolated from a variety of food products. Pathogenicity of *A. hydrophila* is associated with the presence of aerolysin gene (*aer*A) therefore specific and effective tool for its detection is a DNA probe complementary to this gene. Such DNA probe at the sequence 5′ GTCAAGACGGTGGTGGGCTG was designed and used as a sensing element in the presented biosensors. The detection layer of biosensor I was a gold electrode covered with self-assembled monolayer (SAM) consisting of mercaptohexanol and thiolated DNA probe. The detection layer of biosensor II was a carbon paste electrode (CPE) modified with multi-walled carbon nanotubes (MWCNTs) containing covalently immobilized DNA probe. The composition of biosensors detection layers, a way of probe immobilization as well as all parameters influencing hybridization event including preparation of target DNA samples, contamination of non-complementary DNA were carefully investigated. Several electroactive hybridization indicators were examined with both detection layers and two of them were selected for the final determinations: Hoechst 33258 (biosensor I) and daunomycin (biosensor II). Upon hybridization of DNA probes immobilized in the detection layers of biosensors with the target DNA isolated from *A. hydrophila* peak currents were found to increase by 75–135% with the use of biosensor I and 34–92% with biosensor II. Both biosensors were used successfully for the detection of pathogenic strains of *A. hydrophila* in food samples (fishes and vegetables) and it was established that the optimal DNA concentration in analyzed samples for the effective analysis was 2.0 $\mu g\,cm^{-3}$ (biosensor I) and 0.8 $\mu g\,cm^{-3}$ (biosensor II). All results obtained with both biosensors were correlated with results made by polymerase chain reaction (PCR).

1. Introduction

Bacterial contamination of food is one of the major problems of public health both in developed and developing countries. Rapid and sensitive methods of microorganisms' detection and identification are of crucial importance for the food safety. The most common bacteria that cause foodborne diseases are *Campylobacter jejuni*, *Salmonella* spp., *Listeria monocytogenes*. However, referring to the WHO report in epidemiological studies the opportunistic pathogens belonging to the species of *Aeromonas*, *Pseudomonas* and *Mycobacterium* are detected much more often from patients, than those commonly recognized as dangerous [1–3]. *A. hydrophila*, the species of bacteria of the genus *Aeromonas*, receives increasing attention because of its association with human diseases and foodborne infections [3–5]. These bacteria produce several extracellular toxins responsible for the pathogenesis, but the greatest importance is given to aerolysin, cytolytic enterotoxin first described by Bernheimer and Avigad [6]. Aerolysin is soluble, thermolabile single protein molecule with a molecular mass of about 49–52 kDa [3,5]. The gene encoding aerolysin was designated as *aer*A [7]. The sequence of 2346 base pairs (bp) of the aerolysin gene from *A. hydrophila* was published in 1987 [8]. The longest open reading frame extends from 532 to 1989 bp.

The recognition of *aer*A gene fragment can be performed by polymerase chain reaction (PCR). The first scientific work on the detection of aerolysin gene from *A. hydrophila* by PCR was published in 1990 [9]. In the next years there have been subsequent more publications on the application of PCR to detection of this virulence factor. The PCR techniques were applied for *A. hydrophila*

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recognition in many food products like ice-stored freshwater fish [10], processed and ready-to-eat retail food [11] or in a raw milk [12]. Some authors also proposed other methods for the detection of *A. hydrophila*. Arora with coworkers [13] compared the PCR technique (targeting aerolysin gene), outer membrane protein (OMP) based indirect plate ELISA protocol and conventional cultural method for their ability to detect pathogenic strains of *A. hydrophila* isolated from chicken and milk samples. The results of bacteria detection obtained with conventional attitude and PCR approach demonstrated very good comparability. There was also an attempt to identify in PCR reaction products the nucleic acid fragments specific to pathogenic strains of *A. hydrophila* using a microgravimetric (QCM, quartz crystal microbalance) biosensor [14]. Target *aer*A gene fragment were recognized via its hybridization in the sensors detection layer. The detection procedure was quite simple but the preparation of the sensor was complicated. Biotinylated ssDNA probes were immobilized on the streptavidin coated gold surface of a quartz crystal, which was previously modified with thiol and dextran layer. QCM measurements revealed the presence of aerolysin specific nucleic acid fragments in analyzed DNA samples. On the other hand the mechanism of target nucleic acid adsorption in the biosensor detection layer was not verified. Although only complementary DNA fragments were adsorbed on gold surface, the formation of double stranded nucleic acid fragments as a result of hybridization reaction was not confirmed.

DNA hybridization is one of the most important techniques in molecular biology for the detection and analysis of nucleic acids sequences. Specific nucleic acids sequences were detected by single stranded DNA probes complementary to the target DNA. The DNA probes, as a recognition element, were also applied for electrochemical DNA sensor construction [15–17]. The probe was immobilized on the surface of working electrode using different techniques of sorption or covalent attachments. Immobilization of nucleic acids by covalent bonding provided more stable detection layer preventing nucleic acids desorption and resulted in a higher efficiency of the hybridization because of structural flexibility and orientation control of nucleic acids chains [15–19]. The probe immobilized on the electrode surface was able to form double-stranded hybrid with its complementary nucleic acid. The hybridization event was commonly detected by electroactive redox indicators that enable discrimination between single-stranded and double-stranded DNA, newly formed on the electrode surface [15,17,19]. In this analytical approach metal complexes (Ru, Os, Co) – chelated with 2,2′-bipyridyl or 1,7-phenanthroline derivative, daunomycin and methylene blue were frequently exploited.

The most often applied working electrode materials in electrochemical DNA biosensors are gold and carbon paste. Gold electrodes are especially useful thanks to its very good electrical properties (e.g. fast electron transfer important in many oxidation–reduction systems) and great ability to its surface modification with different self-assembled monolayer (SAM) structures [20]. Adequately chosen chemical compounds can adsorb via chemisorption to modified surface and organize (merely by non-covalent interaction between each other) on the electrode surface providing well-defined organic surface with desirable chemical functionalities [21]. The most popular self assembly approach is chemisorption of compounds containing thiol or disulfide group(s) on gold surface. Immobilization of ssDNA probes on gold electrode with SAM monolayer was performed for example by former modification of gold surface with self-assembled monolayer of thiolated compounds with accessible ligands that further covalently bonded with nucleic acid fragments [21,22]. The more convenient approach was direct immobilization of ssDNA probes equipped with mercaptoalkyl linker on its 5′ or 3′-end followed by incorporation into formed SAM monolayer other compounds (e.g. mercaptoalcohols) that provided advisable construction of hybridization biosensor detection layer and prevented non-specific adsorption of nucleic acids [23,24].

A carbon paste consists of a mixture of graphite powder and organic binder, which ensures its stability in an aqueous environment. The electrodes prepared from carbon paste show a low background current and a wide potential window. Additional advantages such as easy way of modification, renewability and low cost make them the frequently used material for the construction of working electrodes [25]. In order to obtain a stable detection layer on the surface of carbon paste electrode the various techniques of covalent attachment of DNA probes were used. The DNA strands commonly were bounded via amino groups of the guanine or one of the ends (5′ or 3′) to the electrode surface modified by active groups [15,26–28]. Immobilization via carboxylic or amino groups seems to be a more interesting coupling method [15,27,28]. Millan and Mikkelsen [27] reported the electrode modification by carboxylic groups. Carbon paste was modified by stearic acid and immobilization was performed in the presence of carbodiimide derivatives making amide bonds between the DNA and electrode surface. However, immobilization process went through deoxyguanosine (dG) residues and only ssDNA enriched with repetitive guanine bases could be attached to the carbon surface. In our previous work [29,30] the method of covalent attachment of DNA probe to the electrode surface through a linker – ethylenediamine was elaborated. In this method the –COOH groups originated also from stearic acid, but the use of the linker allowed a longer distance between the probe and electrode surface, which protected the electrode surface before direct adsorption of the DNA probes in the paste and allowed structural flexibility of the DNA fragments, which consequently resulted in hybridization efficiency [15,31].

Continuous development of nanomaterials provide new opportunities to enhance the accuracy, selectivity and sensitivity of DNA biosensors [15,32]. Many of these benefits could be gained by the use of carbon nanotubes (CNTs). They present high conductivity that enhances the electron transfer between the electrode surface and analytes and they might serve as catalysts in increasing the efficiency of electrochemical reactions, mainly because of enlargement of real surface of electrodes [33–35]. According to Bollo et al. [36] the guanine oxidation signal of double stranded calf thymus DNA was 20 times higher at a CNTs modified glassy carbon electrodes cross-linked with glutaraldehyde (GTA) than at a bare GCE using differential pulse voltammetry. Due to enhanced electron transfer, low resistance and very low electric capacitance of carbon nanotubes could significantly increase the sensitivity of electrochemical signal [35,37].

Large majority of DNA biosensors require sample amplification by polymerase chain reaction (PCR), however, detection of specific DNA sequences without PCR seems a much more useful [38]. In this work a DNA electrochemical biosensors for rapid and reliable detection of pathogenic strains of *Aeromonas hydrophila* was presented. Two of the most popular transducer materials in electrochemical biosensors were applied in our experiments. The fundamental part of the first biosensing device (biosensor I) was a gold electrode modified with mixed self-assembled monolayer of ssDNA probes and mercaptohexanol. The second biosensor (II) based on carbon paste electrode modified by multi-walled carbon nanotubes. The biosensors were meant to detect *A. hydrophila* without PCR amplification.

2. Experimental

2.1. Reagents and solutions

Multi-walled carbon nanotubes (MWCNTs) and multi-walled carbon nanotubes functionalized with carboxyl groups (5% functionalization) (MWCNTs-COOH) with the diameter of