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Application of cell-based biosensors for the detection of bacterial elicitor flagellin

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Abstract

Cell-based biosensors, bioelectronic portable devices containing plant living cells have been used for monitoring some physiological changes induced by pathogen-derived signal molecules called flagellin. The screen-printed electrodes have been adapted for preparation of biosensors. The proton-sensitive thick films have been printed using composite bulk modified with edition of RuO_2. Obtained disposable electrodes were made possible to measure the pH change with well sensitivity and reproducibility. Tobacco cells attached to the electrode surface, cell-based biosensor, can be used for the detection of flagellin, the virulence factor of bacterial pathogen. We culture tobacco cells on the surface of such electrotransducer for several weeks and monitor of potential of cells under flagellin stimulation. The detection of the electrochemical proton gradient across the plasma membrane serves as the analytical signal. The electrode response depended upon H^+ concentration in extracellular solution. It can be conveniently observed on the surfaces of biosensors. Suitable stability and the good response time of constructed biosensors were observed. Future development of these cell-based biosensors could draw advances in selective monitoring of microbial pathogens and other physiologically active components. Moreover, this new method is much faster compared with the traditional microbial testing.

Keywords: Cell-based biosensors; Screen-printed electrodes; Bacterial elicitors; Flagellin; Cell–pathogen interaction

1. Introduction

We live in the era of expanding microbial-diagnostic opportunities in which the development of sensitive and specific methods for the detection of pathogen will play a major role in the prevention of human, animals and plants epidemics. Progress in the biosensor technology provides simple, sensitive and economic determination methods for molecular analysis of DNA and proteins [1–3]. Recently, cell-based biosensors have been considered for new application in molecular diagnostics. A variety of cell types, such as bacteria, yeast, plants, immune cells and neurons, have been used previously to fabricate different devices [4,5]. By using living cells as sensitive elements, the biosensors are able to respond to many biological active agents [6]. In addition, such cells are highly sensitive to environmental conditions and this fluctuation can elicit cellular responses contributing to the electronics signals in the biosensor assay [7]. Therefore, the characterization and control of extracellular factor, such as proton gradient across the plasma membrane, is critical in pathologic pathway. Physiological and biochemical studies have suggested that the plasma membrane H^+-ATPase controls this aspect, and can be used for detection of fast response of plant cells to pathogens or pathogen-derived signal molecules called elicitors. Numerous plant and animals species have been known to respond to pathogen attacks through the interaction with bacterial elicitors [8]. A well-characterized elicitor is bacterial flagellin, which induces changes in cellular concentration of H^+ and the accompanying plasma membrane depolarization [9]. The interaction of this molecule with specific receptor is the first step of a complex intracellular signalling pathway that involves the activation of a mitogen-activated protein kinase (MAPK) and the rapid alkalinization of the extracellular matrix of cells. This pathway in plant cells exhibits

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some analogies to the inflammatory response in human and animals' cells [10].

The extracellular alkalization in response to flagellin in tobacco suspension cultures was the basis for the development of new biosensor system that led to identification and characterization of the bacterial pathogen. The main application of this device is a fast analysis of functional response of cells upon receptor stimulation. Furthermore, cellular responses might be usefully employed as parameters to obtain biochemical information for both signalling and biosensing in cells culture.

Recent advances in thick film technology and electronics have accelerated the development of new generation of biosensors with applications in a wide range of disciplines in clinical diagnostics, environmental monitoring and the pathology [11]. Such DNA biosensors and immunosensors can be applied to detection and identification of microbial pathogens [12,13]. Attractive features of this technology include a simple approach, single use sensor and low cost. Recently, the structure of the screen printing electrodes applied to biosensors has been considerably improved [14]. This technology is especially recommended as a simple and fast method for large-scale production of microelectrodes. In addition, this new device can be implemented for a number of applications ranging from neuron culture analysis to molecular genomic and proteomics.

In this study, a suspension of tobacco cells was attached to the screen printed electrodes and used for detection of flagellin isolated from pathogenic bacteria. The cultured cells on such modified electrodes were applied to monitoring flagellin-containing bacteria. Measurement of the electrochemical proton gradient across the plasma membranes serves as the analytical signal. Thus, prepared cell-based biosensor can be useful for detection of purified flagellin or of crude extract of bacteria. The results demonstrate that this kind of device has potential to monitor changes in living cell over a long term and to evaluate bioactive components of pathogens. Therefore, the objective of the presented research has been to develop a rapid procedure for detection of other bacteria or viruses. This design is expected to be used in future for such detection in the automated procedures.

2. Experimental

2.1. Plant and bacterial materials

Wild-type tobacco plants (*Nicotiana tobaccum*) were propagated from seeds for 14–16 days under 17 h of light (300 $\mu Em^{-2}\ s^{-1}$) at 24 °C and 8 h of dark. Tobacco suspension cells were maintained in Murashige and Skoog medium as previously described [15]. *Agrobacterium tumefaciens* and *Escherichia coli* references strains were obtained from the laboratory collection of the Institute of Plant Genetics, PAS. The bacteria strain was grown by shaking in LB broth at 24 °C overnight.

2.2. Apparatus and reagents

The electrodes were produced using a semi-automatic screen-printer Presco, Model 564 (USA). Potentiometric experiments were performed on an analyzer Autolab PGSTAT (Eco Chemie, The Netherlands) with the system made of screen printed electrodes and an Ag/AgCl (1 M KCl) reference electrode (Mineral, Poland). All media for plant cells cultures were from Sigma-Aldrich Co. (USA). Commercially available pastes: silver-based paste (product no. 7145) and protective UV-cured paste (product no. 5018) both from DuPont (USA) was employed for fabrication of conductive circuit and encapsulation, respectively. Graphite-based paste (Electrodag 412 SS) was from Acheson (The Netherlands). Chemicals used for preparation of supporting electrolytes were from Sigma-Aldrich Co. (USA). Other reagents were of analytical grade and were obtained from POCh (Poland). In all experiments, sterile doubly distilled water was used.

2.3. Preparation of screen-printed electrodes and potentiometric measurements

Recently developed procedure for screen printed electrodes, has been adapted for preparation of biosensors. The proton-sensitive thick films were screen-printed using carbon ink based on RuO_2. The optimum composition of the films was 60% of ruthenium dioxide and 40% of matrix material. All pastes were printed through 68 T mesh polyester screens with 20 μm masking. The fabrication process of the pH electrodes consists of three steps of consecutive printing of silver track (internal contact), modified carbon film and protecting layer (insulation) on the polyester substrate in the form of flexible foil. The silver and carbon layers were cured at 130 °C for 5 and 30 min, respectively. Insulating films were cured for 15 s by UV irradiation using Philips HOK 20/100 UV lamp (2000 W). The sensors were produced in 5 cm × 5 cm sheets of 24 electrodes. The size of single electrodes was 4 mm × 24 mm and the working area of the carbon film was 1.5 mm × 1.5 mm. The thickness of the carbon film was around 15–25 μm. The structure of the electrodes' surface was characterized with a scanning electron microscope (SEM). The calibration of the electrodes with the range of H^+ concentration from pH 4–9 was made using a standard solution. All potentiometric measurements were performed in stirred solutions, at room temperature without special thermostating. The results were used for the investigation of the dynamics of the electrode response and for preparation of calibration graphs. The optimalization of the procedure was performed by reading out in buffer solution (1 M Tris–HCl, pH 5.2). The reproducible results with an RSD of less than 5% ($n = 12$) were obtained.

2.4. Cell cultures and alkalization assays

Suspension cells were maintained in no buffered MS medium. Instead, the medium was adjusted to pH 5.6 with KOH. For routine maintenance, 2 ml of a 1-week-old culture was transferred into 50 ml of medium in 250-ml flasks and maintained on an orbital shaker at 100 rpm in dark at 24 °C. A 2-ml aliquot of cells was transferred into each well of 12-well tissue culture plates (Corning, USA) and allowed to equilibrate on an orbital shaker at 100 rpm for 1 h. Fraction (5 μl) purified flagellin or crude bacterial elicitor extract were added to the cells and changes in pH of the medium were measured every 15 s for

20 min using a Elmetron pH meter with pH minielectrode (Elmetron, Poland).

2.5. Flagellin purification

Flagella from *E. coli* or *A. tumefaciens* were prepared as described previously [9]. The cells were collected by centrifugation (5000×*g*, 20 min) and resuspended in 150 mM NaCl (pH 7.0) in 20 ml polypropylene centrifuge tubes. The flagella were detached from the cells by vortexing for 1 min. Cells bodies and cell debris were removed by centrifugation 3 min at 12 000×*g* and flagella were collected by high-speed centrifugation (2 h at 100 000×*g*). The pellet was resuspended in water and the pH was lowered to 2 by glycine–HCl to dissociate the flagella. After removal of insoluble material by centrifugation (1 h at 100 000×*g*), the supernatant containing the dissociated flagellin molecules was adjusted to pH 7.0 with NaOH. The peptides were transferred to a fresh tube and used directly in experiments or stored at −70 °C for several days. Crude bacterial elicitors were prepared by boiling the suspension for 10 min and removing bacteria debris by centrifugation (5 min at 14 000×*g*).

2.6. Biosensors construction

The cell-based biosensors were constructed under sterile conditions by immobilizing tobacco cells at the surface electrodes. Tobacco suspension cells were centrifuged (10 min at 800 rpm) and then the solution was repeatedly blown out of pipette and doubly washed in MS medium. After cell detachment from the liquid culture, the cells at the density (approximately 100 cells/μl) were mixed with a 0.5% (w/v) low melting point agarose solution in no buffering MS medium at 40 °C. In all cases, the solution was transferred into the surface of a screen printed electrode on the working space of 2.25 mm^2. The electrodes containing components of immobilized living cells in gel-matrix volume 10 μl were obtained. Then, the biosensors were kept in sterile condition in polypropylene centrifuge tubes in MS medium (excluding buffer) at pH 5.2 for several days.

2.7. Experiment design

The sample studied (a solution containing 25 μM of purified flagellin or 10 mg/mL crude bacterial elicitors) and blank control solution (H_2O) were applied on the biosensors. In this application, each individual measurement was performed out at room temperature on the set of 10 biosensors. Potentiometric experiments were controlled by the GPES 4.8 software (Fig. 1).

3. Results and discussion

3.1. Screen printed electrode preparation

New screen-printable carbon-epoxy electrodes were developed. To assemble metal oxide electrodes, several experiments were performed in order to obtain reliable electrodes. Good pH-response of this type electrode has been previously observed and electronics transducers based on them have been reported showed high and reproducibly sensitivity [16]]. The reaction mechanisms of these pH electrodes are discussed elsewhere. The carbon-based ink plays the main role of an electric contact and binds the metal oxide. Other authors have used various metal oxides as sensitive materials [17]. Using the electrode fabrication method presented here, good results were obtained with RuO_2. The pH-response and reproducibility were satisfactory and this was the main reason for the use of these electrodes in biosensor construction. The screen-printed devices obtained were characterized by electrochemical methods and the optimal graphite content and RuO_2. The use of commercial carbon inks with various levels of RuO2 (approximately from 20% to 60%) gives similarly sensitive electrodes. Sensitivity of the carbon surfaces was much more stable than that of those based on glass membranes. In addition, the carbon surface electrodes exhibited nearly the same standard potential (mV) and (mV) sensitivity. Thick-film carbon sensors were more readily available and inexpensive compared with glass components of proton-selective membranes. Calibrations were performed without any conditioning of the sensors before use. The

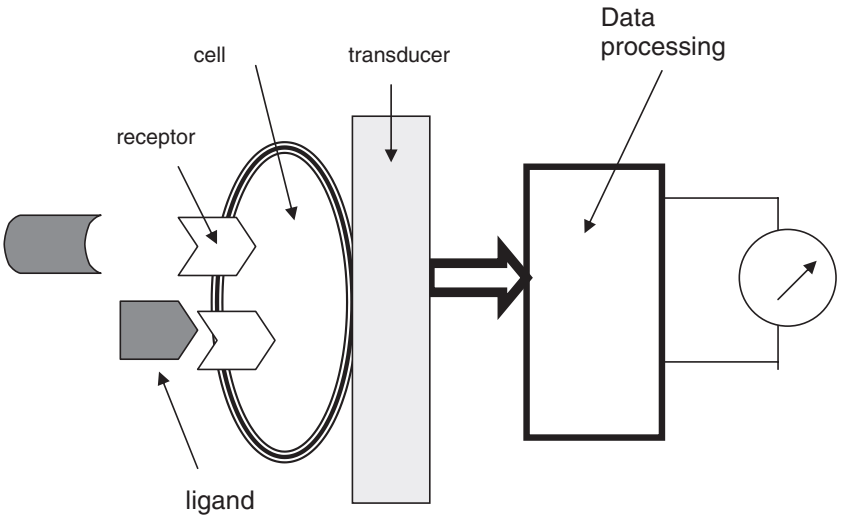

Fig. 1. Scheme of cell-based biosensor used in potentiometric experiments.

RSD obtained for 12 electrodes was in fact lower than 5%. In the case of metal oxide electrodes, a linear, reproducible response with a sensitivity of around 50 mV/pH was obtained (Fig. 2). Moreover, in the course of 2 days immersion of tested electrodes in the plant grow solution (containing living plant cells), no changes in the electrode characteristics were observed. The electrode retained these analytical parameters after several days in wet condition. Good pH-response of these electrodes was previously observed by other authors [16]. The thin-film technology used in this work was characterized by high reproducibly and sensitivity and the electrodes with living cells tested exhibited a fast and linear response to protons in range of 4 to 9 pH. Moreover, the electrodes exhibited nearly the same standard potential and sensitivity (mV) and the drift of the analytical signal was not observed. It was found that the potential of the screen-printed electrode is a linear function of pH in the control buffer solution. The electrodes tested in commercial buffers solution exhibited a fast and linear response to protons in the range of concentrations from 4 to 9 pH. (Fig. 2). The electrodes investigated are useful for construction of cell-based biosensors.

3.2. Cell-based biosensors performances

A basic set of procedures necessary to determine the experimental set-up is introduced here. The practical and theoretical background of immobilization of plant cells was extensively discussed in everywhere [18,19]. The entrapped cells were produced by first mixing a cell suspension with a liquid gel solution. Then the produced solution was transferred onto the surface of the electrodes where the cell growth could start. Immobilization methods in biosensors construction require high cell viability after these procedures. Common immobilization material used in this study was agarose. The concentration of the gel material was from 0.5% to 1%. Solid gel materials were formed on the surface of the electrodes (Fig. 3). Some elements in the design are crucial for the final result. Immobilization of the cell suspension for biosensors requires the thickness of gel-

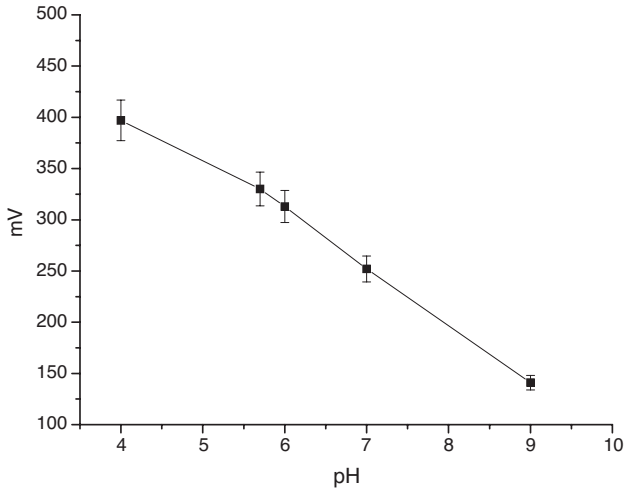

Fig. 2. The electrode tests in range of 4–9 pH. Data are means S.D. (n=12).

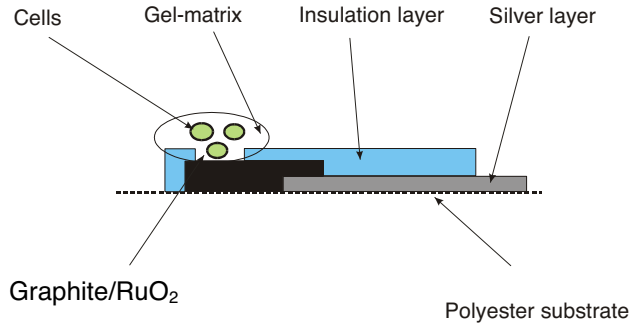

Fig. 3. The scheme of the single cell-based biosensor with gel-matrix.

matrix to be between 0.5 and 2 mm. The microscopic observation confirmed that the tobacco cells were attached to the electrodes' surfaces. To obtain the same total number of cells attached to a given electrode surface, their number was counted under a microscope. The working area of the electrode covered with a approximately 100 cells was 2.5 mm^2. Measurements can be performed at the molecular level. It has also been confirmed that the cell surface characteristics change over the several days of storage (data not shown). This means that the plant cells as particles having stable features during the attachment process start growing on the carbon electrode surface.

3.3. Detection of microbial pathogens by the biosensors

The flagellum is an important virulence factor for bacteria pathogenic to animals and plant [8]. Moreover, many important aspects of plant physiology, including interaction of flagellin with plant cells, seem to be controlled by the proton-pumping ATPase of the plasma membranes. Inactivation of the pump is one of the earliest responses to bacterial flagellin, the resulting external alkalinization (outside of the cell) corresponds to the " peptide-receptor interaction' induced by flagellin, which triggers the cellular response [20]. The well-known extracellular elicitor from pathogenic bacteria used in this study was the

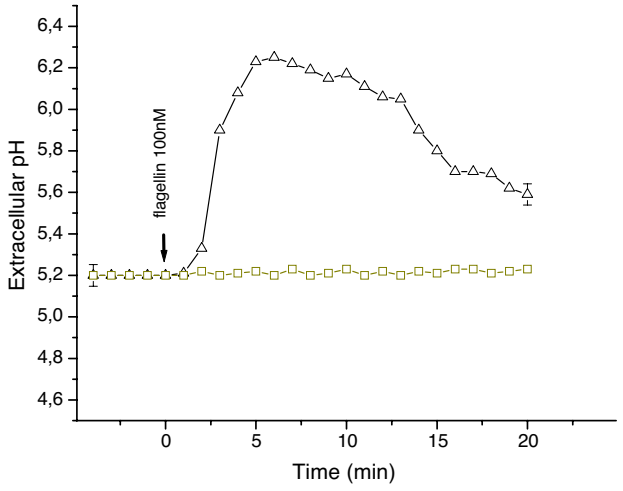

Fig. 4. Extracellular pH alkalinization inducing activity of flagellin isolated from *Agrobacterium tumefaciens* in tobacco cell suspension. Effect of (△) 100 nM flagellin and (□) control (H_2O).

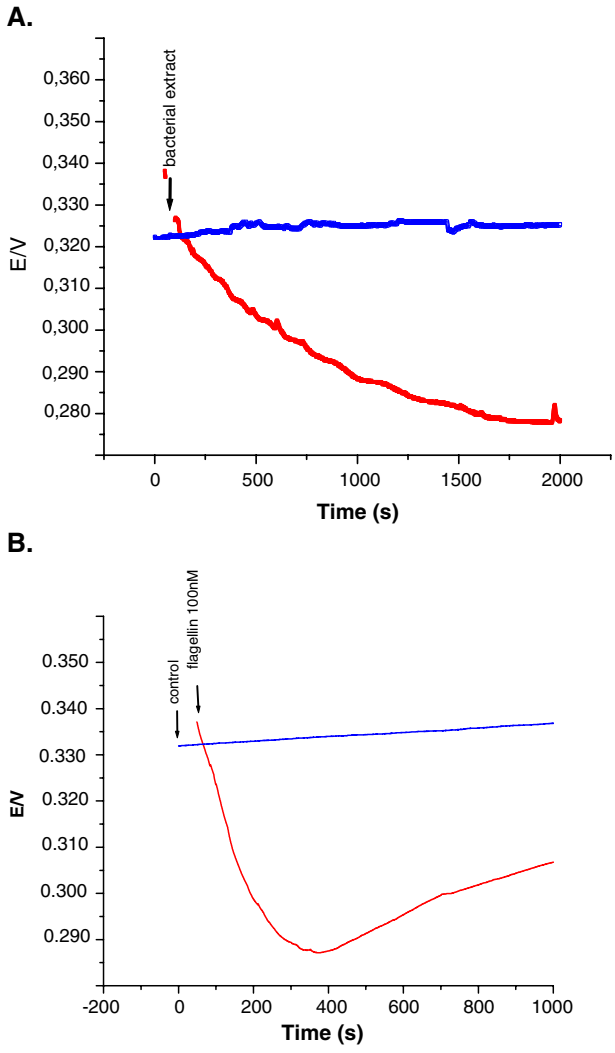

Fig. 5. The cell-based biosensor response to bacterial extract (A) and flagellin (B).

flagellin. Moreover, this peptide is known to elicit a rapid K^+ efflux and concomitant medium alkalization as well as an oxidative burst when added to suspension-cultured plant cells [9]. Tobacco cells used in this study worked as a sensitive and selective chemoreception system for a conserved peptide domain of flagellin. We used medium alkalization to test the effects of flagellin from the bacteria (*E. coli* or *A. tumefaciens*) on suspension-cultured tobacco cells. The alkalization (Δ pH) of the medium begins in the first 5 min in response to the 100 nM flagellin in the range 5.2 to 6.2 pH (Fig. 4). This was similar to the results obtained previously by other authors [9,20]. To further delineate the flagellin determinant perceived by tobacco cells, cell-based biosensors were assayed for activity using purified flagellin and crude bacterial extracts. Cell-based biosensors containing suspension cultures of tobacco show a detectable response to both-flagellin and bacterial extracts (Fig. 5). These results demonstrate that tobacco cells grown on the electrode surface have the highly sensitive perception system for bacterial flagellin (as in culture media). In another experiment it has also been confirmed that the biosensor was sensitive to crude bacterial extract (containing flagellin) but the potential shift was smaller by about 40 mV and the extracellular alkalization was more stable in 30 min (Fig. 5A). The change potential (Δ mV) derived from the cell-based biosensors treated by flagellin was similar to the result obtained for the cells cultured in a liquid medium −50 mV (Fig. 5B). The first and most important reason to use the plant living cells for biosensor construction is to obtain analytical information. In its simplest form, it tells us whether bioactive peptides are present in solution (Fig. 5). Cells with a specific type of receptors can be considered as sensors of ligands, with a sensitivity determined by the binding constant of that receptor/flagellin combination.

4. Conclusions

This paper reports the design of a new device for detection of flagellin employing the specific strain of tobacco cells. The cells have been immobilized on the surfaces of graphite electrodes by gel. The results have shown that plant cells might be immobilized on the electrode surfaces and can interact with the peptide virulent factors from bacteria. Changes in the local pH environment of cell membranes can be detected by potentiometric screen-printed electrodes. Ligand–receptor interactions make it possible to detect the cell response by means of a simple measurement procedure. The suspension cultured tobacco and arabidopsis cells had been earlier used by other authors for flagellin detection. The screen-printed cell sensor presented here is expected o improve the molecular diagnostics in plant pathogen detection. This thin-film technology was shown to be well reproducible and cheap and it can replace more complicated methods used in biotechnology. It is evident from this study that the integrated standard immobilization procedure with electrochemical detection of proton gradient across the plant membrane is less time consuming and more sensitive than the methods usually employed in suspension cultured cells. Additionally, the method proposed needs lower amounts of the samples to be studied and is less time consuming than conventional diagnostics procedures for the detection of *A. tumefaciens*. Finally, the results from this study may lead to the development of rapid and routine tests for monitoring other infectious bacteria. Future work in our laboratory will be focused on direct electrochemical detection of bioactive peptides such as systemin, which bind with a specific receptor and inactivated H^+-ATPase. A wide range of new cell-based biosensors are thus expected to reach the market in the coming years. The detection system using screen-printed electrodes and the simple methods of immobilization of plant cells is hoped to be successfully applied for other bacteria and viruses.

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