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Figure 2

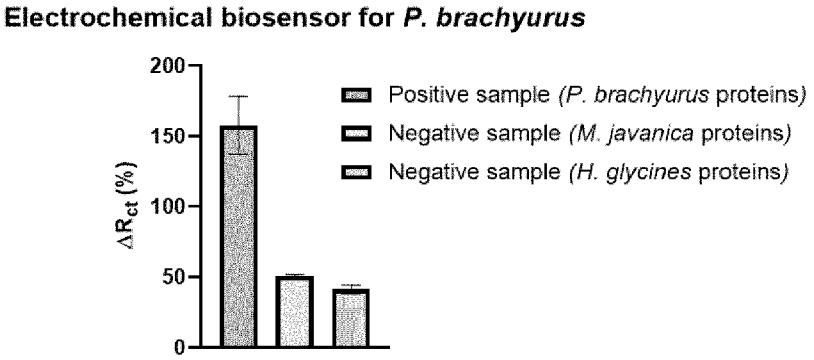

Figure 2 – Bar chart showing the percentage change in impedance of the biosensor constructed for the *P. brachyurus* species. The biosensor was tested with a positive sample (containing the target proteins) and two negative samples (containing proteins from *M. javanica* or *H. glycines* species) .

(57) **Abstract:** The present invention provides a biosensor system comprising one or more electrodes capable of converting a physicochemical signal transduced from a biorecognition layer into an electronic signal; a biorecognition layer comprising biomolecules immobilized onto one or more electrodes capable of binding to the specific analyte of the nematode species as well as a method for detecting simultaneously more than one nematode species.

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Biosensors for the identification of nematode species

Introduction

Plant-parasitic nematodes (PPN) are parasites that in at least one phase of their life cycle use the roots of plants to acquire nutrients. Parasitism can cause stunted growth of plants, root necrosis, and leaf discoloration. Global agricultural losses caused by plant-parasitic nematodes amount to an estimated USD 157 billion annually, significantly impacting the worldwide economy.

The infected fields are mainly treated with crop rotation, where a nematode-resistant cultivar is planted, and with the use of nematicides. However, these strategies are not directed to the treatment of one single species and can be ineffective when the nematode species present in the soil is resistant to the adopted strategy. In addition, the extensive use of nematicides can not only make nematodes resistant but also cause several health problems for the population. Thus, the identification and quantification of nematode species are essential for the adoption of the best strategy and for control to be carried out when the nematode population density is not yet high, avoiding the use of high doses of nematicides.

Identification of nematodes is currently performed using microscopy techniques, in which species differentiation is based on the morphometric characteristics of the specimens, or by molecular analysis using PCR (polymerase chain reaction) for DNA amplification and electrophoresis for the identification of the amplified sequences. These techniques use sophisticated devices that require specialized people to operate them, and detailed steps that confer more time to obtain the results. In this sense, point-of-care devices, such as biosensors, appear as a new tool for the detection of agricultural pests, since the analysis of the sample can be performed in a simpler and faster way.

Biosensors are platforms on which biological molecules, such as enzymes, proteins, and DNA, form a biorecognition layer capable of selectively binding to the molecule to be detected. The interaction between complementary molecules causes physicochemical changes that are converted to a signal measurable by a transducer, such as an electrode. The signal is processed and converted into a physical parameter, e.g. electrical current, capacitance, or impedance. Biosensors can be classified according to the analytical technique used to monitor interactions. Examples are electrochemical, optical, piezoelectric, and electrical biosensors. Electrical and electrochemical biosensors are interesting because they provide fast results, are cost-effective, and can be miniatured for point-of-care detection. These devices have been used in several areas, for example, in the detection of diseases, pesticides, and transgenic food.

Description of the Invention

The present invention provides a biosensor system comprising one or more electrodes capable of converting a physicochemical signal transduced from a biorecognition layer into an electronic signal; a biorecognition layer comprising biomolecules immobilized onto one or more electrodes capable of binding to the specific analyte of the nematode species. The biorecognition layer can be formed by immobilizing biomolecules capable of selective binding to the analyte onto the electrodes. Covalent and non-covalent immobilization methods can be used. For the covalent immobilization of proteins and antibodies on gold surfaces, thiolated molecules such as mercaptopropionic acid (MPA) and 11-mercaptoundecanoic acid (MUA) are typically used. Crosslinking molecules such as carbodiimide (EDC) and N-Hydroxysuccinimide (NHS) also can be used to activate functional groups. To monitor the biorecognition events, impedimetric and capacitive measurements were chosen.

Biosensors using electrical capacitance detection

For capacitive measurements, the non-Faradaic impedance spectroscopy technique is used. This technique consists of applying a potential difference between two metallic plates (electrodes), forming a capacitor. Biosensors operated by this technique are also called capacitive biosensors. The non-Faradaic method is performed using only an electrolyte solution such as KCl and PBS, without redox mediators. In capacitive biosensors, the events on the recognition layer lead to changes in the dielectric constant of the medium (ε) that directly affects the system capacitance, as described in this equation:

$C = \varepsilon\varepsilon_o A/d$

where εo is the vacuum dielectric constant (given by the value 8.85419 pF/m), A is the area of the plates and d is the distance between them. In addition, the geometry of the electrodes affects the detection capacity of these devices, since the greater the εoA/d factor, the greater the change in capacitance. Thus, for the detection of biomolecules, the electrode must have parallel plates very close to each other and a large geometric area. In this sense, interdigitated electrodes (IDE) are the most used for capacitive biosensors. In the typical configuration of an IDE, several metallic plates called fingers are placed in parallel with each pair forming a capacitor. In this configuration, the capacitors are associated in parallel and the total capacitance of the system (C_t) is given by the sum of the individual capacitances (C1, C2, ...) as described by this equation:

$C_t = C1 + C2 + ...$

A capacitive biosensor comprises one or more (preferably two) metallic electrodes which form the capacitor. In one embodiment the electrodes are interdigitated. In this configuration, several metallic plates called fingers are placed in parallel with each pair forming a capacitor.

The capacitive biosensor comprises interdigitated electrodes having at least 10 fingers. The fingers are separated by a distance of 1 to 100 µm.

The electronic signal which may be an electrical current, impedance, or capacitance is analyzed by the data processing device and provided to the user in a numerical or graphical representation, such as graphs or tables. In order to identify the nematode species, multivariate statistical methods can be used, such as multivariate analysis of variance, multivariate analysis of covariance, multivariate regression, principal component analysis, factor analysis, linear discriminant analysis, artificial neural networks. The results of such methods may be shown as score plots.

Biosensors using electrochemical impedance detection

The analytical technique used for these measurements setup is the electrochemical impedance spectroscopy (EIS), which provides information on the electrical properties on the electrode surface by varying the frequency of a low amplitude sine wave. In impedimetric measurements, a fixed potential is applied and the impedance of the system is measured.

In a preferred embodiment, the electrochemical biosensor comprises three electrodes being a counter electrode, a working electrode, and the reference electrode. Biorecognition occurs on the surface of the working electrode, which is responsible for the transduction of the biochemical reaction. The current generated by the system flows between the working electrode and the counter electrode. To monitor the potential of the working electrode, the reference electrode is then used, which must be maintained at a known fixed potential. The electrochemical biosensor may comprise more than one working electrode, one counter electrode, and reference electrodes.

The impedance is dependent on the events that occur on the surface of the working electrode and, for this reason, it can be monitored to determine the presence of the target molecule in the sample.
The excitation wave can be either an alternating potential or an alternating current, so that one of the parameters is controlled and the other is measured. Thus, in pseudo-linear systems, as in the case of an electrochemical cell, the application of a sinusoidal potential results in a sinusoidal current that oscillates at the same frequency. The components of the electrochemical system cause a phase and amplitude shift between these two waves. The system impedance, i.e., the resistance in an alternating current system, is calculated as the ratio between the sinusoidal potential and the response current. In the faradaic method, the measurements are performed using redox mediators, so that the redox reactions between them are monitored. The potassium ferricyanide ($K_3Fe(CN)_6$) and potassium ferrocyanide ($K_4Fe(CN)_6$) redox couple is the most used for this purpose. This EIS

method is so named because the current generated by electrochemical reactions obeys Ohm's law, that is, it is directly proportional to the number of electrons involved in the redox reaction (n), the Faraday constant (F), the electrode surface area (A), and the flow of electroactive molecules (j):

$I = nFAj$

The electrochemical impedance measurements can be represented by the Bode diagram, in which absolute values of impedance or phase angle are plotted as a function of frequency, or by the Nyquist diagram, in which real impedance values are given as a function of their respective imaginary values on a complex plane. The analysis of the Nyquist diagrams allows obtaining information about the components of the electrochemical system, such as the double-layer capacitance (C_{dl}), charge-transfer resistance (R_{ct}), solution resistance (R_{Ω}), and Warburg impedance (Z_w). All of these components form an electrical circuit in the electrochemical system that can be determined using theoretical models. An equivalent circuit is commonly found in electrochemical biosensors and modeled by John Randles. For this configuration, the Nyquist plot is divided into three regions, according to the frequency of the excitation wave. At high frequencies, the capacitor impedance is very low, so that the system can be approximated as an open circuit, which impedance is given only by the resistance of the solution. In the intermediate frequency region, charge transfer processes are predominant. Thus, a semicircle is observed which diameter (R_{ct}) corresponds to the charge-transfer resistance between the electrolyte and the electrode. This parameter is dependent on the configuration of the biorecognition layer and, therefore, it is widely used to describe the processes that occur in it. Finally, in low-frequency regions, the system impedance is governed by the diffusion of electroactive species (Z_w) and the double-layer capacitance (C_{dl}), which behavior is represented in the spectrum by a straight line with a slope.

Therefore, the invention further provides a diagnostic kit comprising (a) a biosensor and (b) a data processing device comprising means for receiving the electronic signal, analyzing the electronic signal, and generating an output of the electronic signal to a user.

In another embodiment a method for detecting simultaneously more than one nematode species is provided comprising the following steps:

(a) incubating an analyte comprised in a soil sample on the biorecognition layer of the biosensor;

(b) applying electrical current to one or more electrodes of the biosensor;

(c) varying the frequency of the current;

(d) measuring the physicochemical parameter with a data processing device.

In one embodiment, different nematode species may be detected simultaneously.

In another embodiment, a method for the preparation of an analyte suitable for the use in the method for detecting simultaneously more than one nematode species comprising

(a) a physical separation of nematode and nematode eggs using sieves aiming at concentrating the nematode and nematode eggs in the soil sample and eliminating the soil and organic matter of the soil sample.

(b) extracting the proteins from nematodes and nematode eggs isolated in step (a) by homogenization.

(c) incubating the extract of nematode protein with the electrodes according to claim 1 for 5 to 60 minutes.

(d) Performing the electrical or electrochemical impedance measurements upon applying an alternate electrical signal to the electrode.

(e) Identifying the nematode species based on the capacitance values compared to a reference signal, using a multivariate statistical method, or by analyzing the percentage change in the electrochemical impedance signal coming from the biosensor electrode.

In one embodiment the method for the preparation of an analyte suitable for use in the biosensor is executed in an agricultural field, greenhouse or glasshouse.

1. Figures

Figure 1 - Score plot of the principal component analysis (PCA) obtained by the combination of two sensory sets - electrodes with organic film and electrodes with SAM - for discrimination of samples without incubation period. The 95% confidence ellipse is shown for each group of samples.

Figure 2 - Bar chart showing the percentage change in impedance of the biosensor constructed for the *P. brachyurus* species. The biosensor was tested with a positive sample (containing the target proteins) and two negative samples (containing proteins from *M. javanica* or *H. glycines* species) .

Figure 3 – Linear dependence between the percentage change in electrochemical impedance and the log of the protein concentration(*M. incognita* species) - Calibration curve obtained by linear regression with adjustment of $R^2 = 0.999$.

A biosensor is a device that allows the identification and quantification of a molecule of interest, the analyte. The biosensor is comprised of a by recognition layer which contains biomolecules such as DNA, enzymes, proteins, and/or antibodies which are suitable to bind selectively to the analyte.

The biosensor further comprises a transducer being one or more electrodes which is capable of converting the physicochemical change occurring in the biorecognition layer by the binding into an electronic signal.

A capacitive biosensor comprises one or more, preferably two metallic electrodes which form the capacitor. In one embodiment the electrodes are interdigitated. In this configuration several metallic electrodes called fingers are placed in parallel with each pair forming a capacitor.

In a preferred embodiment the biosensor comprises three or more electrodes. In a preferred embodiment the biosensor comprises two or more electrodes.

Electrodes, in particular interdigitated electrodes, are usually manufactured using lithography and deposition techniques, e.g. sputtering, in which metals are deposited in the desired configuration on insulating substrates such as glass and silicon.

The electrodes may be disposable gold electrodes on glass substrate. Such electrodes can be manufactured by photolithography.

Atomic force microscopy may be used for the morphological characterization of the working electrodes. X-ray photoelectron spectroscopy technique may be used to analyse the chemical composition of the electrodes and to assess the effectiveness of electrochemical cleaning.

In a preferred embodiment the biosensor comprises three electrodes being a counter electrode, a working electrode, and the reference electrode. Biorecognition occurs on the surface of the working electrode, which is therefore responsible for the transduction of the biochemical reaction. The current generated by the system flows between the working electrode and the counter electrode, which must have a high surface area. To monitor the potential of the working electrode, the reference electrode is then used, which must be maintained at a known fixed potential.

The biorecognition layer can be formed by immobilising biomolecules capable of selective binding to the analyte onto the electrodes. Examples for such biomolecules are fragments of DNA or RNA, antibodies, proteins, protein fragments, secondary metabolites, or saccharides.

Coupling through Protein A or G are examples for non-covalent immobilization.

Covalent immobilisation uses functional substrates such as carbodiimide (EDC) and N-Hydroxysuccinimide (NHS) or mercaptopropionic acid (MPA) and 11-mercaptoundecanoic acid (MUA) molecules. APTES (3-Aminopropyltriethoxysilane) may be used for Iridium Tin Oxide electrodes.

The electronic signal which may be an electrical current, impedance, or capacitance is analysed by the data processing device and provided to the user in a numerical or graphical representation, such as graphs or tables. Such graphs often show the capacitance in relation to the logarithm of the frequency.

In order to identify the nematode species, multivariate statistical methods can be used, such as multivariate analysis of variance, multivariate analysis of covariance, multivariate regression, principal component analysis, factor analysis, linear discriminant analysis, artificial neural networks. The results of such methods may be shown as score plots.

The analyte is one or more biomolecules, such as fragments of DNA, fragments of DNA or RNA, proteins, protein fragments, secondary metabolites, or saccharides which are detected by the biomolecules immobilised in the bio recognition layer. In one preferred embodiment the analyte are species-specific proteins from nematode species.

The biosensor comprises an electrode capable of converting a physicochemical signal transduced from a biorecognition layer into an electronic signal. The biosensor may comprise more than one electrode, preferably one counter electrode, for working electrode, and two reference electrodes. The biosensor may also comprise of interdigitated and electrodes having at least 10, preferably 20, more preferably 50, even more preferably 100, and most preferably 150 fingers. The fingers are separated by a distance of 1 to 100 microns, preferably 2 to 80 microns, more preferably 5 to 50 microns, even more preferably 10 to 25 microns, or most preferably 10

μm. The interdigitated electrodes are manufactured using photolithography technique with sputtering metallization.

The biosensor further comprises a biorecognition layer comprising biomolecules immobilized onto the electrode capable of binding a component of an analyte specific for a nematode species. The biomolecules may be a nucleic acid such as DNA or RNA or antibodies such as polyclonal or monoclonal antibodies.

The recognition of the analyte in the biorecognition layer occurs on the surface of the working electrode, which is therefore responsible for the transduction of the biochemical reaction. The current generated by the system flows between electrodes, for example the working electrode and the counter electrode, which must have a high surface area. To monitor the potential of the working electrode, the reference electrode is then used, which must be maintained at a known fixed potential.

There are several electroanalytical techniques with different operating principles. The amperometric, voltammetric, potentiometric and impedimetric measurements stand out. In amperometric measurements, a fixed potential is applied to the reference electrode and the current generated by the redox reaction of the electroactive species between the working electrode and the counter electrode is measured. When the current is measured during controlled variations of the potential, the technique is called voltammetry. In potentiometric measurements, it is measured the potential difference between the working and reference electrodes, resulting from charge accumulation on the surface of the working electrode. Finally, impedimetric measurements consist of applying a fixed potential and measuring the impedance of the system.

The parameters obtained by these techniques are dependent on the events that occur on the surface of the working electrode and, for this reason, allow determining the presence of the target molecule in the sample.

Voltammetric measurements consist of applying a potential that varies over time. The way in which the variation of the potential on the working electrode occurs in relation to the fixed potential of the reference electrode defines the type of voltammetry. Square-wave voltammetry, differential-pulse voltammetry, and cyclic voltammetry are some examples of voltammetric techniques.

Cyclic voltammetry provides information on the redox potential of electroactive species and the current generated by electrochemical reactions. For that, a triangular wave is applied that linearly sweeps a potential range. When reaching a stipulated potential, the sweep direction is reversed, varying the potential until the cycle is completed. During scanning towards the most positive potential, the electroactive species is oxidized to an E_{pa} potential, with anodic current I_{pa}. In the opposite direction, the species is reduced in an E_{pc} potential, with a cathodic current I_{pc}. The graph generated by the voltammetry measurements is called voltammogram.

In reversible systems, the redox currents (I_p) are proportional to the scanning speed of the potential (v), the electroactive area of the electrode (A), the number of electrons involved in the reaction (n), the concentration of the electroactive species (C) and the diffusion coefficient (D), as described by the Randles-Sevcik equation:

$I_p = (2.69 \times 10^5)n^{3/2}AD^{1/2}Cv^{1/2}$

The variation in the redox potential and the current generated can be indicative of the presence of the analyte and its concentration, enabling the use of this technique both as a qualitative and quantitative method.

The examples are meant to illustrate the invention and are by no means limiting.

Examples

Example 1. Extraction of proteins from nematodes and production of antibodies

Samples of the three selected species containing eggs and juveniles were collected from soils. Samples in an aqueous solution were centrifuged in a first stage for 10 min at 2000 rpm, so that the nematodes and soil residues were deposited at the bottom of the centrifugation tube. The supernatants were removed and a 40 g/75 ml sucrose solution was added; the samples were homogenized and centrifuged for 10 min at 650 g rotation. After this second centrifugation, the nematodes (eggs and juveniles) were suspended in the supernatant while the residues formed precipitates, due to the sucrose density being higher than the density of nematodes. The supernatants containing the nematodes were collected and filtered through a 500 mesh size sieve (0.025 mm mesh opening). In this procedure, the nematodes are retained in the mesh to be collected by inverting the sieve and rinsing with an appropriate volume of water. Finally, the solutions were analyzed using an optical microscope with a 40X magnifying glass to check whether the final samples contained a significant number of nematodes and if the soil residues had been removed.

After removing residues, the proteins were extracted. The samples containing nematodes were processed in a Turrax-type homogenizer for 15 s, three times. Then, to break the cell membranes and extract the proteins, the samples were sonicated in a tip sonicator for 1 h with 20% amplitude. As the sample is heated during this process, they were immersed in ice, which was changed every 20 min of sonication.

The proteins in the homogenate were quantified using the Bradford colorimetric method, which uses the Coomassie brilliant blue BG-250 dye. Samples of the extracted proteins were used to immunize rabbits for aiming the production of polyclonal antibodies.

The immunized serum containing the antibodies was used for the construction of the biorecognition layer of the immunosensors. The extracted proteins were used as positive and negative controls in the detection stage.

Example 2. Fabrication of interdigitated and electrochemical electrodes

The electrochemical and interdigitated electrodes were fabricated using the photolithography technique with sputtering metallization. In the first step of the process, the substrates (BK7 glass) were modified with a photosensitive film, called photoresist, using the spin coating technique and left for 10 min on a hot plate at 90°C for photoresist curing. In the next step, the substrates were exposed to ultraviolet light through an optical mask containing the configuration desired for the device. In this case, a positive photoresist was used, in which

the regions exposed to light were removed by the revealing chemical solution (aqueous potassium hydroxide solution). After the photolithography, the substrates were cleaned with oxygen plasma for removing organic residues.

Metallization was performed in a vacuum chamber and the thickness was measured by a quartz crystal. For the interdigitated electrodes, a 15 nm chrome layer was first deposited for adhesion on the substrate, then a 120 nm thick gold layer was deposited. For the electrochemical biosensor, a 15 nm titanium film was used as the adhesion layer.

Example 3. Cleaning procedures of electrodes

There are two cleaning procedures for the interdigitated electrode. In the first, named KOH, the electrodes were immersed in the 2% KOH solution (m/V) and left in an ultrasound bath for 5 min. The same procedure was performed by immersing the electrodes in ethanol and then in Milli-Q. In the second procedure, called etOH, the electrodes were cleaned only in ethanol and Milli-Q, also for 5 min in an ultrasound bath.

The electrochemical electrodes were cleaned with O_2 plasma using 35 W power and 5 sccm gas flow rate (cm^3/min).

Example 4. Morphological characterization of interdigitated electrodes

Interdigitated and electrochemical electrodes, after cleaning described in example 3, were characterized as for morphology, by atomic force microscopy. Roughness was determined in 4 different electrodes for reproducibility analysis. For interdigitated electrodes, the roughness per area and mean square roughness obtained were 1.24 ± 0.04 nm and 1.58 ± 0.05 nm, respectively. Also, the results revealed that electrodes fabricated by this methodology present similar roughness, with a standard deviation of about 3%. This characteristic is essential for a better organization of the self-assembled monolayer and for the analysis of electrical conductivity to be reproducible.

Example 5. Electrodes functionalization

The capacitive biosensor was constructed by the formation of a self-assembled layer: clean interdigitated electrodes were modified with 1 mM 3-mercaptoproprionic acid (MPA) at room temperature and humid atmosphere for 15 h; then, the electrodes were washed with Milli-Q water and incubated for 1 h with an aqueous solution containing 2 mM 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and 5 mMN-hydroxysuccinimide (NHS), to activate the carboxylic group from MPA molecules. In the next step, the electrodes were modified with the immunized serum for 4 h at 4°C. The blocking procedure of the remaining active sites with bovine serum albumin (BSA), for example, was not adopted in this protocol, since the immunized serum already contains proteins.

Example 6. Electrical measurements - reproducibility and frequency choice

Modified interdigitated electrodes were subjected to 20 consecutive measurements of electrical capacitance to evaluate reproducibility in the frequency range from 10^2 Hz to 10^6 Hz. The capacitance values have shown to be reproducible for almost all frequencies, with a very small variation for the 0.6 x10^6 to 1x10^6 Hz range.

To select a fixed frequency to assess the percentage variation of capacitance and perform multivariate statistical analysis, electrodes modified with immunized serum of *M. incognita* species were incubated in a solution containing 30 µg/mL of protein of *M. javanica* species (negative) for 1 h and in a solution containing the same concentration of protein of *M. incognita* species (positive) also for 1 h. Electrical impedance spectra were collected before and after the described incubations. The measurements were performed in a buffer solution and a 5-minute delay was adopted before the measurements for the organization of the electric double layer. A significant variation in the capacitance after the incubations was observed for frequencies between 10^4 and 10^6 Hz, indicating this region as the one that best discriminates the samples. Considering that there is a small variation in the reproducibility of the measurements for frequencies above 6x10^5 Hz, 10^5 Hz was chosen as a fixed frequency for subsequent analyzes. Through the capacitance spectra obtained for electrodes modified with the immunized serum of *M. incognita* species, samples containing only phosphate buffer (10 mM PBS), , or 30 µg/mL of proteins of *M. javanica* species (negative), or 30 µg/mL of proteins of *M. incognita* species (positive) could be distinguished clearly.

Example 7. Influence of electrode cleaning on selectivity of the electrical biosensor

As described in example 3, two cleaning protocols named KOH and etOH were tested. To assess whether the cleaning procedure affects the ability to discriminate samples and select the most suitable, two sets of electrodes - each one subjected to a cleaning method - were modified with the immunized *M. incognita (Mi)* serum and characterized by capacitance measurements before and after incubations with negative (Mj) and positive (Mi) proteins at 30 μg/mL. Principal component analysis was performed using the covariance matrix for the values obtained at the frequency of 10^5 Hz.

The influence of the variables - in this case, the two cleaning protocols - is analyzed from the loading plot, where each variable is represented as a vector whose intensity and direction determine its influence on the principal components 1 and 2. It is observed that the electrodes submitted to cleaning with KOH show a small intensity in the y-direction and a large intensity in the x-direction, indicating that this variable has a greater influence on the principal component 1 (PC1). On the other hand, the vector that represents electrodes cleaned with etOH has a small intensity on the x-axis and a large intensity on the y-axis; thus, its greatest influence is on PC2. Since PC1 is the component that most discriminates the samples, the cleaning protocol using KOHwas chosen to perform the other studies.

Example 8. Combination of different sensory units

To determine whether variations in capacitance are due to selective antigen-antibody interaction, three sensory sets were evaluated: clean electrodes without modifications, electrodes modified with the organic film without adding the immunized serum, and electrodes with the SAM containing the immunized serum of *M. incognita* species, as described above. For the organic film, the electrodes were incubated for 15 h with 1 mMMPA solution and then for 1 h with 2 mM/5 mMEDC/NHS. The blocking of the carboxylic groups was performed with 0.5% bovine serum albumin (BSA) solution (m/V) for 30 min. For each set, capacitance values were collected before incubation, after incubation with proteins of *M. javanica* species (negative) at 30 μg/mL, and after incubation with target proteins (positive) also at 30 μg/mL. The score plot of theprincipal component analysis obtained by the combination of three sensory sets - cleaned electrodes, electrodes with organic film, and electrodes with SAM showed the discrimination of the three samples applying a 95% confidence interval.

The loading plot for this set of variables shows that there is a negligible contribution from the sets of clean electrodes and with organic film (FO) to the PC1, responsible for the major differentiation of the samples.

Therefore, the discrimination of the samples is due to the specific interaction between the antibodies present in the SAM and its target proteins.

Since the clean electrodes had a low contribution to both PC1 and PC2, an analysis combining only the sensory sets FO and AC was performed. The score plot revealed that when excluding clean electrodes, PC1 increased and was able to discriminate 98.7% of the samples. For this case, adding the scores of the principal components 1 and 2, the value of 100% is obtained. Therefore, the combination of these two sensory sets was chosen. This result shows that the capacitive biosensor was able to identify plant-parasitic nematode species.

Example 9. Electrical detection without incubation

The goal of the biosensor developed here is to detect plant-parasitic nematodes in a simple and fast manner, with the possibility to be performed in the field. Thus, it was studied the possibility of detection without the incubation with the sample. For this, FO and AC electrodes were subjected to capacitance measurements in a buffer solution, after waiting 10 min for the organization of the double electric layer. In a later step, a solution containing non-specific proteins (negative samples) at 30 µg/mL was added to the electrodes; after 10 min of waiting required for the sample to interact with the sensory unit and for the organization of the double electrical layer, electrical impedance measurements were performed in the same solution containing the proteins. The procedure was repeated in a solution containing the target proteins (positive) at 30 µg/mL. The capacitance values were analyzed by the principal component analysis to assess whether the detection performed without an incubation time is also capable of discriminating the samples. The score plot obtained, shown in **Figure 1**, demonstrates not only the biosensor's ability to differentiate samples based on measurements taken without the incubation period, but also shows a higher PC1 score compared to the results of experiments performed with 1 h of incubation. Adding the principal components 1 and 2, discrimination of 100% of the samples is obtained.

For the detection without incubation, the loading plot obtained by the combination of two sensory sets - electrodes with organic film and electrodes with SAM - also showed that PC1 depends almost exclusively on the electrodes that have antibodies, while PC2 is dominated by the values generated from the electrodes modified with organic film. For multivariate statistical analysis, at least two sensory sets are necessary, i.e., two distinct variables. Thus, the ideal model found in this study is the combination of capacitance measurements on FO and AC electrodes, which together can identify all samples.

Example 10. Electrochemical detection

Electrochemical measurements are performed in a miniaturized electronic device. An electrolyte solution

containing 5 mM Ferri/iron in 0.1 M KCl is added to the electrode, covering the entire surface. First, the electrodes are subjected to cyclic voltammetry measurement in the potential range from -0.5 to 0.6 V, at a sweep speed of 50 mV s^{-1}. This step is performed only to pre-activate the electrodes. Then, electrochemical impedance measurements are performed at open circuit potential in the frequency range 10^{-1} Hz to 10^{3} Hz, with an AC amplitude of 10 mV. For the detection tests, these measurements were performed before and after the incubation with the sample for 30 min. The percentage change in R_{ct} resulting from the incubation was calculated for all samples tested. The results showed a much greater variation for the positive samples (containing the target proteins), as seen in **Figure 2**, indicating the selectivity of the biosensor.

Example 11. Electrochemical impedance response as a function of the analyte concentration

The electrochemical impedance response for different concentrations of the target proteins was investigated. A gradual increase in R_{ct} as a function of the concentration of target proteins was identified. A linear relationship between the ΔR_{ct} (%), calculated as the percentage change in R_{ct} after the sample incubation, and the logarithm of protein concentration was achieved as shown in **Figure 3**.

Claims

1. A biosensor comprising
   (a) One or more electrodes capable of converting a physicochemical signal transduced from a biorecognition layer into an electronic/electrochemical signal;
   (b) the biorecognition layer comprising biomolecules immobilized on one or more electrodes capable of binding a component of a specific analyte for a nematode species;
2. The biosensor according to claim 1 wherein the biomolecules are antibodies or any other receptor molecule capable of binding specifically to proteins from certain nematode species
3. The biosensor according to claim 2 wherein the antibodies are covalently bound to the biorecognition layer.
4. The biosensor according to claim 2 wherein the antibodies are non-covalently bound to the biorecognition layer
5. The biosensor according to claims 1 to 4 wherein the biosensor is capable of analyzing more than one analyte simultaneously.
6. The biosensor according to any of claim 1 to 5, wherein the physicochemical signal is electrochemical or electrical.
7. The biosensor according to any of claim 1 to 6 wherein the electronic signal is selected from the group of electrical current, impedance, or capacitance.
8. The biosensor according to any of claim 1 to 6 wherein the signal is optical.
9. The biosensor according to claims 1 to 8 wherein biosensor is a capacitive or impedimetric biosensor.
10. The biosensor according to claims 1 to 8, wherein the biosensor comprises interdigitated or electrochemical electrodes.
11. A diagnostic kit comprising (a) the biosensor according to any claims 1 to 8 and
    (b) a data processing device comprising means for receiving the electronic signal, analyzing the electronic signal, generating an output of the electronic signal to a user.
12. The kit according to claim 11 wherein the physicochemical parameter is selected from the group of electrical current, impedance, or capacitance.
13. A method for detecting simultaneously more than one nematode species comprising
    (a) incubating an analyte comprised in a soil sample on the biorecognition layer of the biosensor according to any claims 1 to 8;
    (b) applying electrical current to one or more electrode of the biosensor according to any claims 1 to 8;
    (c) varying the frequency of the current;

(d) measuring the physicochemical parameter with the data processing device according to claim 11.

14. A method according to claim 13, wherein the percentage change of measured physicochemical parameters is calculated or their values are analyzed using a multivariate statistical method.

15. A method for the preparation of an analyte suitable for use in the method according to claim 13 comprising
(a) a physical separation of nematode and nematode eggs using sieves aiming at concentrating the nematode and nematode eggs in the soil sample and eliminating the soil and organic matter of the soil sample;
(b) extracting the proteins from nematodes and nematode eggs isolated in step (a) by homogenization;
(c) incubating the extract of nematode proteins with the electrodes according to claim 1;
(d) performing the electrical or electrochemical impedance measurements upon applying an alternate electrical signal to the electrode;
(e) identifying the nematode species based upon the impedance values compared to a reference signal, using multivariate statistical method.

16. A method according to 15, wherein the method is executed in an agricultural field, greenhouse, or glasshouse.

FIGURES

Figure 1

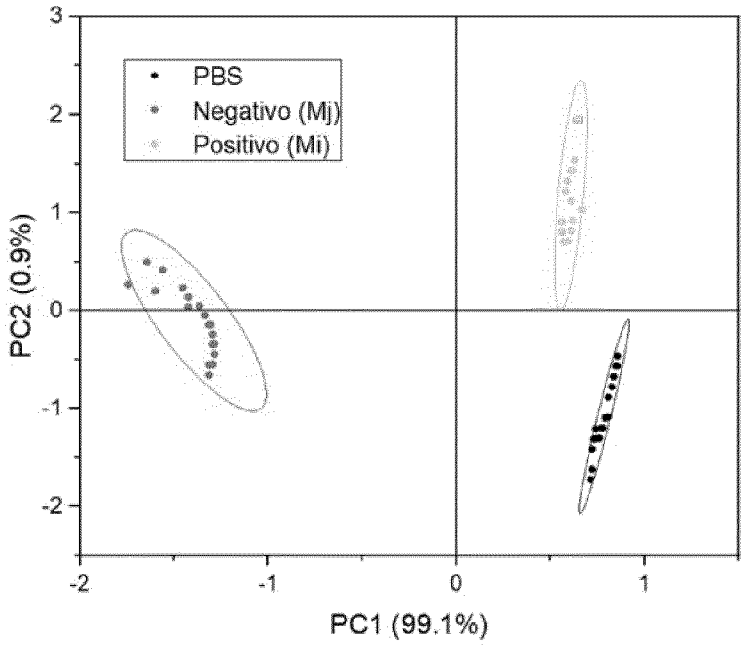


Figure 1 - Score plot of the principal component analysis obtained by the combination of two sensory sets - electrodes with organic film and electrodes with SAM - for discrimination of samples without incubation period. The 95% confidence ellipse is shown for each group of samples.

Figure 2

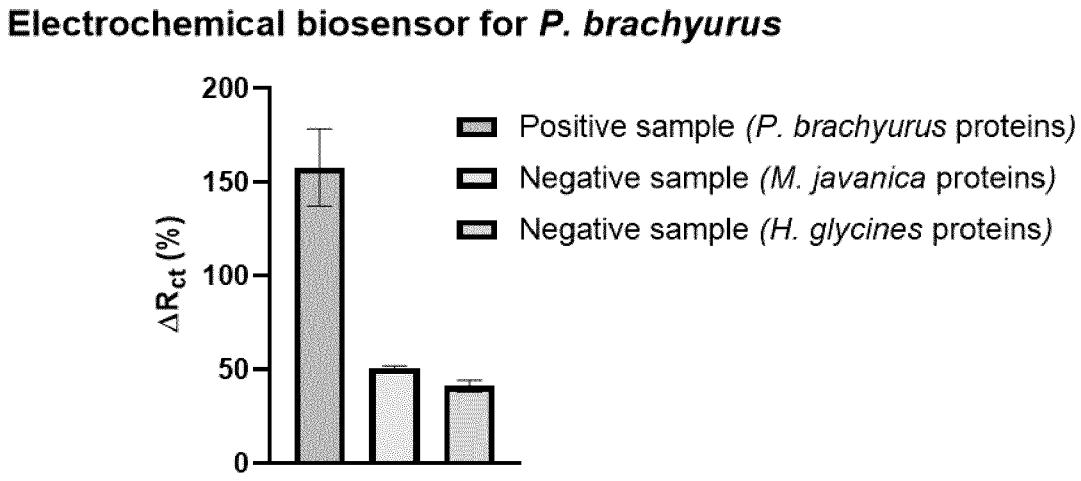
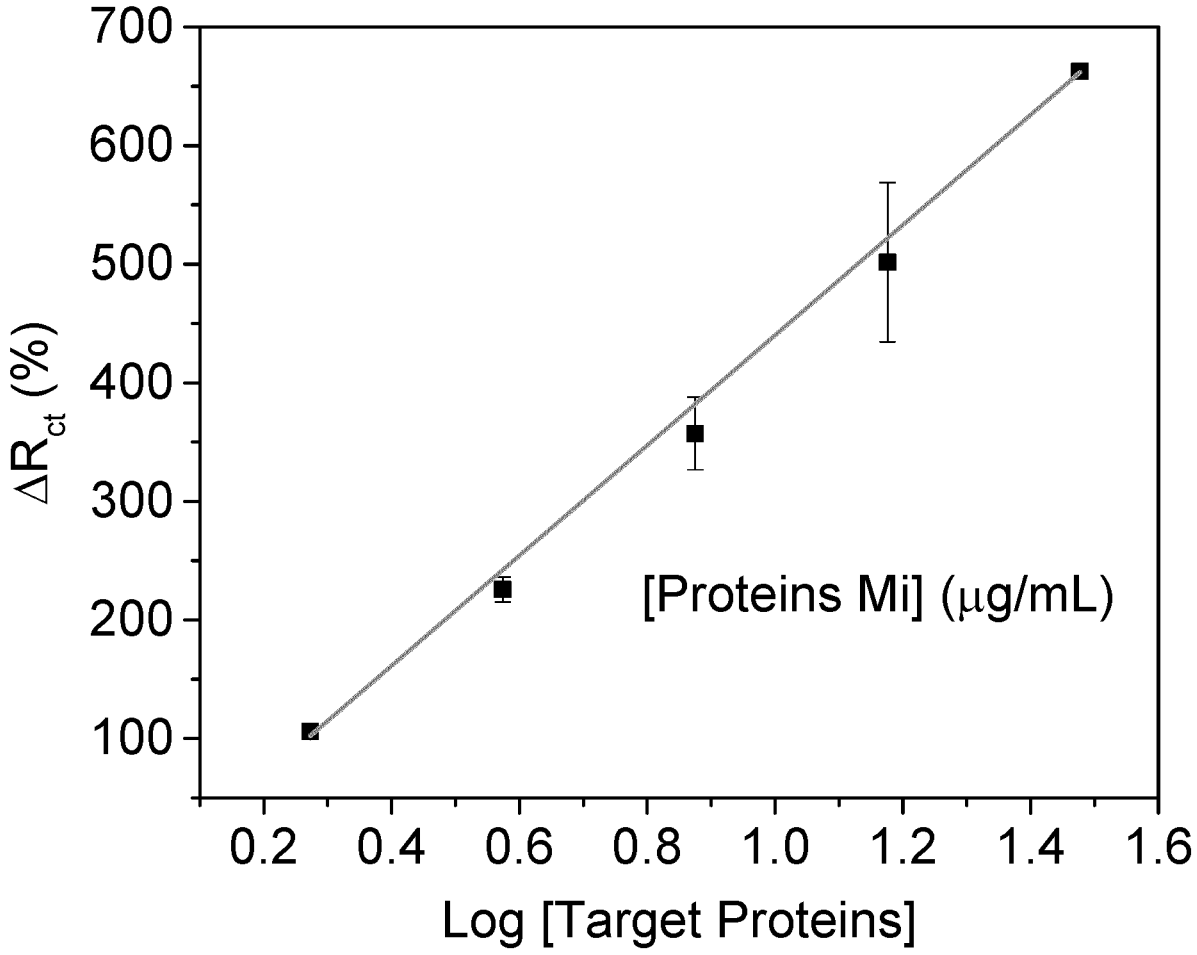


Figure 2 – Bar chart showing the percentage change in impedance of the biosensor constructed for the *P. brachyurus* species. The biosensor was tested with a positive sample (containing the target proteins) and two negative samples (containing proteins from *M. javanica* or *H. glycines* species) .

Figure 3



Equation	y = a + b*x
Plot	B
Weight	Instrumental
Intercept	-24.34746 ± 6.7293
Slope	464.51349 ± 5.9493
Residual Sum of Square	3.68356
Pearson's r	0.99975
R-Square(COD)	0.99951
Adj. R-Square	0.99934

Figure 3 - Linear dependence between the percentage change in impedance and the log of the protein concentration (*M. incognita* species) - Calibration curve obtained by linear regression with adjustment of $R^2 = 0.999$.

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2023/051450

A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N33/53 G01N33/543
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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X	WO 2018/015884 A1 (UNIV KING ABDULLAH SCI & TECH [SA]; UNIVERSITÄT REGENSBURG [DE]) 25 January 2018 (2018-01-25) whole document, in particular p. 6, par. 3, 4; p. 8, par. 1; p. 8, bridging par. - p. 9, par. 3; p. 15-16, bridging par.; p. 18, par. 1; p. 20, 2-3; fig. 9, 10; claims 1-32	1-7,9-12
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Y	whole document, in particular par. 18, 19, 32, 33, 46, 49-51, 83, 120, 131, 158, 164-166, 173, 177, 180; claim 1-56	13-16

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[x] Further documents are listed in the continuation of Box C.

[x] See patent family annex.

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"P" document published prior to the international filing date but later than the priority date claimed

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"X" document of particular relevance;; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance;; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
20 February 2023	01/03/2023

Name and mailing address of the ISA/	Authorized officer
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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2023/051450

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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