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J. Braz. Chem. Soc., Vol. 20, No. 4, 795-801, 2009.
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0103 - 5053 $6.00+0.00

Article

Development of an Electrochemical Immunosensor for *Phakopsora pachyrhizi* Detection in the Early Diagnosis of Soybean Rust

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A ferrugem da soja é uma doença que ocorre nas folhas da soja e é considerada muito agressiva, pois reduz a qualidade do grão final. A identificação precoce do fungo na plantação previne várias perdas para o agricultor bem como para as culturas vizinhas. Neste trabalho, um imunossensor foi desenvolvido sem a utilização de marcadores usando medidas de impedância para detectar a ferrugem asiática nos extratos das folhas contaminadas nos estágios iniciais da doença, antes do aparecimento dos sintomas. O anticorpo anti-micélio do fungo *Phakopsora pachyrhizi* (agente causador da doença) foi imobilizado em substrato de ouro via formação de monocamadas auto-organizadas de tióis e ligações covalentes com a biomolécula. O imunossensor apresentou um limite de detecção de 385 ng mL^{-1}. A otimização das condições experimentais e o bloqueio da superfície para minimizar ligações não-específicas foram realizados. Este estudo proporcionou uma nova perspectiva da utilização do método para o diagnóstico precoce da doença.

Soybean rust is a disease that occurs on soybean leaves and is considered very aggressive, reducing product quality. Early identification of fungus in the plants prevents severe farming losses as well as spreading to neighboring cultures. In this paper, a label-free immunosensor was developed based on impedance measurements to detect Asian rust on soybean leaf extract at the early stages of the disease. The antibody anti-mycelium of *Phakopsora pachyrhizi* fungus (disease agent) was immobilized on a gold substrate *via* a self-assembled monolayer (SAM) of thiols using covalent cysteamine coupling. This immunosensor presents a limit of detection of 385 ng mL^{-1}. The optimization of experimental conditions and surface blocking to minimize non-specific adsorption on the immunosensor response were evaluated. These studies, based on electrochemical impedance spectroscopy (EIS), provide new perspectives on using this method for early diagnosis of soybean rust.

Keywords: immunosensor, SAM, EIS, soybean rust

Introduction

Asian soybean rust is a fungal disease caused by *Phakopsora pachyrhizi* which is a virulent pathogen that can quickly defoliate plants, reduce pod set, pod fill, seeds and, thus, quality, reducing crop yields by 10 to 80%.[1] The disease is disseminated through spores (urediniospores) transported by the wind and spreads rapidly, causing loss of foliar area and a severe reduction in grain yield[2] and, consequently, severe economic losses.

The appearance of the disease is recent in countries that now have large production of soybeans, such as Brazil and United States, but the infection cost was estimated at approximately $1.2 billion for past harvests,[3] a large percentage of these losses resulting from inappropriate use of fungicides.

Symptoms begin on the lower leaves of the plant as small lesions on the undersides of the leaves that increase in size and change from gray to brown.[4] The biggest problem is in the early detection of the fungus infestation because it is performed by a visual method. However, when the fungus is readily visible, the culture is already infected. The lesions can also be misinterpreted as non

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aggressive infections, which are normally found on the crop. The method used to control the pathogen is fungicide application, thus precise and early diagnosis minimizes spore dissemination, decreasing the costs of control and avoiding a continuous environmental contamination.[5]

Thus, farmers need an early identification method that could help them to control disease infestation. Due to their simplicity and sensitivity, biosensors could be effective tools for disease diagnosis and monitoring.[6-8]

Immunosensors are based on the use of an antibody that reacts specifically with a substance (antigen) to be tested. Immobilization of the receptor (e.g., an antigen) on a substrate is convenient for applications of molecular bio-recognition for the detection of a target molecule (e.g., its antibody) present in solution. The specificity of antigen-antibody interactions allows the development of immunosensor devices for clinical diagnostics, environmental monitoring, etc.[9]

Immunosensors offer several advantages such as limited hands-on time, high-throughput screening, improved sensitivity, real-time analysis, possibility of quantification and label-free detection (*i.e.,* antibodies do not need to be labelled with fluorophores, radioisotopes, colloidal gold particles or enzymes for detection of binding events or signal enhancement). Therefore, immunosensors can be successfully applied in agricultural, food, environmental, pharmaceutical chemistry and clinical applications.[10,11]

There are various techniques to evaluate the configuration of immunocomplexes and, among them, Electrochemical Impedance Spectroscopy (EIS) to monitor the binding of antigen-antibody can be emphasized. This is performed in the presence of a redox probe. The formation of immunocomplexes disturbs the double-charged layer at the electrode/electrolyte interface resulting in an increase of thickness and an insulation character to the electrode surface in relation to the redox probe added to the solution. These changes affect the capacitance and the electron transfer at the electrode interface. The great advantage in using EIS as a transducer system is the ability to carry out an immunoassay without needing a tracer, thereby reducing analysis time.[12]

In this paper, an electrochemical impedance immunosensor for the detection of the *Phakopsora pachyrhizi* fungus was developed aiming at the diagnosis of Asian rust on soybean leaves. The sensor was constructed employing the mycelium extracted from spores of contaminated leaves, making it possible to identify the disease at its earliest stages, *i.e.*, before the appearance of symptoms.

Experimental

Reagents and solutions

N-(3-dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride (EDC), cysteamine (CYSTE) and bovine serum albumin (BSA) were obtained from Aldrich, USA. N-hydroxysuccinimide (NHS) was purchased from Fluka, Switzerland. Ethanol was obtained from Synth, Brazil. $K_3[Fe(CN)_6]$ and $K_4[Fe(CN)_6]$ were acquired from J.T. Baker, USA. All chemicals were of analytical grade and were used as received. Phosphate buffer solutions were obtained by mixing solutions of 0.1 mol L^{-1} Na_2HPO_4 and 0.1 mol L^{-1} NaH_2PO_4. A 10 mmol L^{-1} saline phosphate buffer (PBS) at pH 7.4 was prepared by dissolving 0.2 g KCl, 8.0 g NaCl, 0.24 g KH_2PO_4 and 1.44 g $KHPO_4$ in 1.0 L ultra-pure water. All solutions were prepared with deionized water (> 18 MΩ cm, Milli-Q, Millipore).

Apparatus

All electrochemical experiments were carried out in a conventional three-electrode cell at room temperature using a saturated calomel electrode (SCE) and Pt wire as reference and counter electrodes, respectively. The geometric area of the gold working electrode (Metrohm 6.1204.020, Switzerland) was 0.07 cm^2. The impedance measurements were performed using a PGSTAT 30 model from AUTOLAB (Eco Chemie, Netherlands) interfaced with a personal computer.

Antigen

Urediniospores from naturally infected greenhouse soybean plants were obtained from the collection of Embrapa Londrina, PR, Brazil. The spores were collected using a mechanical harvester, and 300 mg of spores was germinated in 300 mL deionized water in a sterile 13 in × 9 in Pyrex baking dish for 24 h at 22 °C. After that the culture was harvested by centrifugation at 6000xg for 15 min, the pellet was suspended in PBS and frozen at –20 °C, after protein quantification.

Polyclonal antiserum

A female New Zealand rabbit weighing 2 kg was subcutaneously inoculated three times at 14-day intervals with aliquots of *Phakopsora pachyrhizi* suspension (0.5 mL) containing 200-300 µg of protein emulsified with an equal volume of Freund's complete adjuvant for the first and incomplete adjuvant for the two subsequent

injections. Ten days after the final injection, blood was collected through cardiac puncture and the sera were kept at –20 °C. Titration and specificity of the collected serum were determined by indirect enzyme-linked immunosorbent assay (ELISA). Preimmune serum was used as a negative control.

Preparation of the self-assembled monolayer (SAM)

The cleaning of the bare gold surface is critically important for self-assembled monolayer formation and should be accomplished systematically.

The gold surface was first polished with a 0.3 µm alumina slurry. After, the electrode was washed with a large amount of deionized water and then sonicated in pure ethanol for 5 min. In a second step, the electrode was cleaned by immersion in a piranha solution (1:3 mixture of 30% H_2O_2/conc. H_2SO_4) for 10 min. Then, the electrode was washed with copious amounts of water. Finally, the gold electrode was electrochemically cleaned in 0.5 mol L^{-1} H_2SO_4 solution, cycling the potential between –0.1 and 1.4 V (*vs.* SCE) for 25 scans.[13] The final cyclic voltammogram was compared to those reported by Finklea *et al.*[14] in order to warrant a clean gold surface to be used each time. The electrode was then immersed for 18 h in 10 mmol L^{-1} cysteamine solution (CYSTE), freshly prepared in ethanol. The modified electrode (Au-SAM) was further rinsed with ethanol to remove physically adsorbed molecules and was used immediately for antibody coupling.

Antibody coupling

For the antibody coupling, 4 µL of the serum containing 47.6 µg mL^{-1} of antibody was diluted in 200 µL of 2 mmol L^{-1} EDC solution plus 200 µL of 5 mmol L^{-1} NHS solution and 600 µL of 0.1 mol L^{-1} PBS buffer solution at pH 7.4. This solution was stored in a refrigerator for 2 hours. This step is used to active the carboxylic groups of antibodies by the formation of NHS ester. After that, the SAM modified electrodes were immersed in the antibody solution and stored at 4 °C for 90 min. The unbound antibodies were removed from the electrode surface (Au-SAM-Ab) using the method cited by Geng *et al.*[15] Finally, the immunosensor was treated with 0.1% of BSA-PBS solution for 30 min to block the nonspecific sites. Then, the electrodes were washed with deionized water (Au-SAM-Ab-BSA).

Impedance measurements

For impedance studies, a sine wave with 10 mV of amplitude was applied to the electrode over the formal potential of the redox couple (0.2 V). Impedance spectra were collected in the frequency range varying from 100 kHz to 100 mHz. Electrochemical impedance spectra were fitted using an Equivalent Circuit contained on the FRA software AUTOLAB (Eco Chemie, Netherlands) and the resistance of charge transfer (R_{ct}) values were determined in a pH 7.4 PBS buffer using 5 mmol L^{-1} of $Fe(CN)_6^{3-/4-}$ as redox probe. Different concentrations of an extract of contaminated soybean leaves (antigen) were injected to study antibody-antigen interactions.

SDS gel electrophoresis

SDS-PAGE according to Laemmli[16] was performed using 12% acrylamide and 5% stacking gels containing 0.1% SDS (reducing conditions). Protein bands were stained with Coomassie brilliant blue R-250. A broad-range protein molar mass marker (Sigma Chemical, Saint Louis, MO, USA) was used for the estimation of protein size.

Results and Discussion

Preparation of the immunosensor

The immunosensor construction and posterior antigen (extracted from contaminated leaves) binding are presented in Figure 1. Firstly, a process of functionalization of the gold surface was conducted through the formation of a cysteamine monolayer based on the strong Au-thiolate bond. Then, the antibodies with previously activated carboxylic groups were immobilized on the surface. Finally, a specific binding event occurred between the anti-mycelium and the antigen of soybean leaves.

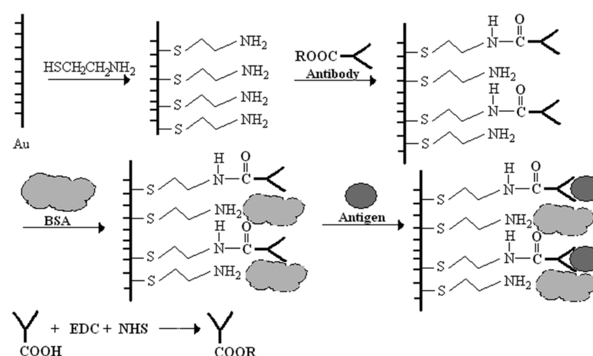

Figure 1. Schematic representation of antibody immobilization by covalent linkage to a gold surface modified with cysteamine.

Electrochemical impedance spectroscopy was an effective tool to investigate the modified electrode with different coating layers in the presence of $Fe(CN)_6^{3-/4-}$ in PBS buffer solution. At the higher frequencies, squeezed

semicircles represent an electron transfer-limited process, followed by a diffusionally limited electron transfer process at the lower frequencies.[17] The diameter of this semicircle in the Nyquist plot, which exhibits the electron transfer resistance (R_{ct}) of the layer, can be used to describe the interface properties of the electrode for each immobilization step.[18,19] As shown in Figure 2, the gold surface modification by cysteamine produces a SAM with a high number of vacancies and, therefore, a small barrier to probe the electron transfer. This behavior of the cysteamine-SAM was expected and verified using other techniques.[20] When the anti-mycelium and BSA molecules were adsorbed onto the SAM, there is an increase in the R_{ct} values because the proteins generate an insulating layer on the modified surface, which makes the redox reaction of $Fe(CN)_6^{3-/4-}$ more difficult.[21,22]

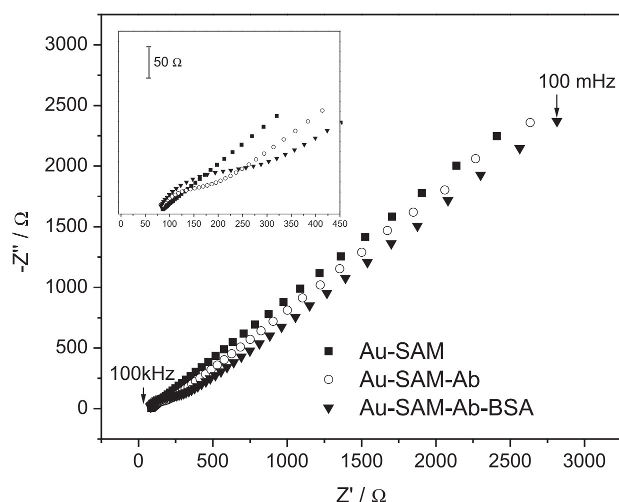

Figure 2. Nyquist plot of sequential immobilization steps onto gold electrodes towards a functional immunosensor in 5 mmol L^{-1} $Fe(CN)_6^{3-/4-}$ in pH 7.4 PBS solution for the frequency range from 100 kHz to 100 mHz with 10 mV of amplitude: SAM formed on gold surface (Au-SAM); anti-mycelium immobilization (Au-SAM-Ab) and after BSA agent blocking adsorption (Au-SAM-Ab-BSA).

Randle's equivalent circuit was adopted to model the physiochemical process occurring at the gold electrode surface:[23] $R_s(C[R_{ct}W])$ where R_s is the resistance of the solution, R_{ct} is the resistance to charge-transfer, W is the Warburg impedance and C is a double layer capacitance.

Antibody immobilization conditions

To provide high sensitivity and good repeatability in the measurements, it is important to find suitable linker compounds to enable packing a high density of antibody on the gold electrode. For this, the incubation time between the anti-mycelium and SAM modified surface was optimized. The electrode surface was coated with 20 µL of 0.8 µg mL^{-1} antibody solution at pH 7.4 for different times. Table 1 shows the results. It is possible to observe that an incubation time shorter than 120 min seems to be insufficient for anti-mycelium immobilization with adequate coverage of the surface. The impedance becomes constant after 120 min and this time was selected.

Table 1. Dependency of incubation time between the anti-mycelium and SAM modified electrode in immunosensor response using 1.5 µg mL^{-1} of antigen in 5 mmol L^{-1} $Fe(CN)_6^{3-/4-}$ in pH 7.4 PBS solution for the frequency range from 100 kHz to 10 mHz with 10 mV of amplitude

Incubation time / min	ΔR_{ct} / Ω
30	61 ± 6
60	74 ± 9
90	92 ± 8
120	123 ± 9
150	125 ± 8

Prior to anti-mycelium immobilization, the carboxylic groups of the antibodies are activated, using EDC and NHS solution. However, the formation of NHS ester can promote cross-linking between the antibodies, producing aggregates. This formation could decrease the free sites on the antibody needed for interaction with the antigen. An experiment was carried out to evaluate aggregate formation after site activation of antibodies by using electrophoresis in polyacrylamide and the results are shown in Figure 3. The

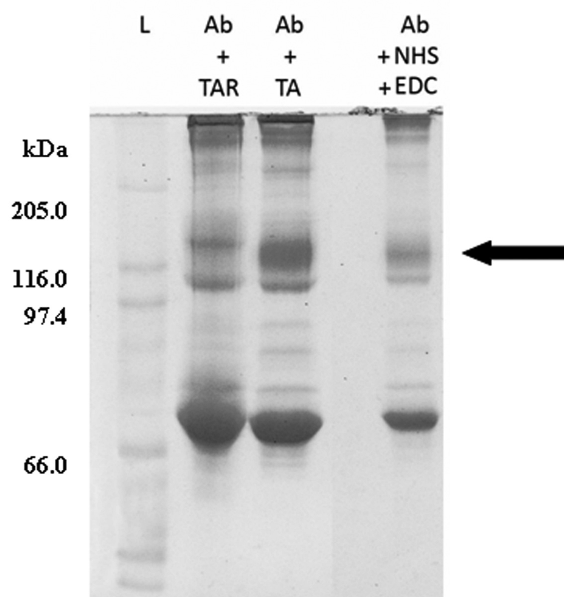

Figure 3. Polyacrylamide gel electrophoresis for the antibody solutions: Line 1 (L) molar mass marker; Line 2 (Ab + TAR) antibody in Lammli reducing sample buffer (with 2-mercaptoethanol); Line 3 (Ab + TA) antibody in Lammli non-reducing sample buffer (without 2-mercaptoethanol); Line 4 (Ab + NHS + EDC) antibody in Lammli sample buffer containing 2 mmol L^{-1} EDC and 5 mmol L^{-1} NHS.

electrophoresis profiles in lines 3 and 4 are very similar, considering the presence of a 155 kDa protein-band (black arrow) referring to the antibody molecules formed by heavy and light chains, which are not possible to observe when aggregates were formed. Even if antibody aggregates are formed, the electrophoresis confirms that there are free antibodies to bind with the surface and, therefore, with the antigens. Moreover, the immobilized antibody on the sensor surface interacts with the antigen with good sensitivity.

The concentration of anti-mycelium incubated onto the electrode surface determines the amount of antigen bound to the immunosensor and, therefore, it is an important parameter for optimization. The antigen / antibody reaction is a phenomenon that is denominated the equivalence zone, that is, only when the antibody and antigen are in ideal dilution can a significant interaction occur between them. In the region of an excess of antibody there is less reaction. Observing Figure 4, it is possible to verify that at concentrations above 0.19 μg mL^{-1} the response becomes almost constant and this concentration was selected for subsequent experiments.

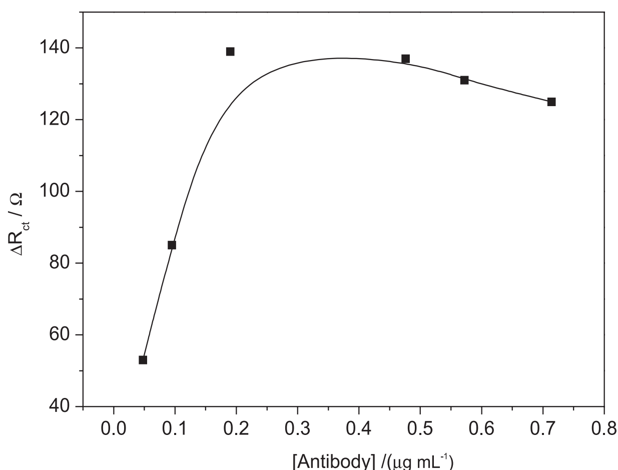

Figure 4. Effect of concentration of anti-mycelium incubated onto the electrode surface in the immunosensor response using 1.5 μg mL^{-1} of antigen in 5 mmol L^{-1} $Fe(CN)_6^{3-/4-}$ in pH 7.4 PBS solution in the frequency range from 100 kHz to 10 mHz with 10 mV of amplitude.

Nonspecific interactions

Usually, nonspecific adsorption is a major problem in label-free immunosensing, since it cannot be distinguished if the increase of R_{ct} was caused by an immunoreaction. One efficient alternative to minimize this effect is the blocking of free reactive sites by a selective layer. In this work 0.05% (m/v) gelatin, 0.1% (m/v) bovine serum albumin (BSA) and 10 mmol L^{-1} glycine were tested as blocking solutions after antibody immobilization. The impedance responses were monitored for each blocking reagent: (*i*) immunosensor blocking and antigen adsorption and (*ii*) immunosensor blocking and healthy soybean leaf extract addition at the same concentration, used as a control. This extract was not reacted with the immobilized anti-mycelium. According to Table 2, the best blocking agent was the BSA solution because the R_{ct} values are very different for contaminated and healthy leaves, indicating that BSA blocks the free sites on the surface, leaving only the available recognition sites for antigen binding.

Table 2. Effect of the blocking agent on the impedance response using an Au-SAM-Ab immunosensor for the antigen with healthy leaf extract used as control in 5 mmol L^{-1} $Fe(CN)_6^{3-/4-}$ in pH 7.4 PBS solution for the frequency range from 100 kHz to 10 mHz with 10 mV of amplitude

Blocking agent	Contaminated leaves ΔR_{ct}^* / Ω	Healthy leaves ΔR_{ct}^* / Ω
0.5% (m/v) Gelatin	148	84
0.1% (m/m) BSA	119	3
10 mmol L^{-1} Glycine	168	97

*the RSD of measurements is about 5.5%.

Optimal immuno-reaction time between anti-mycelium and antigen

The dependency of the impedance shift on the reaction time between the antigen and anti-mycelium is shown in Figure 5. The immunosensor was coated with 20 μL of 1.5 μg mL^{-1} of pH 7.4 antigen solution for different times. The results show that a reaction time shorter than 8 min seems to be insufficient for complete immunoreaction. Above this time, the impedance response is slightly higher, but there is saturation in the response after the second addition of antigen because the complex of the anti-mycelium and soybean leaf extract became unstable. In this way, an interaction time of 8 min was selected for subsequent studies.

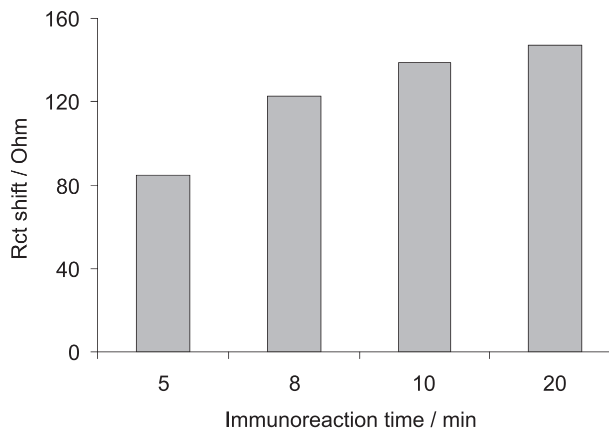

Figure 5. The dependency of the impedance shift on the reaction time between the antigen and anti-mycelium using 1.5 μg mL^{-1} of antigen in 5 mmol L^{-1} $Fe(CN)_6^{3-/4-}$ in pH 7.4 PBS solution for the frequency range from 100 kHz to 10 mHz with 10 mV of amplitude.

Immunosensor performance

Evaluating of the immunoreaction was carried out by exposing the biosensor Au-SAM-Ab-BSA to various concentrations of antigen. The corresponding Nyquist plots of impedance spectra are shown in Figure 6. It was found that the diameter of the Nyquist circle increased on adding antigen, demonstrating that the analyte interacts with the immobilized antibodies.

Figure 7 shows the curve obtained using the biosensor response as a function of antigen concentration. A linear relationship between the ΔR_{ct} and the concentration of contaminated leaf extract was found in the range 0.35-3.5 µg mL^{-1}, having a sensitivity of 292 Ω µg^{-1} mL. The correlation coefficient was 0.995 (n = 10). The detection limit of the proposed immunosensor was measured to be 315 ng mL^{-1}. A recently published paper has described Surface Plasmon Resonance monitoring of the *Phakopsora pachyrhizi* fungus mycelium[24] and the detection limit found was 800 ng mL^{-1}. This information confirms the higher detectability of the proposed method using EIS for the early diagnosis of soybean rust.

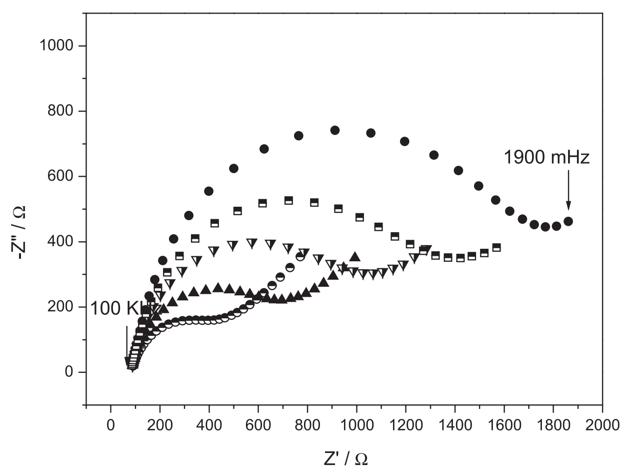

Figure 6. Nyquist plots for impedance measurements in 5 mmol L^{-1} $Fe(CN)_6^{3-/4-}$ in pH 7.4 PBS solution for the frequency range from 100 kHz to 1900 mHz with 10 mV of amplitude for the immunosensor after incubation with different concentrations of antigen from 0.35 to 3.5 µg mL^{-1}.

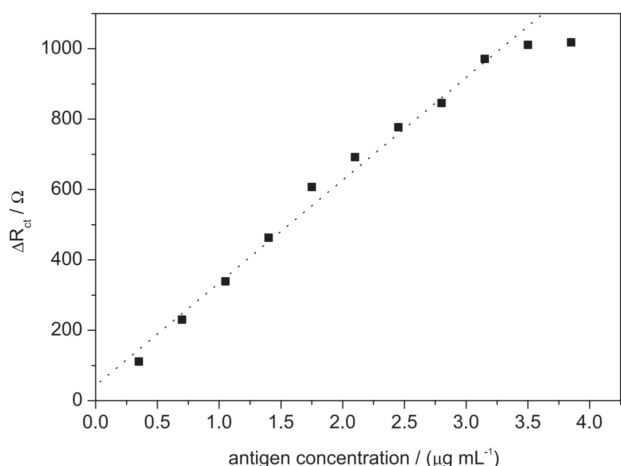

Figure 7. R_{ct} measurements obtained for the immunosensor in different concentrations of antigen in 5 mmol L^{-1} $Fe(CN)_6^{3-/4-}$ in pH 7.4 PBS solution for the frequency range from 100 kHz to 10 mHz with 10 mV of amplitude.

The reproducibility of the biosensor for mycelium of *Phakopsora pachrizi* was investigated with intra and inter-assays precision. The intra-assay of the immunosensor was evaluated with three replicates prepared independently under the same experimental conditions and the inter-assay used the same device for six measurements. The intra and inter-assay variation coefficient obtained for 1.5 µg mL^{-1} of antigen were 6.2 and 5.5%, respectively, indicating acceptable precision and fabrication reproducibility.

Conclusions

In this paper, a label-free impedimetric immunosensor for the detection of mycelium of *Phakopsora pachrizi* was developed by immobilizing anti-mycelium antibodies on self-assembled cysteamine monolayers via covalent coupling on a gold working electrode, presenting good sensitivity. Therefore, this method has potential application to diagnose Asian soybean rust in the initial stages of infection, allowing fast and efficient control against the disease dissemination.

Acknowledgments

The authors thank Embrapa Soybean of Londrina (PR, Brazil) for the donation of the contaminated soybean leaves and FAPESP, CNPq and INCT-Bioanalytic for financial support.

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Received: November 13, 2008
Web Release Date: May 6, 2009

FAPESP helped in meeting the publication costs of this article.