Impedimetric Biosensor Based on a *Hechtia argentea* Lectin for the Detection of *Salmonella* spp.

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Abstract: A sensitive electrochemical detection method for *Salmonella* spp. was described, based on the use of *Hechtia argentea* lectin immobilised on a screen-printed gold electrode. The lectin was extracted from *Hechtia argentea*, a plant belonging to the Bromeliaceae family. The lectin with molecular weight near 27.4 kDa showed selectivity towards D-mannose, contained on the lipopolysaccharide cell wall of *Salmonella* spp. Carbohydrate selectivity of the lectin was measured as a change in impedance with respect to concentration. The binding of the bacteria to the biosensor surface increased impedance with increasing concentrations of *Salmonella* spp., achieving a linear range of detection of $15 - 2.57 \times 10^7$ CFU mL$^{-1}$, with a limit of detection of 5 CFU mL$^{-1}$. Increases in impedance were measured using electrochemical impedance spectroscopy and analysed using Nyquist plots. The biosensor was applied in analysis of hen egg samples, and the results were consistent with those obtained using the official analysis methodology.

Keywords: biosensors; impedance spectroscopy; lectins; *Salmonella*; screen-printed electrodes

1. Introduction

Detection of different bacterial species is important in food safety and public health [1], due to their health implications. *Salmonella* spp. is one of the most frequent pathogens found in foods such as poultry, eggs, dairy products, fruits and vegetables [2,3]. They are facultative anaerobes, and are a Gram-negative bacillus from the family Enterobacteriaceae. *S. enterica* and *S. bongori* can cause disease due to their capacity to adapt to a variety of hosts, including humans [4]. *Salmonella* spp. can cause salmonellosis, affecting millions of people and causing more than 100,000 deaths per year [5].

The detection and identification methods of *Salmonella* spp. are based on the determination of physiological and biochemical markers of the organism. These methods are carried out using different techniques such as traditional culture [6–8], biochemical and serological testing [8–11], immunomagnetic separation [12–14], enzyme-linked immunosorbent assay (ELISA) testing [15–18] and polymerase chain reaction (PCR) [8,19,20]. Although most of these methods are optimal for detection and identification at low concentrations, they are time-consuming and laborious [21].

An alternative to current analysis methods is the use of biosensors, which use selective recognition agents to promote selectivity, reducing erroneous diagnoses with the advantages to provide reliable,
sensitive results in shorter times and less expensive than other methodologies [22]. A biosensor uses a biological recognition element and a transducer [23,24].

Impedimetric biosensors are the most-used alternative for microbial electrochemical analysis, due to their lower limit of detection and feasibility of miniaturization [25].

Among the biological recognition elements, lectins (proteins from plants) have attracted interest for use in biosensors due to their selective interaction with carbohydrates, either in free form or bound to macromolecules. Lectins have a high specificity towards the terminal carbohydrates of glycoproteins and lipopolysaccharides. For this reason, lectin-based biosensors have been used for cancer diagnosis and bacterial and viral analysis [26].

Lipopolysaccharides comprise a family of complex macromolecules, and are the main structural component in the surface of Gram-negative bacteria cell walls [27]. The molecules comprise three distinct regions: lipid A, a central oligosaccharide, and O-specific chains. The enormous structural variation of central oligosaccharides, particularly the O-specific chains between bacterial genera, and even within individual bacterial species, could be useful for analytical purposes [28,29].

*S. enterica* contains repeated units of D-galactose, D-mannose, and L-rhamnose in the O-specific chain [30]. It is reported that the Bromeliaceae family contains lectins selective to D-mannose [31,32].

This work describes the construction of an impedimetric biosensor for the detection of *Salmonella* spp., through the immobilization of a lectin extracted from *Hechtia argentea* (HA) as a recognition agent on a screen-printed gold electrode. *Hechtia argentea* was evaluated as a lectin source, taking into account that it is a plant belonging to the Bromeliaceae family and this species is endemic to Mexico [33].

2. Materials and Methods

2.1. Lectin Extraction and Evaluation

All solutions were prepared in deionised water with resistance not less than 18 MΩ·cm, purified by a Milli-Q system (Millipore, Bedford, MA, USA).

The HA was provided by the Biological Research Center of the Autonomous University of Hidalgo State. HA leaves were dried at room temperature and then pulverized in a mortar. The dried material (10.0 g) was stirred for 60 min in 150 mM NaCl (50.0 mL) (J.T. Baker, Phillipsburg, NJ, USA). The mixture was left for 16 h at 4 °C, and then filtered through gauze. The liquid phase was centrifuged (15 min, 4000 rpm) and stored at −20 °C [34]. Protein content was estimated by the Bradford method using bovine serum albumin (Sigma-Aldrich; St. Louis, MO, USA) as standard [35]. Protein molecular weight was determined by polyacrylamide gel electrophoresis (SDS-PAGE) (11% resolving gel and 4% stacking gel), using Coomassie Brilliant Blue G-250 for protein staining [36].

Lectin presence in HA extracts was confirmed by the evaluation of hemagglutinating activity of the aqueous extract. HA extract (50.0 µL) was mixed with human blood type A, B, and O (50.0 µL each), and the mixture was incubated at 37 °C for 30 min. Agglutination was observed under a microscope at 40× magnification. Negative controls used a 150.0 mM NaCl solution in place of HA extract. Inhibition of hemagglutinating activity was evaluated with the following carbohydrates: N-acetyl-D-glucosamine, N-acetyleneuraminic acid, L-fucose, D-fructose, D-galactose, D-glucose, D-maltose, and D-mannose (all Sigma-Aldrich). Inhibition tests were performed following the reported procedure, adding 0.5 M carbohydrate solution (50.0 µL) for both tests. Negative controls used a 150.0 mM NaCl solution in place of carbohydrate [37].

2.2. Biosensor Construction

The biosensor was constructed on the surface of a low-temperature curing, screen-printed gold electrode (DropSens 220BT, 4 mm diameter, Asturias, Spain). Gold was used as a working electrode, and silver was used as both the auxiliary and pseudo-reference electrodes. A 25.0 mM solution (10.0 µL) of 16-mercaptohexadecanoic acid (MHDA; Sigma Aldrich, St. Louis, MO, USA) in anhydrous absolute ethanol (J.T. Baker) was deposited on the electrode surface. The droplet was dried for 24 h at
25 °C, then the electrode was rinsed twice with ethanol, and left to dry at 25 °C. The surface was then activated with 20.0 mM N-(3-dimethylaminopropyl)-N-ethylcarbodiimide (EDC; Sigma Aldrich) and 5.0 mM N-hydroxysulfosuccinimide (NHS; Sigma Aldrich) solution (10.0 μL each) for 1 h at 25 °C. Then, the biosensor was rinsed with phosphate buffer solution (PBS) at pH 7.4, containing: 137.0 mM NaCl, 3.0 mM KCl, 10.0 mM Na₂HPO₄ and 2.0 mM KH₂PO₄, with 0.5 mM of CaCl₂, ZnCl₂ and MgCl₂. Once the electrode surface was activated, the HA extract (40.0 μL) was applied and allowed to stand for 1.5 h. The electrode was then immersed in a 20.0 mM ethanolamine (Sigma Aldrich) solution (prepared in deionised water) for 30 min, in order to block the still-active positions. The biosensor was rinsed and stored in PBS at 4 °C prior to use [38].

Micrographs of the biosensor surface were obtained by scanning electron microscopy (SEM; Carl Zeiss, model EVO-50, Oberkochen, Germany). The biosensor was evaluated after analyzing a sample containing 10⁵ CFU mL⁻¹ of Salmonella spp.

2.3. Impedimetric Measurements

All impedimetric measurements were carried out in a Metrohm Autolab galvanostat potentiostat electrochemical system (model PGSTAT302N, Amsterdam, The Netherlands), controlled through Nova 2.0 software.

Cyclic voltammetry was used to select the direct current potential used for impedance measurements. Figure 1 shows the cyclic voltamograms of Fe(CN)₆⁴⁻/Fe(CN)₆³⁻ redox couple at different modified electrode surfaces. A reversible Nersitan charge transfer was observed at Au bare surface at 350 mV (vs. Ag) whereas an absence of current peaks for modified surfaces was obtained due to the formation of a coating, indicating an increase in electron transfer resistance.

![Figure 1. Cyclic voltammograms of the electrode surfaces at different stages of biosensor preparation, obtained in 40 μL of 5.0 mM Fe(CN)₆⁴⁻/Fe(CN)₆³⁻ solution, scan rate 20 mV s⁻¹.](image)

Impedance measurements were performed at the formal potential of the Fe(CN)₆⁴⁻/Fe(CN)₆³⁻ redox pair. A direct current potential of 350 mV and an alternating current potential of 5 mV were applied. Impedance was determined by electrochemical impedance spectroscopy (EIS), at 25 frequencies from 0.010 Hz to 100 kHz. A volume of 40.0 μL of either the standard or sample was placed on the biosensor for 15 min at 25 °C, then the electrode was rinsed with PBS solution, and the impedance measurement was performed with 40 μL of the redox probe (5.0 mM potassium hexacyanoferrate (III) +5.0 mM...
potassium hexacyanoferrate (II) trihydrate) [38]. The impedance spectra were plotted in the form of Nyquist plots.

The biosensor was tested employing carbohydrates, which inhibited agglutination, at concentrations between $1 \times 10^{-7}$–$1 \times 10^{-4}$ M. *Listeria monocytogenes* and *Salmonella* spp. were provided by the Food Microbiology laboratory at Autonomous University of Hidalgo State. Bacteria were grown in tryptic soy broth (Bioxon Becton Dickinson, Mexico), and incubated at 37°C for 24 h. Decimal dilutions were made in 0.85% saline solution to obtain a concentration of $10^5$ CFU mL$^{-1}$ of each microorganism, to be analysed by EIS.

To evaluate the proposed biosensor in real samples for comparison with the traditional microbiological culture method, 10 samples of hen eggs were obtained from different commercial establishments in Pachuca, Hidalgo, Mexico. These were kept in polyethylene bags and stored at room temperature from their purchase until the analysis. An egg inoculated with $10^7$ CFU mL$^{-1}$ of *Salmonella* spp. acted as the positive control, and an egg washed with ethanol acted as the blank/negative control. A single egg was placed in lactose broth (LB; Bioxon) (225 mL) for 60 min at room temperature, then the egg was removed, and the broth was incubated for 24 h at 37°C for pre-enrichment. For selective enrichment, 1.0 mL of the broth solution was placed in 10.0 mL of Rappaport-Vassiliadis broth (RVB; Bioxon) for 24 h at 37°C. Two aliquots were taken, the first (40.0 μL) for analysis employing the biosensor, and the second for inoculation in *Salmonella-Shigella* (Bioxon) and brilliant green (Bioxon) for 24 h at 37°C. Both *Salmonella-Shigella* and brilliant green agars are selective media for the growth of *Salmonella* spp. [39,40] (Figure 2).

![Figure 2. Analysis scheme for Salmonella spp. detection. The sample is placed for pre-enrichment in lactose broth (LB) and enrichment in Rappaport-Vassiliadis broth (RVB). (A) Analysis of presumptive colonies, employing the microbiology method, involving inoculation in solid media (*Salmonella-Shigella* and brilliant green agars). (B) Analysis employing the biosensor, using 40 μL of RVB.](image)

3. Results and Discussion

3.1. Lectin Evaluation

The presence of lectin in the HA extract was determined by analysing the agglutination of the erythrocytes [41–43]. Three types of human blood showed agglutination in the presence of HA extract. Agglutination was observed as accumulations of the erythrocytes, which was not shown in their respective negative controls. The agglutination inhibition test for free carbohydrates is based on the affinity of lectin for these molecules [43–46]. The results showed that the carbohydrates that inhibited agglutination were N-acetyl-D-glucosamine, N-acetylneuraminic acid and D-mannose, indicating that the lectin presented active sites that allowed it to interact with these carbohydrates.

The total protein content in the HA extract was 131.7 mg L$^{-1}$. Analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis allowed us to identify the amount of proteins contained in the HA extract, as well as the molecular weight of the protein, when compared to a protein standard of known molecular weight. After staining polyacrylamide gel with Coomassie Brilliant Blue G-250,
a single band near 27.4 kDa was observed, indicating that the HA extract could be used without any additional purification process.

The extracted lectin has a similar carbohydrate affinity compared to other lectins. For example, Cratyli a mollis lectin (31 kDa) showed an affinity for D-glucose and D-mannose [47]. It was used in the development of an impedimetric biosensor based on self-assembled hybrid cysteine-gold nanoparticles for the analysis of S. enterica [48]. Also, a Triticum vulgaris lectin (36 kDa), with an affinity for glycoconjugates with N-acetylglucosamine or N-acetyleneuraminic acid residues, was used for magnetic separation of S. enterica [49].

3.2. Biosensor Impedimetric Evaluation

Impedimetric biosensors are based on the immobilization of a biological recognition element related to the analyte. When recognition occurs on the electrode, changes in the interfacial electron transfer impedance occur. The interfacial changes generated by the recognition processes are detected by EIS [50], and are proportional to the concentration of the analyte [50,51].

The formation of self-assembled monolayers (SAMs) is one of the most-used methods for the immobilization of antibodies in immunoassays. The immobilization of the recognition agent on a surface requires the following characteristics: (1) the recognition element must be oriented with minimum steric impediment to effectively interact with the analyte, and (2) the recognition agent should not lose its ability to interact with the analyte. The main methods of immobilization are the formation of imines with glutaraldehyde and the formation of amides with the addition of EDC/NHS [38,52]. The amide formation offers a high conversion efficiency, mild reaction conditions, and excellent biocompatibility, with little influence on the bioactivity of the target molecules and much cleaner products than other reagents, such as glutaraldehyde [38,53].

In this work, the biosensor was evaluated for measuring the interaction between lectin and carbohydrates that inhibited agglutination in blood. Solutions of N-acetyl-D-glucosamine, N-acetyleneuraminic acid, and D-mannose ($1 \times 10^{-7}$–$1 \times 10^{-4}$ M), were analysed, and an increase in impedance with the concentration of carbohydrates was observed (Figure 3A), while no significant changes were observed during analysis of D-galactose (Figure 3B). The lectin extracted from HA presented an affinity for N-acetyl-D-glucosamine, N-acetyleneuraminic acid and D-mannose. These carbohydrates are contained in the cell wall of some Salmonella spp. [48,54,55] and could be exploited when designing a device for microbiological analysis of Salmonella spp.

![Figure 3](image)

The biosensor was applied for the analysis of independent solutions of a Gram-positive (L. monocytogenes) and a Gram-negative (Salmonella spp.) bacteria (each $10^7$ CFU mL$^{-1}$). The analysis of L. monocytogenes did not reveal any change in impedance (Figure 4A). Although a positive response
was expected in *Listeria* spp., it being a Gram-positive bacterium with a cell wall containing high levels of peptidoglycan, a linear polymer made up of N-acetyl-D-glucosamine and N-acetylmuramic acid that interacts with amino acids [56]. However, since peptidoglycan is not the terminal carbohydrate, there is no interaction with the recognition agent from the HA lectin. An increase in impedance was observed when *Salmonella* spp. was analysed (Figure 4B). This impedance increase can be attributed to the lectin—*Salmonella* spp. interaction; the bacterium contains an external membrane made up of lipopolysaccharides containing different terminal carbohydrates, including D-mannose as the terminal carbohydrate [30].

The interactions that occur in the recognition between a lectin and carbohydrates are hydrogen bonding, metal coordination, van der Waals forces and hydrophobic interactions [26]. In microbial biosensors, the interaction occurs between microorganisms (lipopolysaccharides in the cell wall) and the recognition element (lectin) [57]. Figure 5 shows the recognition scheme for *Salmonella* spp. with the biosensor.

**Figure 4.** Nyquist plots of (A) *L. monocytogenes*, (B) *Salmonella* spp. Both analyses were performed at a concentration of 10^5 CFU mL^{-1}.

**Figure 5.** Recognition scheme for *Salmonella* spp. with the biosensor.
Based on the results of agglutination inhibition and the change in the impedance of the device, it is probable that the lectin contained in HA extract interacts with D-mannose contained in the lipopolysaccharide of Salmonella spp.

Figure 6A shows an SEM micrograph of gold electrodes cured at low temperature, where an irregular surface with small agglomerations acting as single microelectrodes can be observed. The micrograph of the biosensor employed for analysis of 10^5 CFU mL^-1 Salmonella spp. (Figure 6B) shows a layer formation on the electrode surface, and the presence of Salmonella spp. bacteria. This can be attributed to the lectin–carbohydrate interaction.

![Figure 6. (A) SEM micrograph of the unmodified gold electrode surface (DropSens 220BT). (B) SEM micrograph of the surface of the gold electrode modified with Salmonella spp. (10^5 CFU mL^-1).](image)

3.3. Sample Analysis

The biosensor was applied for analysis of hen eggs. The proposed methodology for the EIS analysis involves pre-enrichment, which restores the damaged bacterial cells to a stable physiological condition, then a selective enrichment used to increase the population of Salmonella spp. by inhibiting the growth of other organisms present in the sample, subsequently reducing interferences from other bacteria [6,8,21]. The analysis for Salmonella spp. was constructed using the Nyquist plots, showing that, in a concentration range of 10^3–10^7 CFU mL^-1, there is an increase in impedance with respect to the logarithm of the concentration of Salmonella spp. (Figure 6). The biosensor resistance (ΔR) vs. logarithm of the concentration of Salmonella spp. were linear in the same interval (Figure 7), with a limit of detection of 5 CFU mL^-1 and a limit of quantification of 15 CFU mL^-1 (R^2 = 0.9902). The reproducibility was evaluated analysing with three independent biosensors samples containing 0, 10^5 and 10^6 CFU mL^-1, the relative standard deviation obtained from the analysis was 3.45, 1.69 and 0.90%, respectively. The values obtained were adequate for a biosensor.

Lectins are selective recognition elements for the detection of pathogens, in addition, the collection and extraction processes were not complicated. Lectins were investigated for the detection of some pathogens due to their ability to bonding selectively to the surfaces of viruses and bacteria [58]. Table 1 shows some lectins applied to analyse pathogens, Concanavalin is the most studied lectin and it was used for the detection of Escherichia coli. Currently, the development of electrochemical biosensors based on different lectins for the detection of different pathogens important to human health is an interesting area in biosensing [59].
was not observed using the biosensor. The positive control showed the expected change in impedance.

Salmonella Chemosensors 2020

observed. The results obtained are preliminary, but there must be test more samples, including other samples. For all test samples and the negative control sample, a change in impedance in the analysis achieving a limit of detection of 100 CFU mL⁻¹ carbon electrode, immobilizing specific monoclonal antibodies to Salmonella based on a self-assembled gold nanoparticle monolayer and grafted ethylenediamine on a vitreous comparable with other biosensors based on specific antigens. Yang et al. [65] developed a biosensor compared to other lectins used for bacterial analysis (Table 1). Furthermore, the LOD obtained is limit of detection of 5 CFU mL⁻¹ logarithm of the concentration of that, in a concentration range of 10³–10⁷ CFU mL⁻¹logarithm of the concentration of bacteria [6,8,21]. The analysis for the growth of other organisms present in the sample, subsequently reducing interferences from other condition, then a selective enrichment used to increase the population of analysis involves pre-enrichment, which restores the damaged bacterial cells to a stable physiological

The biosensor was applied for analysis of hen eggs. The proposed methodology for the EIS analysis for 10 hen eggs, including negative and positive control samples. For all test samples and the negative control sample, a change in impedance in the analysis was not observed using the biosensor. The positive control showed the expected change in impedance. The microbiological analysis was congruent, and presumptive colonies of Salmonella spp. were not observed. The results obtained are preliminary, but there must be test more samples, including other analytical matrices, in order to evaluate if the biosensor is robust.

![Nyquist plots](image)

**Figure 7.** Nyquist plots of Salmonella spp. in the concentration range of: (a) 0, (b) 10³, (c) 10⁴, (d) 10⁵, (e) 10⁶ and (f) 10⁷ CFU mL⁻¹.

| Lectin Source | Recognition Carbohydrate | Bacteria | Detection Technique | LOD          | Ref  |
|---------------|--------------------------|----------|---------------------|--------------|------|
| *Arachis hypogaea* | N-acetyl-D-galactosamine and D-galactose | *L. monocytogenes* | Surface plasmon resonance | - | [55] |
| *Canavalia ensiformis* | D-glucose and D-mannose | *E. coli DH5α* | EIS | 75 cell/mL | [60] |
| *Canavalia ensiformis* | D-glucose and D-mannose | *E. coli W1485* | Square wave voltammetry | 25 cell/mL | [61] |
| *Canavalia ensiformis* | D-glucose and D-mannose | *E. coli* | Surface plasmon resonance | 12 CFU/mL | [62] |
| *Cratylia mollis* | D-glucose | *Serratia marcescens, E. coli, S. enterica and K. pneumoniae* | EIS | - | [63] |
| *Ricinus communis* | N-acetyl-D-galactosamine and D-galactose | *E. coli DH5α* | EIS | - | [64] |
| *Triticum vulgaris* | N-acetylneuraminic acid | *E. coli O157: H7* | Surface plasmon resonance | 3 × 10⁵ CFU/mL | [55] |
| *Hechtia argentea* | N-acetyl-D-glucosamine, N-acetylneuraminic acid and D-mannose | *Salmonella spp.* | EIS | 5 CFU/mL | This work |

On the other hand, the biosensor based on lectin obtained from Hechtia argentea is competitive compared to other lectins used for bacterial analysis (Table 1). Furthermore, the LOD obtained is comparable with other biosensors based on specific antigens. Yang et al. [65] developed a biosensor based on a self-assembled gold nanoparticle monolayer and grafted ethylenediamine on a vitreous carbon electrode, immobilizing specific monoclonal antibodies to Salmonella spp. in gold nanoparticles, achieving a limit of detection of 100 CFU mL⁻¹. The proposed biosensor was used to analyse 10 hen eggs, including negative and positive control samples. For all test samples and the negative control sample, a change in impedance in the analysis was not observed using the biosensor. The positive control showed the expected change in impedance. The microbiological analysis was congruent, and presumptive colonies of Salmonella spp. were not observed. The results obtained are preliminary, but there must be test more samples, including other analytical matrices, in order to evaluate if the biosensor is robust.
4. Conclusions

*Hechtia argentea* contains a lectin with a molecular weight near 27.4 kDa that is selective towards N-acetyl-D-glucosamine, N-acetylneuraminic acid, and D-mannose, allowing recognition of *Salmonella* spp. The biosensor achieved a limit of detection of 5 CFU mL\(^{-1}\) and a linear range of detection of 15–2.57 × 10\(^7\) CFU mL\(^{-1}\). The biosensor was applied successfully to the analysis of hen egg samples, and the results obtained were consistent with those obtained from the official analysis methodology. The proposed technique presents advantages over the official methodology for the detection of *Salmonella* spp., such as higher simplicity and shorter analysis time.

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