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AMPEROMETRIC BIOSENSOR FOR *Salmonella* Typhimurium DETECTION IN MILK

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ABSTRACT - This paper reports an amperometric biosensor for rapid and sensitive *Salmonella* Typhimurium detection in milk. The biosensor was assembled from the self-assembled monolayers technique on a gold surface. In this device, polyclonal antibodies were oriented by protein A. The biosensor structure was characterized by cyclic voltammetry and the analytical response was obtained by a chronoamperometry technique using a sandwich system labeled with peroxidase enzyme. The performance of the biosensor was determined and the device was tested in skim and whole milk. The response curve of the biosensor showed a low limit of detection of 10 UFC mL^{-1} and detection time of 125 min. The results of the biosensor performance in milk attested that the device was able to detect *S.* Tiphymurium quickly in a simple laboratory setting and without enrichment step.

KEYWORDS: *Salmonella*; biosensor; electrochemical; milk

1. INTRODUCTION

Salmonella can cause three types of diseases depending on the serotype, salmonellosis (all serotypes except *S.* Typhi and *S.* Parathyphi), typhoid fever (serotype *S.* Typhi), and paratyphoid fever (serotype *S.* Parathyphi). The *Salmonella* detection in food can be carried out by conventional analysis methods based on isolation in culture media, which include steps of pre-enrichment and selective enrichment, followed by isolation on selective and differential media. The species and serotypes confirmation is accomplished by biochemical and serological tests and requires at least five days to reach diagnosis (Andrews et al., 2015).

Currently, there are many alternative methods for *Salmonella* detection applied to immunoassays, nucleic acid techniques, miniaturized biochemical assays and biosensors. Biosensors are a quick and convenient alternative to conventional analytical measures for detection of foodborne pathogens. Different transducers may be used efficiently in biosensors for the rapid pathogen detection (Arora et al., 2013). The electrochemical biosensors are based on potentiometric, amperometric or impedimetric transducers. The amperometric measurements are based on electrical current between

the working and the counter electrode as a function of analyte concentration after applying a constant potential. According to literature, this technique is preferred in the development of many biosensors for *Salmonella* detection (Melo at al., 2016).

The base electrode must present appropriate electrochemical characteristics and have to be compatible with the immobilization method. Gold electrodes have often been applied in biosensors, because gold is an inert metal (unreactive to atmospheric oxygen), non-toxic, and compatible with biomolecules and cell structures. Moreover, thiols adsorb spontaneously on gold and provide organized and stable monolayers (per the Self Assembled Monolayer, SAM technique) (Gooding and Darwish, 2012). Cysteamine is a short chain thiol, which acts as a bridge between the electrode and protein of interest by covalent bonds.

Protein A is widely used in biosensor assembly to orientate antibodies during the immobilization method. It binds the fraction Fc (constant region of the antibody) and allows the Fab fraction (variable region for antigen recognition) to be free to bind to antigens (Branco et al., 2012). This orientation is important for increasing specificity, stability and sensitivity of the biosensor. The choice of the secondary antibody marker is other important step. The use of horseradish peroxidase (HRP) enzyme has been largely used as a marker (Ricci et al., 2012). The HRP enzyme participates in the oxidation-reduction reactions, thereby generating the response measured by the electric current variation in the presence of H_2O_2. Low molecular weight mediators, as hydroquinone, can facilitate the redox reactions due to its high rate of electron transfer speed (Lermo et al., 2009).

The aim of this study was to develop and evaluate an amperometric biosensor from disposable gold electrode for the detection of *Salmonella* Tiphymurium in milk. The performance of the biosensor was evaluated in phosphate buffer and in milk samples previously contaminated with *S.* Tiphymurium in order to establish comparisons between the experimental and the diagnostic conditions.

2. MATERIALS AND METHODS

2.1 Chemicals and biochemical

Horseradish peroxidase (HRP) (250 U mg^{-1}), glutaraldehyde (25%), N-hydroxysuccinimide (NHS), N-ethyl-N′-(3- dimethylaminopropyl) carbodiimide hydrochloride (EDC), cysteamine, protein A, bovine serum albumin (BSA), hydroquinone and hydrogen peroxide were purchased from Sigma Aldrich (St. Louis, MO, USA). The culture medium, brain heart infusion agar (BHI agar), brain heart infusion broth (BHI broth), nutrient agar and nutrient broth were acquired from Difco™ (Becton, Dickinson and Company, Sparks, MD, USA). *Salmonella* Typhimurium ATCC 51812 was purchased from Microbiologics (Saint Cloud, MN, USA) and polyclonal rabbit antisera, *Salmonella* Antiserum Poly A-I & Vi from Difco™. The antibodies were purified by precipitation with $(NH_4)_2SO_4$ at 45% saturation (Green and Hugs, 1995), and the concentration was determined by the Bradford method (1976). The secondary antibody was conjugated to the enzyme HRP according to Avrameas (1969).

2.2 Biosensor assembly

The base for assembling the biosensor was printed gold electrodes Dropsense - C220AT®. The gold electrode surface (Øint=0.5024 cm^2) was modified by using 10 mM cysteamine (cys) for 3 h at room temperature. Thereafter, protein A (prA) was immobilized through covalent bonding after the activation of carboxyl groups with EDC/NHS (Tlili et al 2004). Firstly, a solution containing 2 mM EDC and 5 mM NHS was freshly prepared in an acetate buffer (pH 5.0) and reacted for 30 min. Then, 7.5 mg mL^{-1} of protein A was added in EDC/NHS solution and left to react for 1h. In the next step, the

modified electrode with cys monolayer was incubated in protein A (prA)-EDC/NHS solution for 1 h. The electrode was washed with 0.1 mol L^{-1} PBS (pH 7.4) after each incubation procedure. Finally, the electrode was incubated overnight in an anti-*Salmonella* solution (75 mg mL^{-1}). Non-specified sides of the modified electrode were blocked with 1% BSA for 1 h.

2.3 Analytical response

For the response curve, the analytical response was obtained by immersing the biosensor in 100 µL of *S.* Typhimurium dilutions (0 to 10^6 CFU mL^{-1} in 0.1 mol L^{-1} PBS, pH 7.4) for 1 h. The *S.* Typhimurium dilutions were obtained from sub-culturing of standard strain on nutrient agar at 35 °C for 24 h into 10 mL nutrient broth at 35 °C for 24 h. Bacteria harvesting was obtained from centrifugation at 4500 rpm at 25 °C for 30 min. The material was resuspended in 10 mL of 0.1 mol L^{-1} PBS (pH 7.4). Bacteria concentrations were confirmed by spread plate technique.

The electrode was incubated with labeled secondary antibodies for 1 h. After rinsing with PBS, the electrode was ready for analytical response. The response was determined by polarizing the gold electrode at -75 mV until a stable baseline (steady state) was reached in 120 s. Electrochemical measurements were performed using potentiostat/galvanostat Autolab/ PGSTAT12 and the GPES software (Eco Chemie, The Netherlands), at room temperature.

2.4 Biosensor characterization

Each step of biosensor assembling was electrochemically characterized by cyclic voltammetry studies using solution of 4 mM $K_3[Fe(CN)_6]$ and 1M KCl applying a potential range from -0.30 to 0.75 V and a scan rate of 100 mV s^{-1}. Scanning electron microscopic and ATR-IR spectroscopic analyses were performed. A scanning electron microscope (SEM; Quanta 450 FEG System: FEI Company, USA) was used to characterize the surface morphology of the biosensor. The images were obtained using a scanning voltage of 15 kV. ATR-FTIR analysis of the modified surface was measured directly by pressing the electrode against the ATR crystal in a spectrometer (model FTLA 2000-102, ABB-BOMEN, USA). All spectra were recorded in the range from 600 to 4000 cm^{-1} at 4 cm^{-1} resolution, averaging over 128 scans.

2.5 Milk analysis

The performance of the biosensor was evaluated in samples of whole and skimmed milk previously contaminated with strains of *S.* Typhimurium ATCC 51812. For contamination of samples, *S.* Typhimurium dilutions were used at three different concentrations: a low (10^1 CFU mL^{-1}), an intermediate (10^3 CFU ml^{-1}) and a high (10^6 CFU mL^{-1}) concentration obtained according to item 2.3.

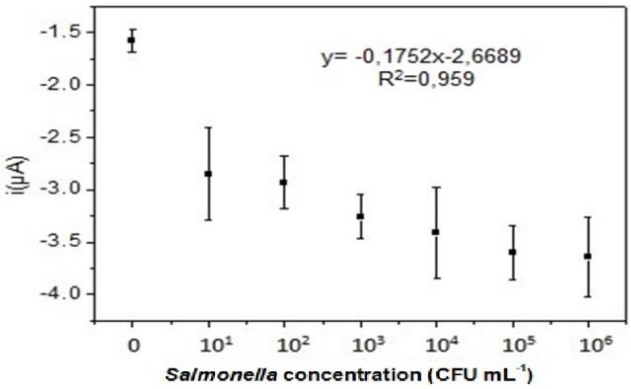
3. RESULTS AND DISCUSSION

3.1 Response curve of the amperometric biosensor

The amperometric response at PBS solution is presented in Figure 1. The biosensor presented a limit of detection (LOD) of 10 CFU mL^{-1}, which was obtained from the equation 1 where, X is the blank response, *t* is the student distribution factor, and SD is the standard deviation.

$$LOD = X + t\,(n\text{-}1,\ 1\text{-}\alpha) \times SD \qquad (1)$$

Figure 1– Response of the biosensor for *S.* Typhimurium, concentrations from 10^1 to 10^6 CFU ml^{-1}, -75 mV potential, PBS solution (pH 7.4) containing 3 mM hydroquinone and 300 mM H_2O_2.

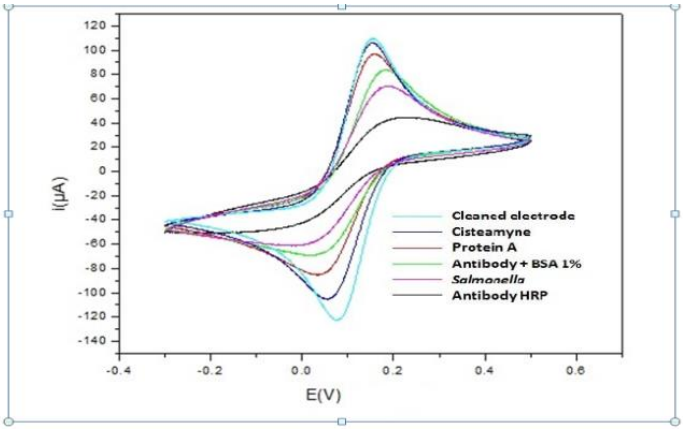


The biosensor developed showed a very low LOD compared to other biosensor for *Salmonella* detection. In the literature are found LODs of 10^2 CFU mL^{-1} (Oh et al., 2004; Mantzila et al., 2008), 10^3 CFU mL^{-1} (Bae et al., 2005), and even higher values. Moreover, the detection time of 125 min makes the current biosensor a very interesting device.

3.2 Biosensor characterization

The cyclic voltammograms demonstrated changes in the electrode surface after each assembly step (Figure 2).

Figure 2. Cyclic voltammograms in 4 mM K3[Fe(CN)6] and 1M KCl solution after each step of biosensor assembly: cleaned electrode; cys monolayer (10 mM); prA (7.5 mg mL^{-1}); anti-*Salmonella* (75 mg mL^{-1}) and BSA (1%); HRP-labeled antibody (75 mg mL^{-1}).

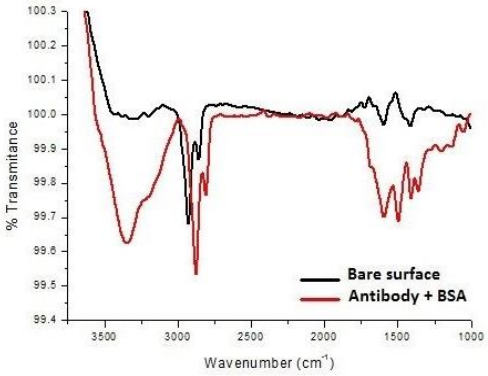


The penetration of the redox probe close to surface electrode in the cys monolayer was slightly reduced. This effect occurs because cys normally forms a thin and well-ordered monolayer due to organized chemisorption on the gold surface. PrA is a single polypeptide chain that results in a small electrical current decrease. The subsequent steps showed a significant decrease in the peak current amplitude of the redox probe, because the experiment involved large-size molecules, such as antibody and *Salmonella* cells.

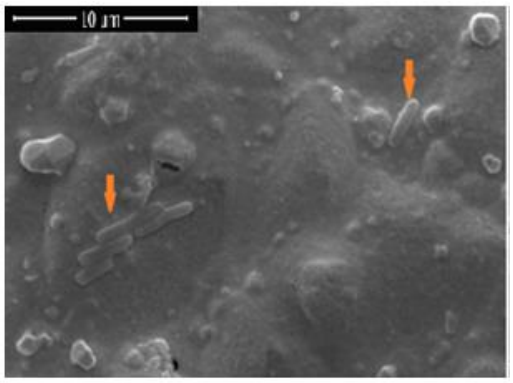
The ATR-FTIR characterization confirmed the surface modification. The modified surface spectrum was compared with the bare electrode spectrum (Figure 3a). The biosensor surface was also studied by scanning electron microscopy (SEM). The image showed *S.* Typhimurium cells on the surface, confirming the antigen-antibody interaction (Figure 3b).

Figure 3- (a) ATR-FTIR spectrum of bare electrode and modified surface. (b) Scanning electron photomicrograph of the biosensor surface after biosensor assembly. The arrows indicate the *S.* Typhimurium captured by the antibody (11000x, 15 kV Quanta FEG).

(a)



(b)

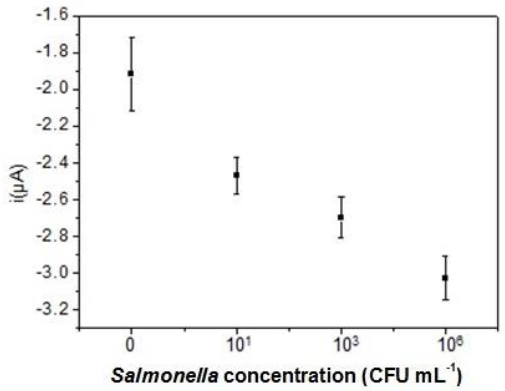


3.3 Analysis of milk samples

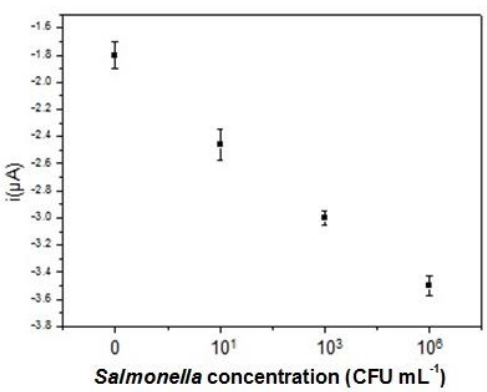
The biosensor detection limit for skim milk and whole milk was kept at 10 CFU mL^{-1} (Figure 4). This result confirmed the biosensor applicability in food. Moreover, a good linearity was noted suggesting a potential for quantitative analysis. The biosensor does not need sample pre-enrichment, which is required for most of the conventional rapid methods for *Salmonella* detection (Lee et al., 2015).

Figure 4 - Amperometric response of the biosensor at different concentrations of *S.* Typhimurium at (a) skimmed and (b) whole milk samples. Results obtained in 10mM PBS buffer (pH 7.4) in the presence of H_2O_2 (300 mM) and hydroquinone (3 mM), potential 75 mV for 120 s.

(a)



(b)



4. CONCLUSIONS

An efficient amperometric biosensor for *Salmonella* Typhimurium detection was developed. The performance of this device is advantageous because it does not require a pre-enrichment step and takes a short time to obtain the results. This aspect is very important for food suppliers that need constant monitoring of food quality. The applicability of this method in milk samples was proven in skim and whole UHT milk.

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