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Development of an impedimetric immunosensor based on electropolymerized polytyramine films for the direct detection of *Salmonella typhimurium* in pure cultures of type strains and inoculated real samples

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ABSTRACT

The development of a faradic impedimetric immunosensor based on electropolymerized polytyramine (Ptyr) films for the detection of *S. typhimurium* in milk is described for the first time. Polyclonal anti-*Salmonella* was cross-linked, in the presence of glutaraldehyde vapors, on Ptyr-modified gold electrodes. The dielectric behaviour of Ptyr films was evaluated with capacitance measurements, while their stability in neutral aqueous solutions was examined with impedimetric measurements. The effect of the concentration of tyramine in the forming solution on both the sensitivity and the dynamic range of the resulted immunosensors was also investigated. The alteration of the interfacial features of the electrodes due to different modification or recognition steps, was measured by faradic electrochemical impedance spectroscopy in the presence of a hexacyanoferrate(II)/(III) redox couple. At samples containing a low initial concentration of 10 cfu mL^{-1} *S. typhimurium*, that actually defines the LOD of the immunosensors, signal changes of 33% and 88% were achieved after 3 and 10 h incubation, respectively. To achieve the working simplicity expected by a biosensor, immunoreaction was performed directly in cultures. This resulted in the elimination of various centrifugation and washing steps, which are used for the isolation of bacteria cells from the culture, thus making the proposed immunosensors promising candidates for on-site applications. Finally, the proposed immunosensors were successfully used for the detection of *S. typhimurium* in experimentally inoculated milk samples.

1. Introduction

S. typhimurium is a Gram-negative foodborne pathogen and it is recognised as the second most common serotype (after *S. enteritidis*) of *Salmonella* found in humans [1]. Due to the inadvertent contamination of foods and water the detection of this pathogen is extremely important for the safety of food products. Bearing in mind that the pathogen has a very high proliferation rate and exists in very complex matrices, there is a need for advanced monitoring methods and techniques.

Although the standard techniques in pathogen detection are sensitive and selective enough, involving enrichment of samples and culture plating procedures, are usually elaborate and need several days for presumptive results and confirmation [2]. Many types of *Salmonella* detection tests have been developed during the last decades, involving enzyme-linked

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immunosorbent assay (ELISA) [3,4] and nucleic acid-based polymerase chain reaction (PCR) technology [5,6]. However, ELISA requires extra enzyme-labelled antibodies, while the PCR based assay is often more labor-intensive than ELISA [7].

A variety of alternative methods have been also reported for the detection of *Salmonella* including the monitoring of oxygen consumption with cyclic voltammetry [8], acoustic wave sensors based on filamentous bacteriophages [9] and indirect fiber-optic biosensors [10]. Other approaches, which are based on quartz crystal microbalance immunosensors [11–13], conductimetric immunosensors [14] and magnetoelastic resonance biosensors [15] have also been proposed.

Of the methods available for the detection of *Salmonella*, impedance microbiology has been successfully used in both standard and real samples by measuring either the impedance of selective growth media or the interfacial impedance of the electrodes [16–18]. However, these methods do not provide the analytical simplifications of biosensors. Similarly, electrochemical ELISA immunosensors [19,20], even though they have been successfully used in real samples, they require extra enzyme labels and intense sample clean up to operate, thus restricting any prospective use of them for on-site testing. On the other hand, electrochemical impedance spectroscopy (EIS)-based immunosensors offer the ability of label-free immunosensing; however, the majority of the published works have not been tested in real matrices, since fundamental issues still exist, and this in turn has brought the reliability of impedimetric immunosensors into question [21].

The aim of this work was the development and the optimization of impedimetric immunosensors specific to *S. typhimurium* that will be able to operate directly in pure type strain cultures and inoculated real samples, without the need to employ time-consuming centrifugation and washing steps for the isolation of the cells, in order to enable their potential use for on-site applications.

2. Experimental

2.1. Chemicals and solutions

Glutaraldehyde (GA) (~25% in water; kept in sealed vials under argon at +4 °C) and bovine serum albumin (BSA) were purchased from Sigma (USA). Absolute ethanol, denaturated ethanol, potassium ferrocyanide and potassium ferricyanide were from Merck (Germany). Tryptone soy broth (TSB) and affinity-purified rabbit polyclonal antibodies against *Salmonella* (~4 mg mL^{-1} in 10 mM PBS pH 7.2) were purchased from LAB M (UK) and Biodesign (USA) respectively. Fresh skimmed pasteurized milk was purchased from the local market. All other chemicals were from Merck and Sigma, and double distilled water (DDW) was used throughout.

A 10 mM phosphate-buffered saline (PBS) solution containing 140 mM NaCl, pH 7.2 was used for the dilution of the antibody stock solution. A 50 mM phosphate buffer (PB) solution pH 7 was used for the activation of amine groups with glutaraldehyde, whereas a 50 mM PBS solution containing 100 mM KCl, pH 7 was used in various washing and post-blocking steps, and as electrolyte of the redox couple in EIS measurements. All buffer solutions were filtered through a 0.2 μm pore size membrane (Millipore, Ireland) and stored at 4 °C for up to 2 weeks.

2.2. Bacteria and colony forming unit (cfu) determinations

The tested bacteria were *Salmonella typhimurium* (4,5:i:1,2) (from National School of Public Health, Laboratory of Microbiology, Athens) and *E. coli* NCTC 9001 (from Public Health Laboratory Service-North, Newcastle, England). The bacteria cultures were grown in TSB at 37 ± 1 °C for 24 h before use, and the number of viable cells was determined by viable count method. Antigen samples were then prepared by serial dilutions of the stock culture with the broth solution.

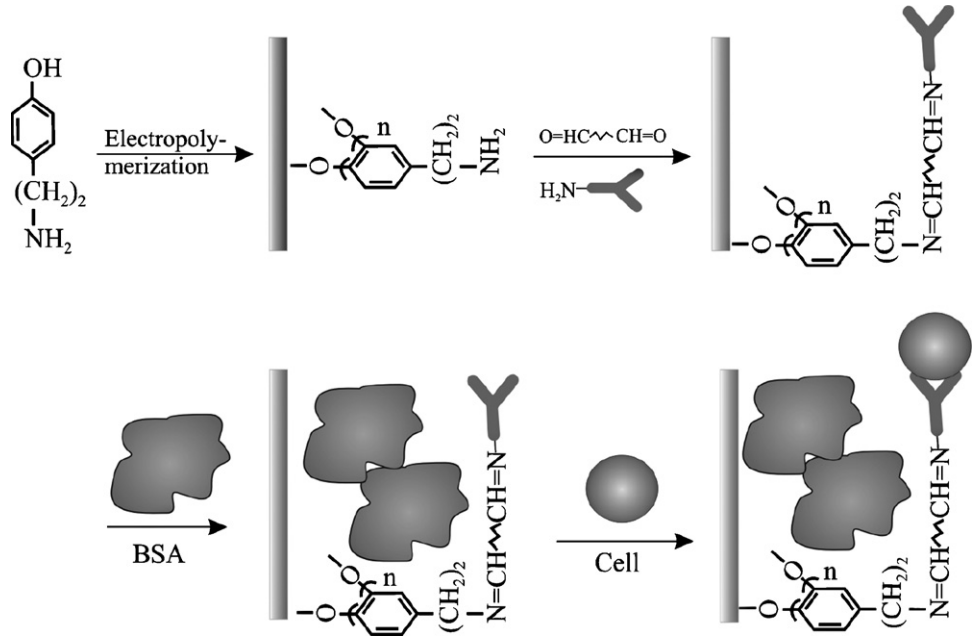
2.3. Apparatus

EIS and cyclic voltammetry (CV) experiments were performed with the electrochemical Analyzer AUTOLAB12/FRA2 (Eco Chemie, The Netherlands) in a one-compartment three-electrode cell. Gold electrodes were used as working electrodes and a platinum wire (diameter of 1 mm and length of 3 cm) served as the auxiliary electrode. The reference electrode was a Ag/AgCl/3 M KCl (IJ Cambria, UK) electrode and all potentials reported hereafter refer to the potential of this electrode. The impedance spectra were recorded over a frequency range of 10^{-1} to 10^{5} Hz, using a sinusoidal excitation signal, superimposed on a *dc* potential of +0.200 V. Excitation amplitude of 10 mV (rms) was used throughout. All faradic EIS measurements were performed in a solution of 5 mM hexacyanoferrate (II)/(III) (1 + 1 mixture) in PBS solution (pH 7) at room temperature. Capacitance measurements were performed in the same buffering system in the absence of the redox probe with an LCR meter (INSTEK 801, Taiwan) at 100 Hz, a zero *dc* potential and *ac* amplitude of 10 mV (rms).

2.4. Formation of electropolymerized films and immobilization of the antibodies

Glassy carbon (3 mm diameter), platinum (2 mm diameter) and gold electrodes (2 mm diameter) were purchased by IJ Cambria (UK). Before use, they polished with Al_2O_3 (0.01 μm grain size), rinsed thoroughly with DDW and sonicated for 1 min in DDW. After polishing, gold and platinum electrodes were cleaned by dipping into a solution of 1 + 1 + 5 (v/v), 25% NH_4OH + 30% H_2O_2 + H_2O for 10 min. Alternatively, gold electrodes were electrochemically cleaned by cycling within the potential window 0–1.6 V with a scan rate 100 mV s^{-1} in 1 M $HClO_4$ or 0.5 M H_2SO_4, until they reach a stable baseline. Then, they were rinsed with DDW and dried with argon.

Electropolymerization of tyramine was performed in an unstirred methanol solution containing 0.3 M NaOH and different concentrations (0.01, 0.025, 0.05 or 0.1 M tyramine) of the monomer (forming solution). The polytyramine-modified electrodes were rinsed with DDW and amine ends were activated in a degassed solution of 2.5% glutaraldehyde in PB solution (pH 7) for 1 h under mild stirring. Then, electrodes were rinsed several times with the same buffer solution to remove the physically absorbed glutaraldehyde. The *Salmonella* antibodies (anti-SA) were introduced onto



Scheme 1 – Tentative view of the different modification and recognition steps. Drawings are not to scale.

the Au/Ptyr/GA-activated electrodes by dropping 10 μL of ~1 mg mL^{-1} antibody in PBS (pH 7.2) and allowed to incubate at +4 °C for at least 12 h in a humidified glass chamber. Alternatively, both activation and immobilization steps were performed simultaneously in a class chamber saturated with glutaraldehyde vapors overnight at +4 °C. After the immobilization of the antibody the electrodes were rinsed with PBS solution (pH 7) to remove the excess of physically bound antibody and immersed in the solution of the blocking agent (1–3 mg mL^{-1} BSA in PBS, pH 7) for 2 h at room temperature to block nonspecific binding sites.

2.5. *Procedures*

2.5.1. *Optimization studies*

The stock culture was serially diluted with TSB and samples of different initial concentrations of *S. typhimurium* over the range of ~10–10^7 cfu mL^{-1} were thus prepared. The 2-mL portions of them were introduced in sterilized glass vials. Ready-to-use immunosensors [fully functionalized (Au/Ptyr/GA/anti-SA) electrodes after their incubation in the solution of the blocking agent] were immersed in the standard culture samples for a specified time interval at room temperature under mild stirring. Immunosensors were then thoroughly rinsed with PBS solution (pH 7) and transferred to the measuring cell.

2.5.2. *Application to milk samples*

The 0.9 mL portions of fresh pasteurized milk were inoculated with 100 μL of the bacteria culture containing 10^7 or 2×10^4 cfu mL^{-1} *S. typhimurium* (positive controls) or *E. coli* (negative controls) and kept under natural conditions for 1 h. Then aliquots (100 μL) of inoculated and non-inoculated (used as blank to evaluate the effect of the matrix components on the measuring signal) milk samples were mixed with 1.9 mL TSB in sterilized glass vials. The rest of the procedure was similar to that followed during the optimization studies.

2.6. *Safety considerations*

All glassware in contact with bacteria must be sterilized for 1.5 h at +121 °C before and after use. Before heating, glassware was immersed sequentially in baths of 0.1 M HCl and denatured ethanol. Waste solutions containing bacteria must be sterilized before disposal.

3. Results and discussions

3.1. *Optimization studies and build up of the immunosensors*

A tentative view of the different modification and recognition steps of the BSA-blocked Au/Ptyr/GA/anti-SA immunosensors is shown in Scheme 1. Electropolymerized polytyramine films have been extensively used as immobilization platforms for the construction of biosensors [23,24]. Their electropolymerization has been shown to occur through the *ortho* position of the phenol group, thus allowing the amino group present on each monomer to be used for the covalent binding of the antibody trough the formation of Schiff bases with the formyl ends of glutaraldehyde [25–27]. In addition, Ptyr films exhibit a remarkable stability over a wide pH range, according to the findings of Wu et al. [28].

The dielectric behaviour of Ptyr films was evaluated by capacitance measurements and CV experiments before and after the formation of the electropolymerized films. Similarly to previous studies [28], CV experiments that performed after

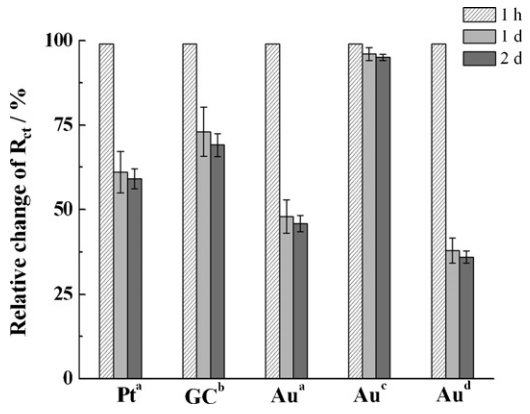


Fig. 1 – Relative signal change of R_{ct} (%) at electropolymerized Ptyr electrodes after 1 and 2 days in PBS pH 7 with respect to the value of R_{ct} observed 1 h after the formation of the film, which taken as 100%. The different cleaning procedures of the electrodes are as follows: (a) polishing and dipping in a mixture of 1 + 1 + 5 (v/v), 25% NH_4OH + 30% H_2O_2 + H_2O for 10 min, (b) polishing, (c) polishing and cycling within the potential window 0–1.6 V with a scan rate of 100 mV s^{-1} in 0.5 M H_2SO_4, or (d) 1 M $HClO_4$. Forming solution, 0.1 M tyramine. Measuring conditions, 10^{-1} to 10^5 Hz at + 0.200 V bias (10 mV rms). Electrolyte, 5 mM hexacyanoferrate(II)/(III) (1 + 1 mixture) in PBS solution, pH 7. Error bars represent 95% confidence limits (n = 3).

the electropolymerization of tyramine in the presence of 5 mM hexacyanoferrate (II)/(III) in 50 mM PBS pH 7, showed no redox peaks (data not shown), thus verifying the formation of the film; however, we did not succeed to measure pure capacitive currents. More specifically, after an electropolymerization process of eight scans, the original capacitance of the bare electrodes was decreased and signal changes of 19%, 45% and 49% were observed at GC/Ptyr, Pt/Ptyr and Au/Ptyr-modified electrodes, respectively, while, in all cases, the recorded phase angle values were lower than 80°.

Based on the above data, and given that faradic impedance measurements are more sensitive [21], further studies were performed with a frequency response analyzer in the presence of 5 mM hexacyanoferrate (II)/(III) redox couple.

The stability of the electropolymerized films in PBS (pH 7) solution was extensively studied at different GC/Ptyr, Pt/Ptyr and Au/Ptyr-modified electrodes by recording their impedimetric spectra over a total period of 2 days. This parameter is of great importance, especially in applications dealing with antibody–antigen interactions, where long incubation times are used. The impedimetric stability of different Ptyr-modified electrodes is illustrated in Fig. 1. Based on the recorded impedimetric signals is obvious that each of the tested Ptyr-modified electrodes has a different conditioning period, which is probably due to the interactions of the electropolymerized films with the active groups exist in each electrode or with the different active groups occur in the same electrode material after a specific treatment. These interactions in combination with the different hydrophilicity of the tested electrode surfaces might also affect the adhesion, the texture or the elasticity of the polytyramine films thus resulting in a different ageing behaviour in each case. The detailed explanation of this behaviour is out of the scope of this work and cannot be elucidated with the data given.

Aiming to develop faradic impedimetric immunosensors over a nonconductive layer, it is obvious that we are interested in achieving a partially covered electrode surface, or in other words, to form a non-compact electropolymerized film in order to ensure a sufficient diffusion of the redox probe to the surface of the electrode, at least during the first steps of the sensors' build up. The degree of coverage can be controlled by either the number of the scans of the electropolymerization process, even though after the first scan the overwhelming part of the film has already been formed [22–27], or the concentration of the monomer in the forming solution.

The effect of the concentration of the monomer on the value of R_{ct} of the Au/Ptyr electrodes as well as on the sensitivity and the dynamic range of the corresponding immunosensors are shown in Fig. 2A and B, respectively. The relative change of the signal of the ready-to-use immunosensors before and after the immunoreaction, ΔR_{ct} (%), which is expressed as $\Delta R_{ct} = \{[R_{ct}$ (after the immunoreaction) $- R_{ct}$ (before the immunoreaction)$]/R_{ct}$ (before the immunoreac-

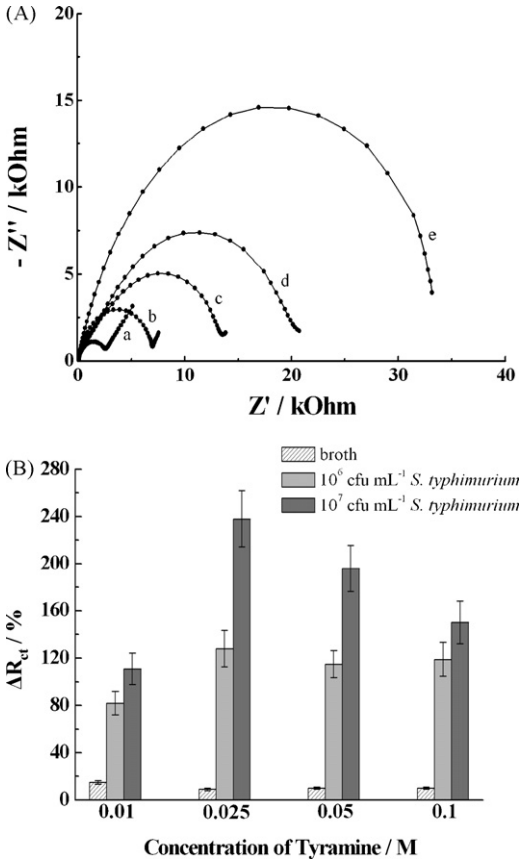


Fig. 2 – (A) Nyquist plots of (a) Au bare and Au/Ptyr electrodes occurred after the electropolymerization of different concentrations of the monomer; (b) 0.01, (c) 0.025, (d) 0.05 and (e) 0.1 M tyramine. (B) Comparative performance of the resulting immunosensors to standard cultures of *S. typhimurium*. Detection time, 3 h. Measuring conditions as in Fig. 1. Error bars represent 95% confidence limits (n = 3).

tion)} × 100 was taken as a measure of the concentration of bacterial cells in standard and real samples. In this study, R_{ct} values correspond to the real impedance component at 0.1 Hz, since attempts to model the impedimetric data as a Randle's or different two-time constants equivalent circuits, showed a poor fit to the models, indicating that the circuit was considerable more complex.

As can be seen in Fig. 2A, R_{ct} values are dependent on the concentration of tyramine in the forming solution. Low concentrations of tyramine lead to the formation of films that apparently provide a less effective barrier to the electron transfer of the redox probe compared with that formed at higher concentrations of tyramine. In addition, by changing the concentration of the monomer we were able to alter the population of the amine groups at each film. The number of the binding groups determines the performance of the immobilized antibodies, as it affects both the amount and the conformation of them over the electropolymerized films. Concentrated solutions of tyramine result in higher loadings of glutaraldehyde, and this may result in turn, in the immobilization of antibodies through multiple binding sites, thus reducing the flexibility (rigidity of binding) and the binding capacity of the antibodies as well as the accessibility of the target analyte to them. As can be seen in Fig. 2B, the best performance in terms of sensitivity and dynamic range was observed at immunosensors developed over Au/Ptyr electrodes occurred from the electropolymerization of a 0.025 M tyramine solution.

Further optimization regarding the efficiency of the immobilized antibodies includes the activation of the terminal amine groups by glutaraldehyde following procedures based on liquid or gas phase. The effect of each activation protocol on the impedimetric behaviour of the resulted Au/Ptyr/GA/anti-SA electrodes is depicted in Fig. 3. The increase of R_{ct} after the activation of the amine group in liquid phase (spectrum 3c) is due to the to the elimination of the positive charge of the free amine groups, which no longer attract the negatively charged redox couple as well as to the spontaneous

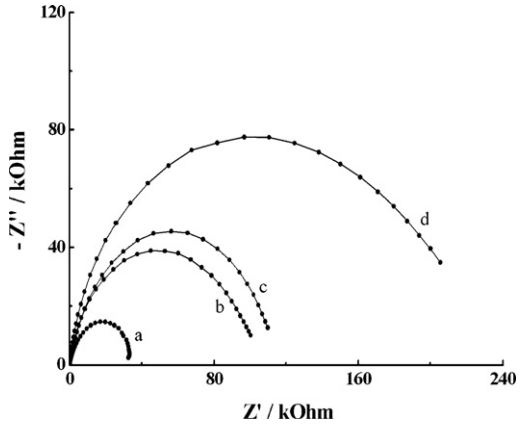


Fig. 3 – Nyquist plots of (a) Au/Ptyr and Au/Ptyr/GA/anti-SA immunosensors occurred from the activation of amine groups in (b) gas and (d) liquid phase. Spectrum (c) corresponds to Au/Ptyr/GA electrodes, an intermediate phase of electrodes (d). Forming solution, 0.1 M tyramine. Measuring conditions as in Fig. 1.

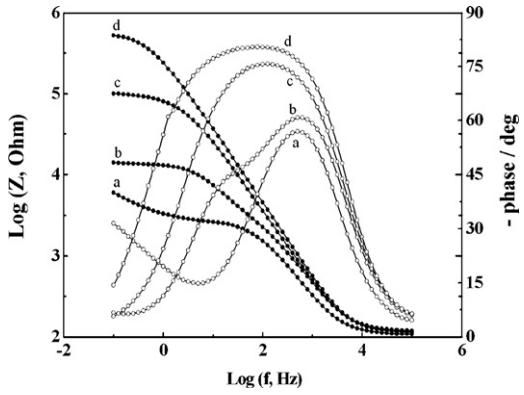


Fig. 4 – Bode plots showing the build up of the immunosensors: (a) Au bare, (b) Au/Ptyr, (c) Au/Ptyr/GA/anti-SA, and (d) after the incubation of (c) in 10^7 cfu mL^{-1} *S. typhimurium* for 3 h. Filled symbols, impedance profile; empty symbols, phase profile. Forming solution, 0.025 M tyramine. Measuring conditions as in Fig. 1.

polymerization of glutaraldehyde in the presence of oxygen [28,29], although the necessary measures for the minimization of the latter effect have been taken. Further increase of the R_{ct} (spectra 3c to 3d) is attributable to the immobilization of the antibody. A similar assessment of the amount of the immobilized antibody is not however possible at the gas phase protocol, as in this case, both the activation and the immobilization steps are proceed simultaneously. Thus, in order to evaluate the two different activation processes, the resulted immunosensors in each case were compared taking as criterion their sensitivity to a standard sample containing an initial concentration of 10^6 cfu mL^{-1} *S. typhimurium*. Best results (data not shown) were taken with electrodes being activated in gas phase and thus this protocol was followed in further studies.

The effect of the bioreceptor loading on the sensitivity of the immunosensors was examined for three discrete antibody concentrations, that is, 0.5, 1 and 2 mg mL^{-1} in PBS (pH 7.2) solution. Higher response was observed at an antibody concentration of 1 mg mL^{-1}, and therefore, further experiments were performed, applying the useful antibody content. The impedimetric response at each modification step and the successful application of the immunosensors for the assessment of antibody–cell interactions is shown in Fig. 4.

3.2. *Performance of the immunosensors*

The effectiveness of the proposed concept, that is, the performance of the immunoreaction directly in the culture sample has been proven and well documented in a previous study of our group [29]. Besides the working simplicity that is provided from the elimination of time-consuming clean up sample procedures, the response of the immunosensors increases with respect to the detection time (incubation time) as a consequence of the simultaneously proliferation of the viable bacterial cells in the tested samples. Subsequently, the response of the immunosensors is practically insensitive to

the presence of dead cells. The increase of the signal with respect of the incubation time is illustrated in Fig. 5. Ready-to-use immunosensors were incubated in standard cultures containing low initial concentrations of 10 and 100 cfu mL^{-1} *S. typhimurium* and signal changes after 3, 6 and 10 h were recorded. After a 3 h incubation time, analytical useful signal changes of 33% and 42%, respectively were achieved and a further increase of them to 88% and 130%, respectively was observed after an incubation period of 10 h. Actually, for an incubation time of 3 h, the limit of detection of the immunosensors is 10 cfu mL^{-1} *S. typhimurium*, while, under the same experimental conditions, signal changes of 66%, 90%, 120% and 190% were achieved at samples containing 10^4, 10^5, 10^6, and 10^7 cfu mL^{-1} *S. typhimurium*, respectively (data not shown).

A key-point however for the successful application of this concept, is to address the nonspecific adsorption of the exotoxins (enterotoxins and cytoxins with a MW ~70–88 kDa [30]) that produced and liberated in the culture medium during the proliferation of Gram-negative bacteria. The effect of the concentration of BSA (blocking agent) on the performance of the immunosensors is illustrated in Fig. 6. Blocking with 2 mg mL^{-1} BSA ensures negligible background (nonspecific) signals, which are due to the components of the culture or to other bacteria exist in the sample. Optimized immunosensors were further tested in milk samples being inoculated with 5×10^4 cfu mL^{-1} *S. typhimurium* (positive controls, P) and *E. coli* (negative controls, N) as well as in uninoculated milk samples used as blank. As revealed from the bar-chart in Fig. 6, the application of the method to real samples results in the appearance of background signals, which are due to the macromolecules (casein's micelles, lipid acids, etc.) exist in the matrix. Using a 3 mg mL^{-1} BSA and at the cost of a lower sensitivity (see Fig. 6), only a minor decrease of the background signal was achieved, and therefore, blocking of the immunosensors with 2 mg mL^{-1} BSA seems to be the best comprise among the elimination of background signals (light gray bars), selectivity (*P*/*N* signal ratio) and sensitivity (gray bars).

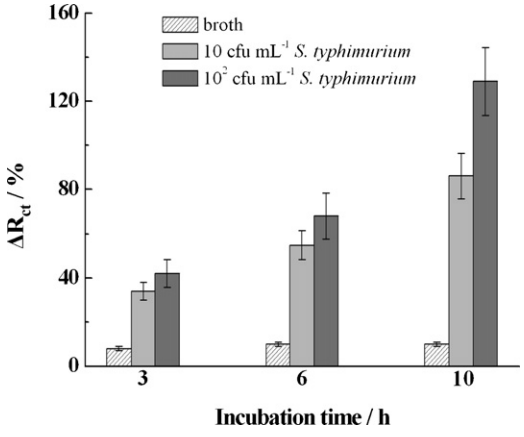


Fig. 5 – Response of BSA-blocked Au/Ptyr/GA/anti-SA electrodes after their incubation in standard cultures of *S. typhimurium* for different time intervals. BSA, 2 mg mL^{-1} Forming solution, 0.025 M tyramine. Measuring conditions as in Fig. 1. Error bars represent 95% confidence limits (n = 3).

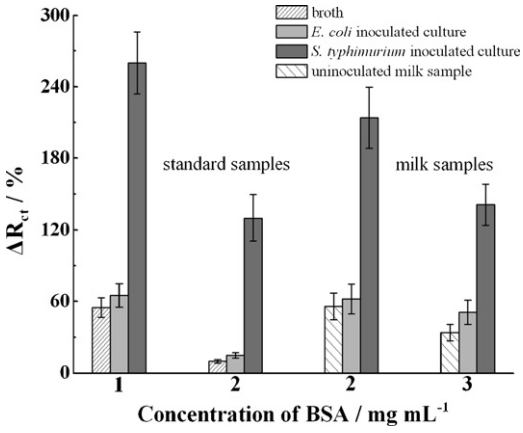


Fig. 6 – Response and specificity of BSA-blocked Au/Ptyr/GA/anti-SA electrodes after their incubation for 3 h in cultures of standard solutions with an initial concentration of 10^6 cfu mL^{-1} *E. coli* and *S. typhimurium* and for 6 h in milk samples inoculated with 5×10^4 cfu mL^{-1} *E. coli* and *S. typhimurium*. Forming solution, 0.025 M tyramine. Measuring conditions as in Fig. 1. Error bars represent 95% confidence limits (n = 3).

The above results certify the applicability of the method in milk samples; however, due to the appearance of background signals, the safe application of the method in samples inoculated with an initial concentration of pathogens as low as 100 cfu mL^{-1} requires longer incubation time intervals (approximately 10 h), in order to receive a signal adequately larger compare with that corresponds to the background signal (data not shown). It should be pointed out however, that this time is practically equal to the total time of analysis. After the enrichment step, a simple impedimetric measurement (it takes about 10 min) is only required for the extraction of the analytical information. On the other hand, in conventional ELISA methods or ELISA-based electrochemical sensors, the enrichment step is followed by a number of time-consuming steps (successive centrifugation and washing steps, incubation with extra enzyme-labelled antibody, etc.) that significantly elongate the total time of analysis.

4. Conclusions

This study employs functional impedimetric immunosensors, based on polytyramine electropolymerized films for the detection of *S. typhimurium* in real samples.

The best performing immunosensors were occurred from gold electrodes treated electrochemically in 0.5 M H_2SO_4, a forming solution of 0.025 M tyramine, and through activation of the terminal amine groups with glutaraldehyde vapors.

BSA-blocked immunosensors can be directly used in pure cultures of type strains and inoculated milk samples for the detection of *S. typhimurium* and thus could be a promising candidate for on-site testing.

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