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Label-free impedimetric biosensor for *Salmonella Typhimurium* detection based on poly [pyrrole-co-3-carboxyl-pyrrole] copolymer supported aptamer.

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ABSTRACT

The Gram-negative bacterium, *Salmonella Typhimurium* (*S. Typhimurium*) is a food borne pathogen responsible for numerous hospitalisations and deaths all over the world. Conventional detection methods for pathogens are time consuming and labour-intensive. Hence, there is considerable interest in faster and simpler detection methods.

Polypyrrole-based polymers, due to their intrinsic chemical and electrical properties, have been demonstrated to be valuable candidates for the fabrication of chemo/biosensors and functional surfaces. Similarly aptamers have been shown to be good alternatives to antibodies in the development of affinity biosensors.

In this study, we report on the combination of poly [pyrrole-co-3-carboxyl-pyrrole] copolymer and aptamer for the development of a label-less electrochemical biosensor suitable for the detection of

S. Typhimurium. Impedimetric measurements were facilitated by the effect of the aptamer/target interaction on the intrinsic conjugation of the poly [pyrrole-co-3-carboxyl-pyrrole] copolymer and subsequently on its electrical properties. The aptasensor detected *S. Typhimurium* in the concentration range 10^2 - 10^8 CFU mL⁻¹ with high selectivity over other model pathogens and with a limit of quantification (LOQ) of 100 CFU mL⁻¹ and a limit of detection (LOD) of 3 CFU mL⁻¹. The suitability of the aptasensor for real sample detection was demonstrated via recovery studies performed in spiked apple juice samples. We envisage this to be a viable approach for the inexpensive and rapid detection of pathogens in food, and possibly in other environmental samples.

Keywords: *S. Typhimurium*, aptamer, Electrochemical Impedance Spectroscopy, poly [pyrrole-co-3-carboxyl-pyrrole], label-free detection.

1.Introduction

The Gram-negative bacterium *Salmonella* is a major food borne pathogen for humans and animals. This pathogen is known as a major factor in gastrointestinal infections and is associated with numerous hospitalisations and deaths all over the world. *Salmonella* can be found in meat, eggs, milk and beverage. Among the over 2500 serotypes of *Salmonella*, one of the most common serotype related to human disease is *Salmonella enterica serovar typhimurium* (*S. Typhimurium*) (Kim et al. 2015; Lee et al. 2015; Ma et al. 2014). Traditional methods of detection of this pathogen are based on the combination of pre-enrichment steps, culturing on *Salmonella-Shigella* (SS) agar plates, and serological validation of suspicious colonies. Although these approaches can provide reliable results, they are quite time consuming and can take up to 5 days to obtain a result. Due to these drawbacks, the development of new and faster approaches for bacteria detection is a core activity in contemporary microbiology (Lee et al. 2015).

Immunological assays such as ELISA can detect *Salmonella* at level of 10^4 - 10^5 cells mL^{-1} ; and lower detection levels can be achieved by coupling ELISA methods with an enrichment step that usually takes between 16 and 24 h (Alakomi and Saarela 2009; Meng Xu et al. 2015). Polymerase chain reaction (PCR)-based methods have been shown to significantly improve the limit of detection down to 5 CFU (colony forming unit) mL^{-1} , but still these approaches require long and often complicated preparation steps, such as cell lysis and, often, DNA separation. Furthermore these methods do not have the ability to determine the viability of the pathogen at the time of the sampling (Zelada-Guilln et al. 2009; Zourob et al. 2008). Despite significant advances, current *Salmonella* detection methods are not fast, easy to perform, reliable or sensitive enough (Lee et al. 2015).

Conductive conjugated polymers (CP) are very attractive and versatile materials for the fabrication of functional interfaces and sensing surfaces. They combine the properties of metals and those of conventional polymer and have found application in various areas including extraction methods (Bagheri et al. 2013), fuel cells (Inamuddin et al. 2009), artificial muscles (Jager et al. 2000) and biomedical engineering (Guimard et al. 2007) as well as in chemosensors (Pattananuwat et al. 2015), biosensors (Li et al. 2015) and functional surfaces for cell stimulation (Svennersten et al. 2011), tissue engineering (Svennersten et al. 2011) and bacterial differentiation (Golabi et al. 2016a).

Among CPs, polypyrrole has found widespread application due to properties such as ease of synthesis in different solvents and at room temperature, good chemical stability and high conductivity. Even more interestingly, polypyrrole can be fabricated via electrochemical approaches, giving a high degree of control over thickness, shape, morphology and hydrophobicity (Balint et al. 2014; Janmanee et al. 2013; Kannan et al. 2011; Peng et al. 2007). However, polypyrrole suffers from the absence of functional groups and so copolymerisation of pyrrole with modified monomers has been explored as a possible route to provide polypyrrole films with new functionalities (Melling et al. 2015).

Copolymers such as poly[pyrrole-co-4-(3-pyrrolyl) butanoic acid, have been used for the fabrication of DNA hybridisation biosensors (Peng et al. 2005). DNA chips, fabricated using poly[pyrrole-COOH-co-pyrrole-DNA probe] copolymer, were used to detect DNA at levels as low as 10^{-15} mole mL^{-1} (Garnier et al. 2007) and the copolymer, poly[pyrrole-co-3-pyrrolylacrylic acid], has been used for the development of impedimetric genosensors (Peng et al. 2007).

Aptamers are short sequences of DNA or RNA that have the ability to bind with high affinity and specificity to their target. They are considered to have several advantages over their corresponding antibodies including small size, ease of synthesis and labelling, low cost of production and the absence of the need for animals to produce them (Zhu et al. 2012).

Electrochemical detection methods are widely used because of their ease of operation, high sensitivity and ready miniaturisation for use in portable device (Zhu et al. 2012). For example, an electrochemical immunosensor based on screen-printed gold electrodes, was developed for the chronoamperometric detection of *S. Typhimurium* down to 20 cell mL^{-1} (Salam and Tothill 2009). Zelada-Guillén et al. demonstrated the real-time potentiometric detection of *Staphylococcus aureus* in pig skin by the use of carbon nanotube/aptamer functionalised electrodes (Zelada-Guillén et al. 2012). Anodic stripping voltammetry was used for electrochemical dual-aptamer-based, sandwich detection of *Staphylococcus aureus* down to 1 CFU mL^{-1} (Abbaspour et al. 2015). Finally, a target-induced aptamer displacement strategy has been applied for the detection of *E. coli O111* via differential pulse voltammetry (Luo et al. 2012).

Label-free electrochemical detection is a very attractive option and has been demonstrated by the use of electrochemical impedance spectroscopy. For example an electrochemical impedance aptasensor for *S. Typhimurium* was constructed based on graphene oxide/gold nanoparticles and

was able to detect the pathogen in the concentration range 2.4×10^3 CFU mL⁻¹ (Ma et al. 2014). Furthermore, a screen-printed interdigitated microelectrode, in combination with magnetic-bead sample separation was applied for the rapid detection of *E. coli* O157:H7 and *S. Typhimurium* in food samples (Meng Xu et al. 2015). In addition, Labib et al, developed an aptamer-based impedimetric sensor with the ability to detect *S. enteritidis* down to 600 CFU mL⁻¹ in 10 minutes, and to distinguish it from other Salmonella species, including *S. typhimurium* and *S. choleraesuis* (Labib et al. 2012a). The same authors developed an impedimetric sensor via the self-assembly of thiolated aptamer onto a gold nanoparticle-modified screen-printed electrode. The tunable specificity of the aptamers, allowed live and killed bacteria be distinguished (Labib et al. 2012b).

Unfortunately however, all the label-free electrochemical approaches described above required the presence of a redox probe, more often ferro/ferricyanide, in the measuring solution. In the study presented herein, we report on the use of the poly[pyrrole-co-3-carboxyl-pyrrole] copolymer-aptamer bioconjugated for the development of a label-free electrochemical biosensor for the detection of *S. Typhimurium* based on the intrinsic variation of the electrical properties of the polymeric surface, without the need for the addition of redox probes. The potentiality of the proposed aptasensor was demonstrated via calibration, selectivity and recovery experiments, which demonstrated its specificity, sensitivity and applicability for the detection of *S. Typhimurium* in a food sample (apple juice).

2. Experimental

2.1. Materials

Analytical grade reagents without further purification, except for pyrrole monomer (vacuum distilled prior to use), were used in this work.

Pyrrole-3-carboxylic acid (Py-COOH) was purchased from Fluoro chem (Hadfield United Kingdom) and lithium perchlorate (LiClO₄) from Acros. N-hydroxysuccinimide (NHS), N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) and Pyrrole (Py) were obtained from Sigma-Aldrich (Stockholm, Sweden). PBS (pH 7.4) tablets were supplied by Medicago AB (Uppsala, Sweden).

The six bacterial strains used in this study were: *Escherichia Coli* (CCUG 3274, CCUG53375, and CCUG10979), *Enterobacter aerogenes* and *Citrobacter freundii*) and *S. Typhimurium*. The three *E.*

Coli strains were acquired from the Culture Collection, University of Gothenburg, Sweden, the other strains were provided, in kind, by Linköping University Hospital.

Aptamer with 5'-NH₂-ttt ggt cct tgt ctt atg tcc aga atg cga gga aag tct ata gca gag gag atg tgt gaa ccg agt aaa ttt ctc cta ctg gga tag gtg gat tat-3' sequence (N 45 in the original manuscript) was obtained from a previous work reported by Joshi A et al. (Joshi et al. 2009). The synthetic aptamer was purchased lyophilised from biomers.net (Germany). On arrival, the aptamer was reconstituted to a 100 µM stock solution with filtered (0.2 Micron filter) Milli-Q water, divided in small aliquots and stored at -20 °C until use.

All solutions were prepared in high purity water obtained from a Milli-Q RG system (Stockholm, Sweden).

2.2. Apparatus

An Ivium Stat.XR electrochemical analyser coupled with dedicated software (Ivium, Eindhoven, Netherlands) was used for electrochemical measurements, film deposition and cleaning of the gold surface. Attenuated total reflectance (ATR-FTIR) measurements were done with a PIKE MIRacle ATR accessory with a diamond prism in a Vertex 70 spectrometer (Bruker) using a DTGS detector; all measurements were performed at room temperature under continuous purging of N₂. IR spectra were obtained at 4 cm⁻¹ resolution and 32 scans between 4000 and 800 cm⁻¹. The Scanning Electron Microscopy (SEM) micrographs were taken using a Leo 1550 Gemini SEM operating at 6.0 keV. A Shimadzu UV-1601 PC spectrophotometer was used to obtain the concentration of cultured bacteria (600nm). Contact angle measurements were carried out using a CAM 200 optical contact angle meter (KSV Instrument, Helsinki, Finland).

2.3. Preparation of the film

Gold disk electrodes (internal diameter 2 mm from CHI Instruments, USA) were polished on polishing pad micro clothes with 1.0, 0.3 and 0.05 µm alumina powder (CHI Instruments, USA) to a mirror finish and then cleaned in a water ultrasonic bath. After the mechanical cleaning, the surface was immersed for 60 s in cold piranha solution (3:1 H₂SO₄ / H₂O₂) followed by an electrochemical activation step in H₂SO₄ (0.5 M) solution by cyclic voltammetry (25 cycles).

A copolymer of Pyrrole-3-carboxylic acid and Pyrrole (1:4 ratio in 100 mM LiClO₄ aqueous solution) was synthesised using a potentiostatic (constant potential) method at 0.7 V (vs Ag/AgCl,

KCl 3 M reference electrode) until the consumed charge reached 5 mC cm^{-2} . The prepared film was washed with deionised water and stored at 4°C until use.

For SEM and FT-IR images, the same procedure was applied on $5 \times 10 \text{ mm}$ Au chips produced by sputtering a $2000 \text{ \AA}$ Au film onto a Ti coated ($100 \text{ \AA}$) Si wafer.

2.4. Immobilisation of aptamer onto polymeric film

The presence of the carboxylic group in the poly[pyrrole-co-3-carboxyl-pyrrole] copolymer films allowed the covalent immobilisation of the aptamer (Chei et al. 2008; Tlili et al. 2005).

Functionalisation of the copolymer with aptamers was carried out by immersing the film in $40 \text{ }\mu\text{L}$ aqueous solution containing 50 mM NHS and 200 mM EDC for 30 min , in order to activate the carboxylic group present; this was, followed by incubation of the activated film with $20 \text{ }\mu\text{L}$ of a $2 \text{ }\mu\text{M}$ aqueous solution of the amino-functionalised aptamer for 60 min .

2.5. Preparation of bacterial solution

The six bacterial strains were cultured separately in Tryptic Soy Broth (TSB) for 18 h , at 37°C under gentle (170 rpm) shaking. The overnight cultured bacterial cells were then collected by centrifugation (5 min at 5000g), the supernatant discarded and the pellet resuspended in sterile phosphate buffered saline (PBS) solution ($\text{pH } 7.4$). Following three cycles of washing (accordingly to the procedure described above) the bacterial solutions were diluted to 10^8 CFU mL^{-1} (concentration calculated according to Mc Farland turbidity standards); these solutions were used as the working stock bacterial solutions. Serial dilutions ($10^2 - 10^8 \text{ CFU/mL}$) of standard stock solutions were prepared with LiClO_4 100 mM for measurement.

2.6. Impedance measurement

The impedance measurements were recorded from 10 kHz to 0.5 Hz , at amplitude of 5 mV , in 100 mM LiClO_4 solution without any redox probe. All measurements were performed at a bias potential of 0 mV vs the OCP (open circuit potential) of the film.

Measurement in the presence of the redox probing molecule (5 mM Ferro/Ferri equimolar solution) were also attempted; this resulted to be less reproducible and noisier (data not shown) making in this way favorable the measurements in 100 mM LiClO_4 solution alone.

2.7. Bacteria measurements

Detection of bacteria was performed accordingly to the following protocol: aptasensor were incubated for 45 min in the LiClO₄ 100 mM solution containing the bacteria or in the 1 to 100 diluted apple juice. Following a 15 min wash in LiClO₄ 100 mM the EIS spectra were recorded accordingly to protocol described in section 2.6. Calibration curve were performed by exposing the aptasensor to increasing concentrations from 10² to 10⁸ CFU mL⁻¹ of *S. Typhimurium*. For each concentration measurements were performed following the protocol described above. Following recording the EIS spectra the aptasensors were put in contact with the solution containing the following higher concentration to be investigated.

Please insert Scheme 1 here

3. RESULTS AND DISCUSSION

3.1. Characterisation of Copolymer

3.1.1. Scanning electron microscopy (SEM)

Fig.1 shows the SEM images (low and high resolution) of a polypyrrole (PPy) (Fig. 1A and Fig. 1B) and poly[pyrrole-co-3-carboxyl-pyrrole] copolymer (Fig. 1C and Fig. 1D) films synthesised according to the protocol described in the Experimental Section. Clearly the two surfaces showed different morphology; poly[pyrrole-co-3-carboxyl-pyrrole] copolymer film presented a smoother structure when compared with the PPy (Guimard et al. 2007). The PPy film was characterised by the well-known globular and compact structure of PPy. The smoother surfaces recorded in the case of the poly[pyrrole-co-3-carboxyl-pyrrole] copolymer film could be due to the reduced polymerisation speed (ca. 2.5 times slower than those of PPy) (Xu et al., 2005; Street et al., 1983; Golabi et al. 2016b) and to the presence of the COOH that introduced into the copolymer chain sterical and electrostatic hindrances (Korri-Youssoufi and Yassar 2001) resulting in a more planar, more flat linear chain.

Please insert Fig1 here

3.1.2. ATR-FTIR spectroscopic measurements

Copolymer formation was further confirmed by ATR-FTIR spectroscopic measurements (Fig. 1E). The poly[pyrrole-co-3-carboxyl-pyrrole] copolymer (blue line) showed a peak at 1680 cm^{-1} , which is associated with the C=O stretching mode of carboxylic groups (Peng et al. 2005) and which is clearly absent in the PPy film (red line). Furthermore a shift to higher frequencies of FT-IR spectrum of poly[pyrrole-co-3-carboxyl-pyrrole]copolymer was recorded; this was an indication of the reduced rotational freedom in the polymer probably due to the changes in the polymer backbone associated with the presence of COOH in it.

3.1.3. Cyclic voltammetry measurements

Electrochemical characterisation of the prepared films was performed using cyclic voltammetric and impedimetric measurements. Cyclic voltammograms of the prepared films were recorded in LiClO_4 100 mM solution; these experiments allowed a deeper understanding of the redox properties of the films (Fig 2A). The poly[pyrrole-co-3-carboxyl-pyrrole] copolymer showed two distinct couples of redox peaks ($-0.5/-0.6\text{ mV}$ and $-0.15/-0.16\text{ mV}$) attributed, respectively, to the pyrrole-COOH unit and to the pyrrole monomer oxidation/reduction (Chei W. et al 2008). On the other hand, when cyclic voltammetric experiments were recorded for the PPy film, only a pair of redox peaks at -0.15 V and -0.16 V was recorded. Another significant difference was that the poly[pyrrole-co-3-carboxyl-pyrrole] copolymer showed a quasi-reversible behavior, while the PPy demonstrated reversible behavior; these changes in redox properties could be associated with the reduced conductivity of the film, loss in polymer chain conjugation (Garnier et al., 2007), when the monomer with the COOH group was incorporated in the film (Chei W. et al 2008). This difference recorded in the voltammetric spectra confirms the successful electrodeposition of the poly[pyrrole-co-3-carboxyl-pyrrole] copolymer and that this has a block-polymer form (Korri-Youssoufi and Yassar 2001).

Please insert Fig2 here

3.1.4. Contact angle measurement

Contact angle measurements were used to study the wettability properties of the surface. The poly[pyrrole-co-3-carboxyl-pyrrole] copolymer showed a reduced contact angle when compared

to the PPy film (Table 1S). This reduction in contact angle is a clear indication of the increased hydrophilic character of the copolymer. This increased hydrophilicity was ascribed to the presence of the carboxylic acid groups and to their ability to form hydrogen bonds with the water (Truong et al. 2011)

3.1.5. Impedance measurement

Impedance measurements showed a reduced intrinsic conductivity (increase in resistance) of the poly[pyrrole-co-3-carboxyl-pyrrole] copolymer when compared to the PPy film (Fig 1S). The presence of the COOH groups in the copolymer chain is expected to introduce a sterical and electrostatic hindrance that is expected to affect the conjugation in the polymeric film. Alteration of the π - π conjugation in PPy films has been shown to have a significant effect on the film conductivity (Garnier et al., 2007).

3.2. Aptamer immobilisation

The Au electrodes modified with poly[pyrrole-co-3-carboxyl-pyrrole] copolymer film were used to develop aptasensors via covalent immobilisation of the aptamer onto the surface using EDC/NHS chemistry (methods section). In this step high concentration of amino modified aptamer (2 μ M) was used with the aim of saturating with aptamer the copolymer. Immobilisation of aptamer onto the surface was confirmed by contact angle measurement (Table 1S) and electrochemical impedance measurements (Fig 2B). The presence of phosphate groups in the aptamer structure was considered, responsible for the increased hydrophilicity of the poly[pyrrole-co-3-carboxyl-pyrrole] copolymer-aptamer surface, which was clearly highlighted by the reduced contact angle value (Table 1S). This result was confirmed by EIS measurement (Fig 2B). A general circuit was utilised to model the impedance spectrum (inset Fig 2B). In the proposed circuit, previously described by Tlili et al (Tlili et al., 2005), R_s represents the solution resistance, R_{ct} is the charge transfer resistance at the copolymer-aptamer/electrolyte interface, and CPE is the constant phase element accounting for the charge capacitance at the copolymer-aptamer/electrolyte interface. The model was shown to fit adequately the response obtained for the different surfaces investigated including poly[pyrrole-co-3-carboxyl-pyrrole] copolymer and poly[pyrrole-co-3-carboxyl-pyrrole] copolymer-aptamer bioconjugate and for the sensor response

upon detection of *S. Typhimurium* at the different concentration investigated. This could be clearly seen from Fig 2S where example of the fitting results are presented.

The value of R_{ct} changed significantly following aptamer immobilisation, passing from ca. 78 Ω for the poly[pyrrole-co-3-carboxyl-pyrrole] copolymer, to ca. 1118 Ω for the poly poly[pyrrole-co-3-carboxyl-pyrrole] copolymer-aptamer bioconjugated film. This result was consistent with previous report where the aptamer attachment was associated with an increase in the resistance of the polymer (Zhu et al. 2012).

Please insert Fig 2 here

3.3. Detection of bacteria

Different concentration of bacteria solutions (10^2 - 10^8 CFU mL⁻¹) were prepared by dilution of bacterial stock solutions in LiClO₄ 100 mM. The choice of LiClO₄ was dictated by the fact that incubation (45 min) in PBS (the buffer in which the aptamer was selected) (Joshi et al., 2009) resulted in a significant alteration of the conductivity properties of the poly[pyrrole-co-3-carboxyl-pyrrole] copolymer-aptamer film, as clearly indicated by the EIS measurements reported in the Supporting Information (Fig 3S).

The suitability of LiClO₄ solution for bacteria detection was investigated by comparing the EIS results before and after the incubation of poly[pyrrole-co-3-carboxyl-pyrrole] copolymer-aptamer-films with 100 mM LiClO₄ solution with (10^6 CFU mL⁻¹) and without *S. Typhimurium*. As can be seen from Fig 4S, when bacteria were absent, there was no significant change in the impedimetric response. On the other hand, when bacteria were present, a clear increase in the impedance response was recorded (Fig 2B). Interaction between the aptamer and bacteria is expected to induce significant changes in the poly[pyrrole-co-3-carboxyl-pyrrole] copolymer-aptamer-films electrical properties by altering significantly the environment in close proximity with polymeric chains (Peng H., et al., 2009; Tlili et al. 2005). Several phenomena have been shown to alter the intrinsic conductivity properties of CPs including: (i) alteration of the π - π conjugation (Garnier, F. et al., 2007) due to the steric hindrance generated by the introduction, following biorecognition, in the polymer body of the bulky bioorganism, (ii) changes in the interfacial double layer due to the charge properties of the pathogens (Zelada-Guilln G. et al., 2009) and (iii) reduction in the mobility

of the dopant (ClO_4^-) due to the electrostatic repulsion induced by the pathogens (Thompson L.A. et al., 2003).

In order to confirm the role of the aptamer in the performance of the biosensor, two control experiments were performed. In the first experiment, poly[pyrrole-co-3-carboxyl-pyrrole] copolymer film without immobilised aptamer were incubated with (10^4 CFU mL^{-1}) bacterial solution. In the second experiment, a biosensor modified with an *E.Coli*-specific aptamer (Bruno 2014), was incubated with the same concentration of bacteria. The results of these control experiments (Fig 5S and Fig 6S, respectively) clearly showed limited response, probably due to physical absorption of bacteria onto the polymeric surface, when compared to those recorded with the proposed *S. Typhimurium* aptasensor.

3.4. Optimisation of incubation time for *S. Typhimurium* detection

Prior to evaluating the analytical performance, in terms of response/concentration and selectivity, the influence of the incubation time on the aptasensor response was evaluated. In order to explore this, aptasensors were incubated with bacterial solution (10^6 CFU mL^{-1}) for different incubation times (Fig 7S). In order to minimise the variability that can be recorded between different aptasensor the relative variation of Rct ($\Delta\text{Rct}/\text{Rct}_0$) was used as analytical response (Nasef et al., 2010). This value was calculated as follows:

$$\Delta\text{Rct}/\text{Rct}_0 = (\text{Rct}_{\text{after}} - \text{Rct}_0)/\text{Rct}_0 \quad (\text{eq. 1})$$

where $\text{Rct}_{\text{after}}$ is the value of Rct after recognition of the pathogen and Rct_0 is the value recorded for the poly[pyrrole-co-3-carboxyl-pyrrole] copolymer-aptamer-films before the incubation with the solution containing the pathogen.

As it can be seen from Fig 8S, the analytical response increased until 45 min when it started to level off. A longer incubation time (60 min) did not result in a significant increase in the response; subsequently 45 min was selected as incubation time for the following experiments.

3.5. *S. Typhimurium* calibration curve

Calibration curves were built following the protocol described in the material and method section (section 2-7). Typical aptasensor responses are illustrated in Fig 3A and a calibration plot is presented in Fig 3B. The relative resistance was shown to increase linearly ($y = 0.1566x -$

0.0382 equation with $R^2 = 0.99$) with the logarithm of the *S. Typhimurium* concentration within the investigated concentration interval (range of 10^2 – 10^8 CFU mL⁻¹) with a LOQ of 10^2 CFU mL⁻¹ and a LOD (3 time the standard detection of the blank experiment: incubation in the absence of *S. Typhimurium*) of 3 CFU mL⁻¹. Error bars in the calibration indicate the reproducibility (3 measurements), over 2 days, performed using 3 different aptasensors. Reproducibility of the aptasensor was calculated over the full range of concentration; this resulted in an average RSD of 5.2% (n=3 for each of the 7 concentrations used in this calculation and listed in Table 2S).

3.6. Selectivity of the aptasensor

Three different strains of *E. Coli*, *Enterobacter* and *Citrobacter* were utilised to investigate the specificity of the aptasensor. Solutions containing 10^6 CFU mL⁻¹ of each bacterium were incubated with the aptasensor for 45 min. As it can be seen from Fig 3C, the relative resistances (calculated accordingly to eq. 1) for the five investigated bacteria strains are much lower than those recorded for the *S. Typhimurium*, confirming the specificity of the aptasensor. Interestingly is the fact that for interfering strain resulted in a loss of Rct; the reason for these results are not clear but they might reside in the intrinsic charge and morphological properties of the different pathogens.

Please insert Fig 3 here

3.7. Real sample measurement

Real sample measurements were carried out in spiked (10^2 and 10^6 CFU mL⁻¹) filtered and diluted apple juice. Dilution of the apple juice (1 to 100) was performed using LiClO₄ 100 mM as dilution media. The results of the recovery studies are summarised in Table 1. Concentrations of bacteria in the spiked samples were calculated by fitting the relative resistance (calculated accordingly to eq. 1) variation in the calibration curve of Fig. 3B. Clearly the aptasensor worked in diluted spiked real samples. Reproducibility of the detection was calculated for both spiked concentration (n=3); this resulted in an average RSD of 11%.

Please insert Table 1 here

4. Conclusions

In this study the successful fabrication of the poly [pyrrole-co- 3-carboxyl-pyrrole] copolymer and its conjugation with a *S. Typhimurium* specific aptamer was achieved. Chemical (FT-IR) physical (electrochemical and contact angle) characterisations were used to confirm the synthesis of the designed bioconjugate film. The use of the poly [pyrrole-co-3-carboxyl-pyrrole] copolymer-aptamer bioconjugate as sensing surface in electrochemical impedance label-free aptasensor was also investigated. The proposed method sensing surface was demonstrated to allow sensitive detection of *S. Typhimurium* in a fast and sensitive way by taking advantage of the changes, following aptamer pathogen interaction, in the electrical properties of the poly [pyrrole-co-3-carboxyl-pyrrole] copolymer without the need of the addition of any reporting redox probe. This significantly simplifies the assay opening in this way new possibility for fast in situ screening of environmental and food products. The developed label-free aptasensors was shown to respond linearly over the *S. Typhimurium* concentration range 10^2 - 10^8 CFU mL⁻¹ with a LOQ of 10^2 CFU mL⁻¹ and a LOD of 3 CFU mL⁻¹. The aptasensor was also shown to be highly selective for *S. Typhimurium* when compared to the detection of other possible model interfering strains (*E. coli* 375, *E.coli* 797, *E.coli* 3274, *Enterobacter*, *Citrobacter*). Finally the aptasensor was demonstrated to allow the fast (45 min) label-free detection of *S. Typhimurium* in spiked apple juice. Despite further work on the stabilisation of the sensor surface will be required, the authors envisage this as a viable approach for inexpensive, simple and rapid detection of pathogens in food and possibly environmental samples.

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SCHEME, TABLES AND FIGURE CAPTIONS

Scheme1. Pictorial representation of the proposed approach for aptasensor fabrication.

Table 1: Summary of recovery studies in 1:100 diluted, in LiClO₄ 100 mM, apple juice.

Fig 1. SEM image (low and high resolution) of PPy (A and B) and [pyrrole -co- 3-carboxyl -pyrrole] copolymer (C and D) deposited onto Silicon based Au film. FT-IR spectrum of (E) [pyrrole -co- 3-carboxyl -pyrrole] copolymer (blue line) and PPy (red line) films.

Fig 2. (A) Cyclic voltammogram of [pyrrole -co- 3-carboxyl -pyrrole] copolymer (red line) and PPy (blue line) in LiClO₄ 100 mM at 50 mVs⁻¹. (B) EIS measurements of [pyrrole -co- 3-carboxyl -pyrrole] copolymer film before (red line) and after (blue line) attachment of aptamer and after incubation with 10⁶ CFU mL⁻¹ solution of *S. Typhimurium* (green line) (measurement solution LiClO₄ 100 mM). The impedance spectra were recorded from 10 kHz to 0.5 Hz, at 0 mV vs (OCP), amplitude of 5 mV in LiClO₄ 100 mM solution.

Fig 3. (A) Impedance measurement for different concentrations (10²– 10⁸ CFU mL⁻¹) of *S. Typhimurium*; (B) Calibration curve obtained by fitting the results from 3 consecutive calibration curves. (C) Selectivity evaluation of the aptasensor over the detection of six model pathogens 3 *E. Coli* strains, *Enterobacter* and *Citrobacter* (10⁶ CFU mL⁻¹) Measurement and capture solution: LiClO₄ 100 mM. Error bars (Fig. 3B and Fig. 3C) were obtained by calculating the SD of 3 measurements performed each with a different aptasensors. The impedance spectra were recorded from 10 kHz to 0.5 Hz, at 0 mV vs (OCP), amplitude of 5 mV in LiClO₄ 100 mM solution.

Scheme 1

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Nominal Concentration CFU mL ⁻¹	Experimentally obtained Concentration CFU mL ⁻¹
10 ²	1.4x 10 ²
10 ⁶	4.1x10 ⁶

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Table 1

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Figure 1

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Figure 2

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Figure 3