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Three-dimensional impedimetric aptasensor for detection of *Escherichia coli* O157:H7

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Abstract

Microbial safety of drinking water constitutes a major concern in countries at all levels of economic development. Thus, rapid, sensitive and cost effective methods of pathogenic bacteria detection, like common like *Escherichia coli* O157:H7, which can cause important diseases, is highly required.

In this work an impedimetric transducer modified with *E. coli* specific aptamer is studied. To enhance the sensitivity a three-dimensional interdigitated electrode array (3D-IDEA) impedimetric transducer in which the electrodes separated by insulating barriers was used. In this sensor, chemical reactions at the surface of the barrier provoke electrical charge redistribution which causes changes in surface conductivity. A DNA aptamer that recognizes specifically the outer membrane proteins of the *E. coli* O157:H7 was selected as the biorecognition moiety.

Here we report a novel label-free impedance aptasensor for detection and quantification of pathogenic *E. coli* O157:H7 with a low detection limit, good selectivity and short detection times. The developed sensor shows a linear response ($R^2=0.977$), proportional to the logarithm of bacterial concentration present in the sample, with a limit of detection (LOD) of about 10^2 cfu·mL⁻¹. No response was registered when the aptasensor was incubated with other bacterial strains, confirming the selectivity of suggested method. Additionally, the possibility of the sensor regeneration is shown, so that the detection may be performed several times with the same sensor. Moreover, suitability of the aptasensor for bacteria detection in real samples was demonstrated with a new approach involving bacteria pre-concentration.

1. Introduction

Water is a natural resource and is essential to sustain life, making its total economic value immeasurable. The quality of water, whether it is used for drinking, irrigation or recreational purposes, is significant for health in both developing and developed countries worldwide. So, water plays a crucial role in economic development and social welfare, being a key factor in health, food production and poverty reduction (Odonkor and Ampofo 2013; Snozzi 2001).

The presence of pathogenic bacteria in water is one of the main causes of human infection diseases and their detection with suitable, sensitive and fast methods is of great importance (Saxena et al. 2015). *Escherichia coli* is the predominant member of the facultative anaerobic portion of the human colonic normal flora and its presence in environmental samples, food or water usually indicates fecal contamination, lack of hygienic practices and storage conditions. So, the presence of this microorganism in water indicates that there is a high risk of the presence of fecal-borne bacteria and viruses, many of which are pathogenic (Edberg et al. 2000; Heijnen and Medema 2006; Odonkor and Ampofo 2013)

Among the different strains, the enterohemorrhagic *E. coli* O157:H7 can produce from slight to life-threatening diarrhea, urinary tract infections, respiratory disease or pneumonia (Croxen et al. 2013). The strain O157:H7 bacteria produce verotoxins and is considered as the major causative agent of hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS), which causes acute renal failure in children and can result in morbidity and mortality in adults (Lim et al. 2010; Saxena et al. 2015). Thus, the illness is associated with the consumption of contaminated food, including unpasteurized milk or fresh products like leaf lettuce and apples, as well as water that has not been properly disinfected (Solomon et al. 2002). Therefore, the presence of these bacteria is a major concern in food industry and water treatment. Additionally, the infection dose of enterohemorrhagic *E. coli* is in the range of 10 – 100 colony forming units organisms, low compared with other virulence types (Nguyen and Sperandio 2012), thus highly sensitive, rapid and selective detection methods of *E. coli* O157:H7 are highly required.

Conventional methods for bacteria detection as microbiological culturing and isolation require enrichment steps and involve long processing times, with a delay of two or three days to obtain the results, lack of sensitivity and require trained personnel (Lazcka et al. 2007). Enzyme-linked immunoassay (ELISA) in a 96-well microtiter plates gives the possibility to simultaneously analyze a great number of samples but its sensitivity sometimes is insufficient. The most commonly used methods are based on molecular techniques, like polymerase chain

reaction (PCR), in which the required time is shorter compared to traditional methods, but it uses a complex set-up, expensive reagents and equipment (Ahmed et al. 2014; Croxen et al. 2013; Velusamy et al. 2010). Therefore, highly sensitive, fast and low cost analytical technologies are requested. In this sense, biosensors, devices that integrate a biological recognition element and a physical transducer that generate a measurable signal proportional to the concentrations of analytes (Su et al. 2011), are promising tools for bacteria detection.

Different biomolecules may be employed as the recognition element in biosensors for bacteria detection, including enzymes, antibodies, nucleic acids or lectins (Velusamy et al. 2010), among which, antibodies are the mostly used owing to their high selectivity and affinity with target molecules (Sadik et al. 2009; Wang et al. 2012). Nevertheless, antibodies production is based on clone technology, involving a considerable cost. Additionally, their instability in a certain range of pH and temperature, short shelf life, easy degradation and the difficulty to be reused generate a problem for their application in robust biosensors development (Jarczewska et al. 2016; Luo et al. 2012; Torres-Chavolla and Alocilja 2009; Wang et al. 2012).

Aptamers are artificial oligonucleotide sequences of 30 to 100 nucleotides of DNA or RNA molecules that bind to specific ligands with high affinity and can be easily synthesized with high specificity to a certain target molecule. They are produced *in vitro* from pools of random nucleic acids sequences through the selection evolution of ligands by exponential enrichment, SELEX process (Tombelli et al. 2005; Wu et al. 2014)]. Binding of aptamer to a particular target depends on several factors, including conformational structure, electrostatic interactions, hydrogen bonds or van der Waals forces (Stoltenburg et al. 2007). Aptamers show binding affinity and specificity comparable to those of antibodies and they present important advantages like better reproducibility in chemical production, stability under a wide range of pH conditions, resistance in harsh environments without losing their bioactivity, small size and low production cost (Torres-Chavolla and Alocilja 2009). Moreover, in the course of their chemical synthesis it is easy to modify the aptamer sequence introducing specific active terminal groups that help to immobilize effectively these molecules on different transducers, thus forming biosensors, commonly named as aptasensors (Ravalli et al. 2016). In recent years a large number of publications have been focused on the development of aptamer-based biosensor for detection of pathogenic bacteria: *Salmonella* (Sheikhzadeh et al. 2016), *Staphylococcus aureus* (Shahdordizadeh et al. 2017), *Listeria* [(Sidhu et al. 2016), and also *E. coli* (Luo et al. 2012).

More concretely, in the case of *E. coli* O157:H7 a great number of optical and electrochemical biosensors have been reported in the literature (Xu et al. 2017), however only few works are focused on the use of aptamers. Zelada-Guillén *et. al* (Zelada-Guillén et al. 2010) developed a potentiometric aptamer-based biosensor with single-walled carbon nanotubes (SWCNT) to detect *E. coli* O157:H7 in different complex samples, like milk and apple juice. Wu and co-workers (Wu et al. 2012a; Wu et al. 2012b) presented different rapid colorimetric detection systems improving in the course of their work the limits of detection from 10^5 cfu·mL⁻¹ (Wu et al. 2012a) to 10^4 cfu·mL⁻¹ (Wu et al. 2012b). Demirkol and Timur (Demirkol and Timur 2016) reported a sandwich aptamer-based fluorescent assay for analysis in a microtiter plate format that permits to detect selectively the enterohemorrhagic *E. coli* down to 10^2 cfu·mL⁻¹. Khang *et al.* (Khang et al. 2016) for *E. coli* O157:H7 monitoring developed a chemiluminescent biosensor based on graphene oxide (GO)/iron nanocomposites modified with an aptamer. The reported detection limit was 4.5×10^3 cfu·mL⁻¹. Recently, Burrs et al. (Burrs et al. 2016) have presented the development of platinum nanocauliflower-graphene hybrids on paper for amperometric detection of pathogenic *E. coli* O157:H7 using a RNA aptamer. However, the majority of the reported aptasensors require indirect methods that complicate the detection process. Here we present a direct label-free impedance based biosensors to detect *E. coli* DSMZ 17076, a non-pathogenic bacteria phenotypically identical to *E. coli* O157:H7.

Electrochemical impedance spectroscopy (EIS) permits to study changes occurring on the solid/liquid interface on the surface of electrodes produced by physical, chemical or biological interactions (Katz and Willner 2003). The impedance measurements may be carried out in a faradaic or nonfaradaic modes. In the first case, the presence of a redox couple that discharges on the electrode surface is required. The charge transfer resistance of this electrochemical reaction is the main parameter that is affected by the surface biochemical reactions. In the nonfaradaic mode, in the absence of charge transfer, a transient current flows across the interface that mainly depends on the interfacial capacitance. This mode of impedance measurements is considered as a more amenable method for direct biosensing applications (Daniels and Pourmand 2007). Different kind of electrodes can be used as impedimetric transducers for bacterial detection, but interdigitated electrode arrays (IDEA) present certain advantages, like small size, increased signal-noise-ratio and fast establishment of a steady state (Varshney and Li 2009), compared to other electrodes systems.

Planar IDEA devices are formed by a pair of comb-like metal electrodes on a planar insulating substrate by conventional micro-fabrication techniques. In this case, impedance is measured

between the two electrodes and depends on the solution conductivity and the interfacial properties of the electrodes: interfacial capacitance (Berggren et al. 2001) and surface conductivity (Guimerà et al. 2015). While traditional macro-electrodes with large surface area can be used to carry out measurements of interfacial capacitance, in micro-scale transducers the surface charge also plays an important role (Guimerà et al. 2015). In this sense, to improve the sensitivity of standard IDEA sensors a three-dimensional interdigitated electrode arrays (3D-IDEA) impedance transducer, in which the electrodes are separated by insulating barriers, was proposed (Bratov and Abramova 2013b; Bratov et al. 2008b). The 3D-IDEA devices are highly sensitive to changes in the surface charge at the solid/liquid interface produced by chemical and biochemical reactions (Bratov et al. 2012), and have been demonstrated useful in label-free detection of bacteria (Hoyos-Nogués et al. 2016) and bacteria endotoxins (Brosel-Oliu et al. 2017).

In this work, we report a novel label-free impedimetric aptasensor for the direct detection of *E. coli* O157:H7. The surface of the 3D-IDEA transducer was initially modified with a mercaptosilane to covalently immobilize a *E. coli* specific 5' disulfide-modified DNA aptamer by means of a thiol/disulfide exchange reaction (Rogers et al. 1999). The biofunctionalization strategy is schematically presented in Figure 1. The impedimetric measurements allow registering the presence of target bacteria at different concentrations with low limit of detection in a short time. Moreover, the feasibility of the aptasensor was validated with real samples introducing a pre-concentration step with filtration system developed in our group. Finally, the regeneration of the aptasensor after the detection of bacteria has been also demonstrated, confirming the great potential of its application. The present study suggests a rapid and attractive method for bacterial detection especially in water quality analysis in environmental samples.

[Figure 1]

2. Experimental

2.1. Electrodes fabrication

The interdigitated electrode array was formed on a silicon wafer covered with a 2.5 mm thick thermal silicon oxide layer. As an electrode material a highly conductive tantalum silicide (TaSi_2) was deposited using magnetron sputtering. This layer was patterned using conventional lithography giving as a result interdigitated electrodes with 216 digits of 3 μm width and 3 μm gap between the adjacent electrodes. The aperture between the electrodes is 1.4 mm and the

total length between the electrodes is 301 μm . The wafer was covered by a 4 μm thick low pressure chemical vapor deposition (LPCVD) silicon dioxide in which electrode digits and contact pads of the transducers were opened by deep reactive ion etching (DRIE). Thus, 4 μm high barriers with nearly vertical walls separating the electrodes digits were formed. Complete technology of sensor fabrication is presented elsewhere (Bratov et al. 2008b) and the resulting sensor is shown in Figure 1A .

2.2. Reagents, solutions and materials

DNA aptamer sequence 5'- S-S- ATCCGTCACACCTGCTCTGTCTGCGAGCGGGGC GCGGGCCCGCGGGGGATGCGTGTTGGCTCCCGTAT-3' used in this study against *E. coli* O157:H7 was selected from previous work (Demirkol and Timur 2016; Wang et al. 2012). The aptamer was purchased in lyophilized form from Sigma-Aldrich (Spain). Prior to use the aptamer was purified by elution through Illustra NAP 10 columns for a DNA gel filtration with Sephadex G-25 DNA grade (GE Healthcare Life Science, Spain) using deionized DNase free-water. DNA concentration was determined accurately by measuring the absorbance at 260 nm at room temperature by means of a spectrophotometer ($\epsilon = 650900 \text{ L}\cdot\text{mol}^{-1}\cdot\text{cm}^{-1}$) (DU 730 Life Science UV/Vis Spectrophotometer from Beckman Coulter). Stock solutions were divided in aliquots and stored at -20°C until use at desired concentration.

Phosphate buffer solution (PBS) (0.01 M phosphate buffer, 0.0027 M KCl and 0.137 M NaCl, pH=7.4) was used as the buffer solution to prepare the aptamer during the immobilization process and as a supporting solution to perform the bacterial detection assays. For the surface treatment 3-mercaptopropyl-trimethoxysilane (MPTES) was purchased from Sigma-Aldrich. All the solutions were prepared with deionized MilliQ water (18 MOhm $\cdot\text{cm}$) which was also used for cleaning and rinsing processes.

2.3. Preparation and characterization of aptasensor

Prior to use, 3D-IDEA sensors were cleaned with isopropanol for 10 minutes, rinsed with water and dried under nitrogen flow. Afterwards, the surface was treated with (3-mercaptopropyl) trimethoxysilane (MPTES) by means of vapor-phase silanization. During silanization sensors were placed in a custom-designed cell and maintained at 50 $^{\circ}\text{C}$ for 1 h under a controlled atmosphere of mercaptosilane. This method was adapted from previously reported protocol (Wieringa 2000).

The covalent immobilization of 5' disulfide-modified DNA aptamer on the 3D-IDEA surface was performed via the mercaptosilane layer using a thiol/disulfide exchange reaction described

elsewhere (Rogers et al. 1999). For this 3D-IDEA sensors were immersed into a 20 nM solution of aptamer dissolved in PBS (pH=7.4) overnight at room temperature with slight agitation. The complete biofunctionalization strategy is schematically presented in Figure 1.

In order to optimize the immobilization process of aptamer, different concentrations were tested (see Supplementary Information, Fig. S1) and 20 nM was selected to perform the detection experiments. Finally, the electrodes were rinsed with KCl 10^{-5} M solution to remove adsorbed PBS salts to reduce their possible effect on the impedance measurements.

2.4. Circular dichroisms (CD) and molecular absorbance measurements

The CD spectra were recorded on a JACSO spectropolarimeter J-815. The 2 μ M aptamer samples were prepared in 10^{-5} M KCl and in standard PBS, and their spectra were registered at 25 °C in the range of 220-320 nm, with a scanning speed of 100 nm/min, a response time of 4 sec, data pitch of 0.5 nm and a band-width of 1 nm (Benabou et al. 2016).

2.5. Bacteria cultivation and detection experiments

E. coli DSMZ 17076 strain, a non-pathogenic but phenotypically similar to the enterohemorrhagic *E. coli* O157:H7, was employed to perform the detection assays. Bacteria were grown overnight at 37 °C in Luria-Bertani (LB) medium broth before each assay. The cells were harvested by centrifugation at 9000 g for 10 minutes, then washed three times in sterile PBS (pH=7.4) and re-suspended in a fresh PBS solution at desired concentrations to perform the detection assays. After each test bacterial concentration was determined by colony counting in LB agar plates, incubated for 24 h at 37 °C.

E. coli detection assays were performed by incubation of aptasensors in solutions with bacterial concentration ranging from 10^1 to 10^5 cfu·mL⁻¹. At least five different concentrations were used for the assay. 3D-IDEA sensors biofunctionalized with the aptamer were immersed in 100 mL of bacteria suspensions in PBS solution under stirring conditions to maintain a homogeneous distribution of bacteria. The aptasensors immersed into PBS solution without bacteria and non-functionalized 3D-IDEA in solutions with the highest concentration of *E. coli* were employed as controls. The experimental setup for bacterial detection assays is detailed elsewhere (Hoyos-Nogués et al. 2016).

To test the selectivity of the detection method, cross reactions were studied using the bacterial strains *E. coli* K12, *Salmonella typhimurium* ATCC 14028 and *Staphylococcus aureus*

ATCC 6538. In this case, the same procedure was used for bacteria samples preparation. All the different bacteria samples were adjusted to the same concentration of 10^5 cfu·mL⁻¹ in PBS to compare the selectivity of the proposed biosensor. All the experiments were done in sterile conditions to prevent contaminations.

In the case of real drinking water samples, prior to be analyzed with the impedance-based aptasensor, they were subjected to a filtration pretreatment, as described in detail in Supplementary Information, to avoid possible interferences from unknown sample components.

2.6. Impedance measurements

A QuadTech 7600 Plus, a highly precision LCR Meter analyzer, was employed for all impedance measurements in a 100 Hz – 100 kHz frequency range with 100 mV (amplitude) voltage excitation. The measurements were performed in a nonfaradaic mode and no DC voltage bias was applied during the impedance measurements. Impedance data treatment and equivalent circuit fitting was performed using the Z-Plot/Z-View software package (Scribner Associates, Southern Pines, NC, USA). All experiments were done at least on three biosensors under the same conditions.

Impedance measurements were carried out at controlled room temperature in 10^{-5} M KCl solutions. To guarantee the reproducibility of the bulk solution conductivity for each single measurement a fresh portion of solution was used and its conductivity of $2.50 \mu\text{S}\cdot\text{cm}^{-1}$ was controlled with a commercial conductimeter (EC-Meter GLP 31+, Crison).

3. Results and discussion

3.1. Characterization of the aptasensor by impedance measurements

The electrochemical impedance spectroscopy (EIS) technique used in this work is an effective technique to study electrochemical systems analyzing their complex electrical resistance, sensitive to surface phenomena and changes in bulk properties (Lisdat and Schäfer 2008). Therefore, it is a powerful tool to monitor electrode surface modifications in biosensing processes (Li et al. 2014; Maalouf et al. 2007) and it was employed in this work also to characterize each step of the biosensor fabrication. Impedance changes were studied in a non-faradaic mode with IDEA in low conductivity KCl solutions in the absence of redox probes. Under this measurement conditions surface conductivity plays an important role due to surface charge presence (Bratov and Abramova 2013b). This effect is more pronounced in 3D-IDEA sensors, in which the major part of the electrical current between the electrodes goes close to the surface of the barrier separating the electrodes and not through the surrounding bulk solution (Bratov et al. 2008b). This permits to improve the sensitivity of electrodes to biochemical reactions that occur on the sensor surface, thus monitoring the biofunctionalization steps and the detection performance.

The impedance response of the studied system may be presented by an equivalent circuit shown in Fig. 2A, formed by the following components: R_C is the contact resistance introduced by wires and collector bars of the thin film electrodes; C_{IDS} is the geometrical (stray) capacitance between two electrodes; R_S is the resistance between two electrodes of the array; and CPE_{DL} is a constant phase element representing the capacitance of the electrical double layer at the electrode-water solution interface (Bratov et al. 2008a). As previously reported (Bratov and Abramova 2013a), R_S is a parallel combination of the bulk solution resistance (R_{BULK}) and the surface resistance (R_{SURF}) (Fig. 2A), but under the experimental conditions used it is not possible to distinguish these two elements in the impedance spectra. However, if the bulk solution conductivity remains fixed, as in our experiments, we may attribute the changes in R_S to the surface resistance changes produced by surface reactions and modifications (Bratov and Abramova 2013a; Brosel-Oliu et al. 2015).

Fig. 2B presents experimental impedance spectra as a Nyquist plot (Z' vs Z'') that allows to observe the formation of a semicircle at high frequencies, corresponding to the resistance R_S in parallel with the geometrical capacitance. The intercept with Z' axis on the left side gives the R_C values, while the intercept on the right side gives the value of $R_C + R_S$, where $R_S = (R_{BULK} +$

R_{SURF}). The linear response at low frequencies in the Nyquist plot is produced by the CPE of the interfacial capacitance.

[Figure 2]

3.2. Aptamer immobilization and optimization of incubation time for *E. coli* detection

In order to determine the optimal aptamer concentration for the sensor surface functionalization, 3D-IDEA was chemically modified via vapor-phase silanization with MPTES. The aptamer modified with a disulfide group at 5' terminal was selected to be covalently attached to the sensor surface, grafted with mercaptosilane, via thiol/disulfide exchange reaction between the disulfide terminal of oligonucleotide and the sulfhydryl group of mercaptosilane (Rogers et al. 1999). To ensure the completeness of the chemical reaction the electrodes were maintained overnight in the aptamer solution.

To confirm the importance of the aptamer concentration on the performance of impedance-based biosensor, aptasensors were modified in the aptamer solutions of different concentrations and then were incubated in the presence of 10^4 cfu·mL⁻¹ of *E. coli*. Figure S1 of Supplementary Information shows that in the presence of *E. coli* the impedimetric response increases with the concentration of immobilized aptamer with a maximum at 20 nM of aptamer solution employed. In the absence of aptamer practically no response to *E. coli* presence was observed, demonstrating that there is no nonspecific binding of bacteria on the sensor surface. Therefore, the aptamer concentration of 20 nM was selected as optimal for sensor functionalization and was used in the following experiments.

Aptamer immobilization on the surface and its subsequent interaction with *E. coli* produced significant changes in the impedimetric response, affecting R_s and C_{DL} values of the equivalent circuit. Bacterial presence on the functionalized interdigitated sensor surface modifies the double layer interfacial capacitance (C_{DL}) and the CPE α parameter due to the formation of additional layers over the electrode digits surface. However, the ΔR_s , associated with the surface conductivity due to the surface charge presence, show higher reproducibility and a larger scale variation and was selected as the main parameter to monitor the aptasensor response.

The impedance sensor response within bacterial detection assays was expressed as variations in R_s :

$$\Delta R_s = R_s^{E.coli} - R_s^{aptamer} ,$$

where $R_s^{aptamer}$ is the resistance of 3D-IDEA biofunctionalized with the aptamer and $R_s^{E.coli}$ is the resistance values after the sensor interaction with *E. coli* cells .

Prior to the aptasensor sensitivity and selectivity characterization, the required incubation time was evaluated. One of the important parameters of biosensors is the response time, the time during which a sensor reaches the stationary signal level at a certain concentration of analyte in a sample. The optimal incubation time of aptasensors was evaluated in a $10^5 \text{ cfu} \cdot \text{mL}^{-1}$ *E. coli* bacterial solution by periodic measurements of impedance spectra to register the evolution of R_s (Figure S2). It was observed that impedance response increases significantly reaching a stationary phase within 30 minutes. Thus, this time interval was chosen as the suitable incubation time to guarantee the completeness of bacteria reaction with the aptasensor, and, therefore, it was employed in the following experiments.

3.3. Study of the aptamer stability by CD and UV experiments

The stability of the aptamer as a biorecognition moiety is essential in the performance of the proposed impedance based biosensor. Aptamers, compared with other biorecognition elements, present certain advantages widely described in the literature (Torres-Chavolla and Alcocilja 2009), like the small size, cost effectiveness or resistance to harsh conditions. However, to preserve its bioactivity an aptamer should maintain intact its secondary structure. The aptamer employed in this study is composed by different loop-hairpin structures that allow to interact properly with the target molecules (Bruno et al. 2010). To ensure that under experimental conditions of this study the aptamer does not change its proper structure required for biorecognition, circular dichroism and molecular absorption measurements were done. CD is a spectroscopic method that allows to study the conformation of proteins and nucleic acids in solution, and is widely applied due to its sensitivity to structural changes in biomolecules (Martin and Schilstra 2008). Thus, this technique was used to analyze possible changes in the aptamer structure. These experiments were carried out by conditioning the aptamer in the physiological pH buffer (PBS), employed during the immobilization step and as the medium for *E. coli* detection, and in a low electrolyte concentration solution (KCl 10^{-5} M), used during impedimetric measurements. Results of CD and molecular absorption measurements are detailed in the Supplementary Information and are summarized in Figure S3. As follows from the obtained data, in PBS solution the aptamer conformation remains nearly intact, however, slight changes in CD spectra were observed in KCl 10^{-5} M solution. Therefore, the aptasensor contact with KCl was reduced to minimum (1-2 min), only to

perform impedance measurements and to ensure that the aptamer structure is not affected. All the rest of processes and bacteria assays were carried out in PBS.

3.4. *E. coli* detection with the aptasensor

The sensitivity and the limit of detection are the most critical parameters in the performance of a biosensor for its applicability (Zourob et al. 2008). To evaluate the sensitivity of the proposed aptasensor bacteria detection was performed after 30 minutes of sensors incubation in samples with different *E. coli* concentrations, ranging from 10^1 to 10^5 cfu·mL⁻¹, as explained in the experimental section. After incubation the sensors were extracted from the bacterial solution, rinsed with 10^{-5} M KCl solution to eliminate PBS salts from sensor surface and, thus, to reduce their possible effect on impedance measurements. Finally, their impedance spectra were measured in 10^{-5} M KCl.

Moreover, two experiments were employed as controls. In the first case, to confirm the absence of non-specific bacteria adsorption on the initial, not functionalized sensor surface, electrodes were incubated in the solution with the highest (10^5 cfu) bacterial concentration. On the other hand, sensors grafted with aptamer, in parallel with bacteria detection experiments, were immersed in a PBS solution without bacteria for the same period of time to guarantee the stability of the impedance response.

The results revealed that variation on the impedance response (ΔR_s) is directly proportional to the logarithm of bacterial cell concentration. Figure 3 shows the linear correlation, $\Delta R_s = S \cdot \log C + R_o$, between ΔR_s and the logarithm of *E. coli* concentration in the range of $10^1 - 10^5$ cfu·mL⁻¹ with a good correlation coefficient of 0.977 and a sensitivity, S , of 25.91 ± 0.19 K Ω per bacteria concentration decade. In the case of controls, no impedance changes were observed, demonstrating the effectivity of proposed aptasensor and the absence of nonspecific interactions. The limit of detection (LOD) of the aptasensor was 2.9×10^2 cfu·mL⁻¹. It was calculated using the method reported by Shrivastava *et. al* (Shrivastava and Gupta 2011) as $LOD = X_{b1} + 3S_{b1}$, in which X_{b1} is the mean signal value of the blank in the absence of bacteria, and S_{b1} is the standard deviation in the blank. The limit of detection is comparable with other previously reported biosensors and is in the range of the *E. coli* O157:H7 infection dose of 10-100 cfu·mL⁻¹, which is of great importance taking into account the pathogenicity of this bacteria.

[Figure 3]

3.5. Analysis in drinking water samples

The real sample analysis was performed in water directly collected from the tap and spiked with bacteria at 10^5 cfu·mL⁻¹. As previously reported, the quality of drinking water for bacteriological safety is essential in many applications like human consumption, food preparation, washing or irrigation. To reduce the possible matrix effect of the analyzed water samples a previous filtration step was performed to retain bacteria in a specially designed filtration system. The system and methodology are presented in more detail in Supplementary Information (Figure S4). This filtration system may be employed for the treatment of different liquid samples in a short time (5 minutes for 100 mL of drinking water) and is of great interest to reduce the possible matrix effect of real samples on the sensor response. Furthermore, it has to be considered that this strategy is very useful to capture bacteria that are in low concentration in large volumes, which it is a recurrent problem in control of water-related infections, when the volume of samples to analyze may be considerable.

After the filtration, bacteria were recovered and transferred to PBS supporting solution to perform the detection assays. Transaction of the recovered bacteria into a fresh PBS and sensor incubation in this solution allowed to eliminate possible contamination and interference from “unknown” sample and to maintain the aptamer in the ideal conformation to detect bacteria and ensure a good response in the presence of bacteria. Figure S5 shows the differential response between solutions in the absence of bacteria and in water samples inoculated with 10^5 cfu·mL⁻¹. Applying earlier performed calibration (see Fig. 3) to thus determined ΔR_s value of 10.70 ± 1.96 K Ω we obtain between 5.53 and 4.02 log $C_{E.coli}$, that it is about 10^5 and 10^4 cfu·mL⁻¹ of the *E. coli* concentration. Taking into consideration the filtration system recovery rate of 92.3 ± 1.2 %, determined concentration is very close to that of the sample.

3.6. Selectivity of the aptasensor

To ensure the selectivity of the proposed aptasensor in specific binding of target bacteria *E. coli* O157:H7 experiments were performed in samples with different bacterial strains, two gram-negative, *E. coli* k12 and *Salmonella typhimurium*, and one gram-positive, *Staphylococcus aureus*, all adjusted at the same final concentration of 10^5 cfu·mL⁻¹. Moreover, a blank without bacteria (only PBS) and the specific *E. coli* O157:H7 at 10^5 cfu·mL⁻¹ were analyzed in parallel. The test of specificity was carried out incubating the aptasensors in each of bacteria samples for 30 minutes, measuring their impedance response in each case.

As it can be observed in Figure 4, the sensor shows high selectivity to *E. coli* O157:H7, as in all other cases after the incubation of the aptasensor in different bacteria samples the observed impedance changes were insignificant. In the case of the blank without bacteria and the *E. coli* K12, the increase in impedance is almost imperceptible. However, in the presence of *Salmonella* and *S. aureus* the response is slightly higher. These results show that the selected aptamer immobilized on the sensor surface maintains its specificity in reaction with the *E. coli* O157:H7, giving significant ΔR_s increase, demonstrating the affinity for this bacterium. This may be considered as a validation of the selectivity of the proposed aptasensor.

[Figure 4]

3.7. Sensor regeneration

In the development of biosensors is essential to reduce costs per analysis and to do this regeneration strategies are particularly important (Goode et al. 2015). In our case, experiments on regeneration of the aptasensor after its reaction with bacteria were performed. This can be done by provoking dissociation of the complex of aptamer and the *E. coli*, but it is important to guarantee that the aptamer recovers its structure necessary for the biorecognition after this treatment.

To break the interaction aptamer-*E. coli* the sensors were firstly immersed in distilled water at 80 °C for 30 minutes to release the bacteria from the aptamer. Higher temperatures provoke the aptamer denaturation and the loss of its tertiary structure, resulting in the lack of affinity for bacteria. Afterwards the sensors were immersed in a PBS solution to restore the appropriate aptamer conformation. It should be taken into account that the interaction between the terminal DNA aptamer and the functionalized electrode surface is produced by a covalent interaction through a disulfide covalent bond. This bond is quite strong and the applied increase of temperature does not affect it, so the aptamer is retained immobilized on the surface.

To perform regeneration tests the sensors were incubated in 10^5 cfu·mL⁻¹ *E. coli* samples and then were subjected to regeneration process as presented above. In parallel, to check if the process affects the aptamer, the same experiments were made with sensors incubated in PBS in the absence of bacteria. Figure 5 shows the impedance changes observed after each incubation and regeneration step. As demonstrated previously, the changes in resistance R_s as a result of *E. coli* interaction with the aptasensor can be employed as the reference parameter

to monitor the recovery of the sensor parameters. Firstly, the aptasensor was used to detect bacteria and, as previously observed, in the presence of *E. coli* an increase in R_s was registered. The control aptasensor stored in PBS showed no R_s changes. Secondly, after the first regeneration step, a decrease in R_s was produced with its final value coinciding with the control test without bacteria. This indicates that the bacteria are removed from the sensor surface and that the treatment does not affect the aptamer properties. The same process of bacteria detection was repeated showing subsequent recovery of the sensor response. Therefore, these results validate the efficiency of the regeneration methodology employed for the developed aptasensor.

[Figure 5]

4. Conclusions

In this study an innovative label-free aptasensor based on a 3D-IDEA impedimetric transducer has been introduced for the detection of the *E. coli* O157:H7, a well-known pathogenic fecal-borne bacteria.

The surface biofunctionalization with the aptamer and the detection of bacteria was characterized by EIS technique. The DNA aptamer was covalently immobilized on the 3D-IDEA surface grafted with mercaptosilane. Additionally, the effect of the assay conditions on the stability of the aptamer was investigated by means of circular dichroism technique, demonstrating that the aptamer in PBS remains stable, while in 10^{-5} M KCl some changes in its structure occur. This permitted to optimize the assay protocol to assure the aptamer stability.

The studied aptasensor showed a linear relationship ($R^2=0.977$) between the impedance changes and the logarithm of *E. coli* concentration in a broad range from 10^1 to 10^5 cfu·mL⁻¹ with a LOD about 2.9×10^2 cfu·mL⁻¹. Moreover, the selectivity of proposed aptasensor has been validated with other bacterial strains, demonstrating high selectivity for the target *E. coli* O157:H7. The developed aptasensor allows to detect *E. coli* bacteria in PBS solutions in a very short time of only 30 minutes.

To study the applicability of the developed sensor to perform bacteria detection in real samples, tap water samples spiked with *E. coli* were analyzed. To reduce possible sample matrix effect on the sensor response a promising pre-concentration system for bacteria retention was employed that may be used for bacteria extraction from other liquid samples. Obtained results show satisfactory precision of the developed detection protocol.

Finally, it was shown that the sensor may be used multiple times. The developed regeneration protocol is based on a simple temperature treatment in water.

In summary, coupling of aptamers with 3D-IDEA biosensors has resulted in the implementation of a rapid, label-free, sensing platform with high sensitivity and selectivity for the detection of the pathogenic strain *E. coli* O157:H7. The methodology proposed in the performance of this label-free biosensor can be potentially employed for the development of other rapid and selective aptasensors as promising tools for detection of other pathogenic bacteria in water samples.

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Figure captions

Figure 1. 3D-IDEA sensor surface image and its schematic representation (A) and different biofunctionalization steps (B).

Figure 2. Electrical equivalent circuit used for impedance spectra fitting (A) and Nyquist plots of 3D-IDEA after biofunctionalization and after detection of *E. coli* O157:H7 measured in KCl 10^{-5} M solution (B).

Figure 3. Calibration curve of impedance response, ΔR_s , expressed as a function of the logarithm of *E. coli* O157:H7 concentration from 10^1 to 10^5 cfu·mL $^{-1}$.

Figure 4. Evaluation of selectivity of the aptasensor for the detection of different bacteria strains (*E. coli* O157:H7, *E. coli* K12, *Salmonella typhimurium* and *Staphylococcus aureus*) and control in PBS without bacteria.

Figure 5. Impedance changes (ΔR_s) after the detection process of *E. coli* (10^5 cfu·mL $^{-1}$) and the two consecutive regeneration steps with aptasensors (n=3).

Three-dimensional impedimetric aptasensor for detection of *Escherichia coli* O157:H7

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Highlights:

- A novel impedance-based aptasensor for detection of *E. coli* O157:H7 is proposed.
- The limit of detection of the biosensor is around 10^2 cfu·mL⁻¹ with a detection time of 30 minutes.
- The selectivity of the developed aptasensor is demonstrated in regard to other bacterial strains.
- Regeneration protocol for the aptasensor, to be employed more than once, has been developed
- The methodology proposed can be potentially employed for the development of other rapid and selective aptasensors.

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05/06/2017

Dear editor,

On behalf of the authors I would like to submit a revised version of the manuscript: "Three-dimensional impedimetric aptasensor for detection of *Escherichia coli* O157:H7" based on the work carried out in Instituto de Microelectronica de Barcelona, Centro Nacional de Microelectronica (IMB-CNM), CSIC, Campus UAB, 08193 Bellaterra, Barcelona, Spain for publishing in Biosensors and Bioelectronics.

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The work presented has not been published previously and is not under consideration for publication elsewhere. Its **publication is approved by all authors** and by the responsible authorities where the work was carried out (IMB-CNM, CSIC).

If accepted, it will not be published elsewhere including electronically in the same form, in English or in any other language, without the written consent of the copyright-holder.

All authors have materially participated in the research and/or article preparation and have approved the final revised version of the article.

All the authors confirm that there are no known conflicts of interest associated with this publication.

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Figure(s)

A

3D-IDEA

B

Figure(s)

A

B

Figure(s)

Figure(s)

Figure(s)

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