Carbon nanotube-based aptasensors for the rapid and ultrasensitive detection of bacteria

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Abstract

In this paper we present a new generation of potentiometric biosensors based on carbon nanotubes (transducer layer of the biosensor) and aptamers (sensing layer of the biosensor) for the ultralow and selective detection of microorganisms. We show that with these aptasensors we were able to detect a few CFU of the target bacteria almost in real-time, both in buffered and in real samples.

1. Introduction

Effective prevention of infectious diseases caused by bacteria is one of the current public concerns. The detection of bacteria has remained for years based on standard microbiological methods [1]. These methods have been a typical practice in clinical diagnostics as well as in ensuring the safety of food and water to be consumed for almost one century. They make use of specific media for selective enrichment and culturing of bacteria, followed by further isolation steps, and finally confirmatory biochemical tests are also needed. The process duration depends on the target pathogen, but in most of the cases a confirmatory result can take from a few days to even weeks. In recent years, several research groups have tried to attain detection systems within much shorter response times while achieving very low concentrations. For instance methods based on ultrafast polymerase chain reaction (PCR) [2] can detect bacteria in a few

minutes with limits of detection of a few colony-forming units (CFU). However, the techniques based on nucleic acid sequences detection usually require preprocessing steps for DNA extraction, amplification and detection, what makes the overall procedure expensive and complicated. Other recent strategies are the use of functionalized gold nanoparticles that bind to the bacterial surface [3], positron emission tomography (PET) imaging [4] or bacteriophage amplification coupled with matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry [5], although in all cases pretreatment steps or complex and expensive equipment are required.

Our research group developed a few years ago potentiometric ion-selective electrodes (ISE) based on carbon nanotubes [6]. Crespo et al. [6] showed that single walled carbon nanotubes (SWCNT) can act as efficient ion-to-electron transducers in potentiometric analysis. The notable charge-transfer capability between heterogeneous phases of SWCNT together with their remarkable double layer capacitance [7] explain their transducing behaviour. SWCNTs are also easily deposited over many surfaces making them ideal for solid contact electrode design [8]. Since ISEs can only be used for the analysis of ions and small analytes, in a further step we removed the polymeric membrane that makes ISEs selective to a target analyte and we substituted the membrane by a suitable molecular receptor to detect microorganisms. Aptamers were selected as molecular receptors in the construction of a new generation of carbon nanotubes-based potentiometric aptasensors. Aptamers are highly suitable receptors for the selective and high proficiency detection of a wide range of molecular targets, including bacteria [9]. Moreover, aptamers can self-assemble to carbon nanotubes via π-π stacking interaction between the nucleic acid bases and the carbon nanotubes walls [10]. With these aptasensors we have been able to successfully detect ultralow concentrations of the target analyte in virtually real-time.

In this paper we describe the way of construction of these carbon nanotubes-based aptasensors, we present the main results we have obtained with their use and we finally discuss the advantages and limitations, envisaging some of the trends for the future.

2. Material and methods

Unless otherwise indicated, all the reagents (>99.5% purity) were purchased from Sigma-Aldrich (Tres Cantos, Spain).

2.1 Single walled carbon nanotubes (SWCNTs) and aptamers

Single walled carbon nanotubes (HeJi, Zengcheng, China) with >90% purity, 150 μm average length and 1.4-1.5 nm diameter were oxidized in a silica furnace chamber at 365 °C, with synthetic air flow-rate of 100 cm^3min^{-1} during 90 min, in order to selectively remove the amorphous carbon. Subsequently, SWCNTs were refluxed in 2.6 M nitric acid for 4 h to oxidize the metallic impurities remaining from the synthesis. The carbon nanotubes became carboxylated after this latter oxidation step. The SWCNTs in nitric acid solution were filtered and thoroughly rinsed with water to remove the remaining acid. The filtered SWCNTs were dried overnight at 80°C, and stored under dry conditions.

Specific aptamers for each microorganism were purchased from Eurogentec (London, UK) with the following sequences:

5'-GGG AAC AGU CCG AGC CUC ACU GUU AUC CGA UAG CAG CGC GGG AUG AGG GUC AAU GCG UCA UAG GAU CCC GC-3' for *Salmonella Typhi* [11].

5'-GGG AGA GCG GAA GCG UGC UGG GUC GCA GUU UGC GCG CGU UCC AAG UUC UCU CAU CAC GGA AUA CAU AAC CCA GAG GUC GAU-3' for *Escherichia coli* [12].

5'-GCA ATG GTA CGG TAC TTC CTC CCA CGA TCT CAT TAG TCT GTG GAT AAG CGT GGG ACG TCT ATG ACA AAA GTG CAC GCT ACT TTG CTA A-3' for *Staphylococcus aureus* [13].

All the aptamers were modified with a $-(CH_2)_5NH_2$ at the 3'-terminal. Once received, the aptamer was resuspended in MilliQ water and stored at -80 °C until needed.

2.2 Preparation of the aptasensors

The solid contact electrode was made of a 3 mm diameter glassy carbon cylindrical rod (HTW Hochtemperatur-Werkstoffe GmbH, Thierhaupten, Germany) covered by a Teflon jacket of 7 mm diameter. The surface of the glassy carbon was successively polished using 25 and 1 μm grain size polishing alumina (Buehler, Lake Bluff, USA) before the SWCNTs layer was deposited by spraying on the same surface. For the spraying process, we used sonication for 30 minutes at 0.5 s^{-1} in a tip sonicator (Ultraschallprocessor UP200S, Dr. Hielscher, Teltow, Germany) to prepare a solution of 25 mg of purified SWCNT dispersed in 10 mL of MilliQ water containing 100 mg of sodium dodecyl sulphate (SDS). 10 mL of the SWCNT/SDS/H_2O solution was sprayed under a high temperature (200°C) air blow, and washed with MilliQ water to progressively remove the SDS (Fig. 1a). Residual SDS was finally removed selectively by

heating at 280 °C with an air flow rate of 100 cm^3min^{-1} for 1 h (the Teflon jacket was temporarily removed during this step). The spraying process produced a 30 μm homogeneous layer of SWCNTs on the top of the glassy carbon rod that completely covered the whole surface (Fig. 1b).

To covalently link the aptamers to the SWCNTs, first of all the carboxylic groups that are on the sidewalls of the deposited SWCNTs were activated using a solution of 100 nmol of N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC)[1] and 25 nmol of N-hydroxysuccinimide (NHS)[2] in a 50 mM 2-(N-morpholino) ethanesulfonic acid (MES) buffer pH 5 for 30 minutes [14, 15]. After this step, the electrodes were soaked overnight into 500 μL of a 1 μM aptamer solution, which also consisted of phosphate buffer solution (PBS) pH 7.4 (1.7 mM) and cetyltrimethylammonium bromide (CTAB)[3] 0.2 mM. PBS was prepared sterilely using a 1:100 dilution of a 0.17 M stock solution of corresponding amounts of KH_2PO_4 and Na_2HPO_4, with pH adjusted as required. Finally, the sensors were washed in MilliQ water and stored in PBS 1.7 mM pH 7.4 until needed. This well-known carbodiimide-mediated chemistry was followed to form stable amide bonds between the carboxylic moieties on the sidewalls of the SWCNTs and the primary amine spacer on the 3' end of the aptamers (Fig. 1d).

2.3 Potentiometric analysis

Potentiometric analysis was performed by real-time measurements of the electromotive force (EMF) between the terminals of a two-electrode system (Fig. 1c) consisting of the carbon nanotube-based aptasensor as the working electrode, and a double junction reference electrode (Ag/AgCl/KCl 3 M containing a 1 M LiAcO electrolyte bridge, type 6.0729.100, Metrohm AG, Herisau, Switzerland) as the reference electrode at isothermal conditions (22 ± 0.5 °C) in a water-jacketed glass cell under constant stirring conditions (300 rpm). A high-input impedance voltmeter (1015 Ω, model EMF-16, Lawson Laboratories Inc, Malvern, PA, USA) was used in all the cases to measure the difference in electromotive force. The changes on EMF were automatically measured at periods of 10 seconds. The electrolyte used in the cell was 5 mL of PBS 1.7 mM pH 7.4. The EMF value was recorded automatically with the software provided by

[1] EDC is used is used in the activation of the carboxylic groups at the sidewalls of the carbon nanotubes via the formation of an O-acylisourea intermediate during the covalent functionalization of carboxylated nanotubes with NH_2-modified aptamers.

[2] NHS is used in the activation of the carboxylic groups at the sidewalls of the carbon nanotubes via the displacement of the O-acylisourea intermediates in order to form a stable NHS-ester. Such an intermediate can be further displaced by the NH_2-moieties of the modified aptamers in the formation of amide bonds between the aptamers and the carboxylated nanotubes.

[3] CTAB is a positively charged surfactant that is used to reduce the electrostatic repulsion between the remaining non-activated carboxylic groups at the sidewalls of the carbon nanotubes and the negatively charged phosphate groups of the aptamer backbone at pH 7.4 during the carbodiimide-mediated functionalization process.

the company. In all cases, the amount of bacteria detected by the potentiometric measurements was simultaneously standardized in quintuplicate using the agar plate count technique [1].

2.4 Microorganism culturing

All the bacteria strains were cultured following standard microbiological techniques, using in all cases sterilized materials, solutions and culturing media adequate for each type of microorganism. All the culturing media were purchased from Becton, Dickinson and Company (Sparks, USA). Lyophilized strains of all microorganisms were purchased from Colección Española de Cultivos Tipo (Valencia, Spain).

Lyophilized pure strains of *Salmonella Typhi* (CECT 409), *Lactobacillus casei* subsp. *casei* (CECT 4180), *Escherichia coli* (CECT 675), *Staphylococcus aureus* (CECT 4630) and *Staphylococcus epidermidis* (CECT 231) were reactivated with NaCl 0.85 % and further incubated in broth for 24-48 h at 37 °C. The pellet was then transferred to selective agar medium and sub-cultured for 24-48 h at 37 °C in order to confirm the purity of the strains. The selective medium used in this latter step depended on the cultured bacteria: mannitol salt agar was used for the selective isolation and enumeration of *Staphylococci*; tryptic soy agar was used in the enumeration and cultivation of pure strains of *Salmonella Typhi*, *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Escherichia coli*; tryptic soy broth was used in the enrichment and cultivation of pure strains of *Salmonella Typhi*, *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Escherichia coli*; xylose-lysine-deoxycholate agar was used for isolation, differentiation and confirmatory growth of the *Salmonella* group; MacConkey agar was used for isolation, differentiation and confirmatory growth of *Escherichia coli*; and lysogeny broth and agar (Luria-Bertani medium) was used for maintainance, enumeration and growth of *Escherichia coli* strains. Characteristic colonies were transferred to non-selective broth and agar media and cultured for 24-48 h at 37 °C. Colonies obtained in agar cultures were then transferred to glycerol/ tryptic soy broth 20:80 and stored at -20 °C until needed and reactivated by inoculating the bacteria in 10 mL of sterile broth medium at 37 °C for 24 h. Bacteria grown in non-selective broth was used for different purposes (e.g. testing of biosensors, inoculation of samples).

Agar-supported colonies of *Escherichia coli* (CECT 4558) were transferred to MacConkey agar medium and incubated for 24-48 h at 37 °C in order to confirm the purity of the strains. Characteristic colonies were transferred to non-selective Luria-Bertani broth and agar media and cultured for 24-48 h at 37 °C. Colonies obtained in agar cultures were then transferred to glycerol/Luria-Bertani medium 20:80 and stored at -20 °C until needed and reactivated by

inoculating the bacteria in 10 mL of sterile broth medium at 37 °C for 24 h. Bacteria grown in non-selective broth was used for different purposes (e.g. testing of biosensors, inoculation of samples).

For the bacteria enumeration assays, testing of aptasensors and preparation of standard solutions, the selected bacteria were inoculated in 10 mL of sterile broth medium at 37 °C for 24 h. Then, the bacteria samples were centrifuged at 6000 rpm for 15 minutes and the supernatant was discarded. The precipitate was resuspended in 10 mL of sterile buffer (PBS 1.7 mM), and the resulting solution was 1:10 diluted eightfold to provide a series of 10^{-1} to 10^{-8} stock solutions of bacteria. Each stock solution was quantified with the standard plate count method by triplicate, [1] in appropriate culturing agar medium for each specific bacteria.

3. Results

3.1 Detection of microorganisms in buffered samples

Aptasensors based on carbon nanotubes as ion-to-electron potentiometric transducers and aptamers as biorecognition elements were demonstrated as a completely novel biosensing platform with great capabilities in real-time pathogen detection at zero-tolerance levels in phosphate buffer solutions. In this case a RNA aptamer of 71-mer specifically tailored for the molecular recognition of type IVB pili of *Salmonella enterica* serovar Typhi (also known as *Salmonella* Typhi, or ST) [11] was used as biorecognition elements chemically linked to the transducing layer of nanotubes.

The potentiometric response of the aptasensor was found to be immediate after each inoculation with a range from 0.2 CFU mL^{-1} (1 CFU in 5 mL PBS) to 10^6 CFU mL^{-1} of ST (Fig. 2a), with a sensitivity of 1.87 mV/decade [16]. The response time is around 60 s after each inoculation, indicating a fast affinity equilibrium between the aptamers and ST. Considering the results obtained for a set of 5 different sensors, a linear relationship existed between the EMF response and the logarithm of the bacteria concentration up to 10^3 CFU mL^{-1}. The slope considerably decreases at higher levels, reaching a plateau at concentrations above 10^6 CFU mL^{-1} (Fig. 2b). This can be explained by the progressive saturation of the available binding sites. The signal corresponding to the lowest concentration of bacteria (0.2 CFU mL^{-1}) is high enough to be distinguished from the instrumental limit of detection delimited by three times the standard deviation of the noise (standard deviation of noise = ± 0.08 mV). After each set of potentiometric measurements, the aptasensors were regenerated by dissociating the aptamers from the bacteria in NaCl 2 M for 30 minutes, and then reconstituted by conditioning in PBS. With this regeneration all the electrodes were able to detect the minimum concentration of

bacteria for at least 3 months. This regeneration was proven to be successful in all the aptasensors reported in this paper.

Selectivity was tested against increasing concentrations of *Escherichia coli* and *Lactobacillus casei* (Fig. 2c). In both cases high concentrations of these microorganisms did not produce any significant potentiometric response. Control experiments (Fig. 2c) also confirmed that both aptamers and carbon nanotubes are needed to obtain the potentiometric response and that this response was not originated by unspecific adsorptions.

3.2 Detection of microorganisms in real samples

After the detection of microorganism in buffered samples, the logical step was the extension to real samples. A potentiometric aptasensor was addressed against a non-pathogenic strain of *Escherichia coli* (*E. coli* CECT 675) as a surrogate for pathogenic *Escherichia coli* O157:H7 in liquid food matrices such as milk or fruit juice, following the report of a RNA aptamer of 81-mer against this microorganism [12]. The pathogen was first detected using the developed aptasensors in phosphate buffer solutions and the performance parameters were adequately evaluated. In order to assess the applicability of this aptasensor into real samples with complex matrices, a sample pre-treatment protocol was designed, developed, validated and deployed into real fruit juice and milk samples looking for the elimination of undesired electroactive species within the original matrix, which otherwise may lead to incorrect or inaccurate results in biosensing experiments. The pre-treatment procedure includes three steps for the consecutive filtration, washing and elution of the target microorganism while the compatibility with the developed aptasensor was evaluated by the analysis of real samples with known loads of the target bacteria.

Similarly to the results obtained for the detection of ST, there was an immediate and evident change in the instrumental response at all concentrations upon the addition of *E. coli* to buffered samples [17]. In this case the stabilization time was a bit longer than for ST and while additions of low concentrations (4 CFU mL^{-1}) were stable after 120 seconds of the sample addition, higher values (10^4 CFU mL^{-1}) needed around 10-20 minutes. In any case, analysis of samples with concentrations of bacteria higher than 10^4 CFU mL^{-1} can be made but the exact amount of bacteria cannot be found. For the three aptasensors tested the range was linear for the interval from 4 to 10^4 CFU mL^{-1} with a sensitivity of 2.00 mV/decade. The instrumental limit of detection, measured as the average plus three standard deviations of the instrumental noise, was 63.4 μV for all the sensors tested, so higher changes in the EMF response should be easily resolved. More interesting are the results about selectivity in which the aptasensors showed an excellent intra- and inter-species selectivity (Fig. 3a). Selectivity was tested by exposing the

aptasensor to stepwise concentrations of *Escherichia coli* CECT 4558, *Lactobacillus casei* and *Salmonella enterica* in the same range of concentrations than the ones used for E. coli CECT 675 in PBS.

The real samples used were semi-skimmed milk and apple juice purchased in a standard supermarket. The samples were contaminated with *E. coli* (CECT 675) and were stored at 4°C before use. 1-2 mL of the samples (the volume depended on the specific sample analysed) were filtered and washed with PBS (minimum 10 mL) with an on-line filtration system (sterile cellulose acetate filters 13 mm diameter and 0.45 μm pore size, GE, Brussels, Belgium) in order to remove the charged species that are usually present in real samples whilst also keeping the total amount of microorganism cells on one side of the filter. The retained microorganisms were then easily eluted by passing 6-8 mL of PBS in the opposite direction of the initial filtering. The outcoming solution of eluted bacteria in PBS was injected directly into the measuring vessel [17]. The total time of the filtration-washing-elution process remained in the order of less than one minute for PBS and juice samples to two minutes in the case of milk. When this procedure was applied to real samples containing microorganisms we were possible to detect 12 CFU in 2 mL of milk (6 CFU mL^{-1}) and 26 CFU in 1 mL of apple juice (Fig. 3b).

Staphylococcus aureus (*S. aureus*) was also selected as a target molecule to be detected in pig skin, as a surrogate for human skin. The aptamer used in this case was a 88-mer DNA-aptamer reported by Cao et al. [13]. Before analysing real samples, performance parameters of the aptasensor were also established in PBS. These aptasensors were not able to achieve the low concentration levels of the aptasensors developed for ST and *E. coli*. Fig. 4a shows that the first clear potentiometric change was observed at a concentration of $8x10^2$ CFU mL^{-1} [18]. A response time between 6 and 11 minutes (90% of the total response, see inset in Fig. 4a) was observed for the concentration range $8x10^2$ CFU mL^{-1} to 10^8 CFU mL^{-1} and the signal was stable for at least one hour after the addition of the sample, which is enough time to perform any sample analysis with this type of aptasensor. The obtained sensitivity was also lower than in the previous cases (0.36 mV/decade), Fig 4b. Selectivity assays carried out with *E. coli* and *Staphylococcus epidermidis* show no change in EMF in the range between 0 and 10^8 CFU mL^{-1}.

For the detection of *S. aureus* in pig skin, we previously inoculated *S. aureus* over 2x2 cm of freshly excised dorsal pig skin. We then rubbed the inoculated pig skin with one sterile cotton swab moistened with sterile PBS, and this swab was introduced in a tube containing sterile PBS in order to transfer the recovered microorganisms to the solution by intense shaking and later detect the *S. aureus* present in an aliquot of this solution using the developed aptasensors. With this swab-based protocol, the average recovery rate of *S. aureus* is of 15%, what shows that *S.*

aureus can be detected in solid samples but that this protocol should be improved in order to correctly detect microorganisms in this type of samples.

4. Discussion

While the carbon nanotube-based aptasensors presented in this paper allows for the detection of ultralow concentrations of ST and *E. coli*, the detection of *S. aureus* does not reach the same low values of concentration. This may be due to the the thick polysaccharide layer of poly-N-acetylglucosamine on the surface of *S. aureus* and the low abundance of antigens that are externally exposed and available to biorecognition elements. This particular structure of *S. aureus* seriously limits the development of aptasensors for direct electrochemical detection, and only a few electrochemical biosensors have been developed to date for the detection of *S. aureus* with detection limits ranging between 10^4 and 10^5 CFU mL^{-1}. Probably because of this particular structure the response time of our aptasensor is also affected, and while low concentrations of *E. coli* and ST are detected in 60-120 seconds, a response time between 6 and 11 minutes is obtained for *S. aureus*.

The sensitivities are quite low for all the reported aptasensors, but since the instrumental noise is very low, probably due to the outstanding transducing abilities of carbon nanotubes, very low concentrations of microorganisms are able to detect. While the low instrumental noise can be probably attributed to the transducing abilities of carbon nanotubes, the excellent selectivity (intra- and inter- species) has to be assigned to aptamers. Depending of the purpose of the analysis, this can be an enormous benefit or a disadvantage. For instance, it is a benefit when testing a single serovar of a bacterial species but can be a drawback when we are interested in detecting the presence of microorganisms at the species or even genus level. In this latter case, the simultaneous use of several aptamers, isolated considering the different species –and serovars- should be considered. Another drawback of the use of aptamers is that only some aptamers against microorganisms are reported in the literature, but the use of improved Selex procedures for new aptamers selection is constantly increasing this number [19].

Successful detection of microorganisms in real samples such as milk or fruit juice was also accomplished. The pretreatment process needed to isolate the charged species in the real samples from the targeted microorganism was very simple and non-expensive, and it did not add a significant time to the overall process of analysis. When the aptasensors were applied to real solid samples (bearing in mind than in this case the detection was of such a challenging microorganism as *S. aureus*) the obtained recovery values were far from the ones obtained in liquid samples, showing the need for improved pretreatment protocols for solid samples.

This potentiometric biosensing platform also exhibited some limitations and drawbacks. The sensing capability for each individual aptasensor depended on the success of several critical steps. As an example, an appropriate deposition of the carbon nanotube transducing layer on the surface of the supporting electrode was a challenging step that depends on individual skills. In the case that the nanotubes layer was not adequately deposited, leaching of carbon nanotubes present on the more external layers would represent a serious limitation for aptamer-functionalized sensors since the recognition layer may be lost after the functionalization step. Additionally, the functionalization procedure also depends on technical skills, such as the adequate preparation of the solutions involved during the functionalization steps or the adequate management of materials. Therefore, the rate of functional aptasensors has remained variable so far and depended on the technical skills exhibited during the preparation of the sensors.

The cost of the aptamer synthesis was additionally a limitation. As an example, 1 μmol of a RNA oligomer of about 50-100 bases may cost between 500-1600 euro according to the year 2012 market prices. This factor is critical, especially when the functionalization of nanotubes with aptamers yields to a low rate of functional aptasensors. However, the exponential increase on the number of patents and published scientific articles related to aptamers observed since the year 2002 may be translated into a decrease on market prices in a future. This same phenomenon was observed in recent years with carbon nanotubes, which can be nowadays acquired at reasonable prices.

5. Conclusions

We have presented in this paper potentiometric aptasensors based on carbon nanotubes as ion-to-electron transducers and aptamers as biorecognition elements that can be used in the real-time detection of pathogens. This generation of aptamer-based aptasensors can be customized for the detection of different pathogens and different aptasensors were prepared using different aptamers, addressed against their corresponding specific pathogenic targets: *Salmonella* Typhi and *Escherichia coli* as gram-negative bacteria models and *Staphylococcus aureus* as a gram-positive bacteria example. Detection of the corresponding targets was successful in all the cases and their performance parameters were estimated.

Aptasensor miniaturization would represent an opportunity area for improving this new generation of aptamer-based potentiometric biosensors in a near future. The advantages of miniaturization are evident in terms of cost, since a less amount of aptamers and nanotubes would be therefore needed. As an example, our group is currently developing the first examples

of single-use potentiometric sensors in a miniaturized planar format or using non-expensive substrates such as paper [20, 21]. The combination between the aptasensor technology and the reduced planar format of potentiometric sensors may be translated into single-use potentiometric aptasensors in a future, thus eliminating the need of aptamer regeneration steps in pathogen detection. However, the possible repercussions on aptasensor performance when the sensor surface is dramatically reduced have not been explored so far.

Finally, the possibility to expand the biosensing platform for the detection of other targets than bacteria is a very interesting research area for the future. As an example, the real-time detection of whole infectious agents such as viruses or parasites would represent a very important advance in pathogen biosensing.

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Figure captions

Figure 1. a) Carbon nanotube deposition on the surface of a glassy carbon electrode; b) ESEM (environmental scanning electrode microscope) image of the previously deposited layer of SWCNTs on the top of the glassy carbon electrode, showing a lateral view and a surface view. The lateral view shows the homogeneous height of the layer and the surface view shows that the layer completely covers the whole surface; c) Experimental set-up for potentiometric measurements; d) Schematic illustration of the potentiometric aptasensor including the covalent functionalization of carboxylated SWCNTs with the specific aptamer following carbodiimide-mediated chemistry and the interaction between aptamer and the target bacteria.

Figure 2. a) Potentiometric response of the aptasensors exposed to stepwise concentrations of ST. Values are the final concentration of bacteria. Inset is an amplification for the inoculation step at 0.2 CFU mL^{-1} to show the fast response (time is in seconds); b) EMF response versus decade of concentration of ST. The solid line is the linear regression fit and the equation below was obtained for that range. Error bars are the standard deviation of the response obtained at same concentrations for 5 different sensors. Error values in parenthesis are the standard deviation for the different regression equations obtained for 5 different sensors; c) EMF response versus recorded time, for stepwise concentrations of bacteria. Solid vertical lines represent inoculation with increasing amounts of bacteria. 1: carbon nanotube sensor functionalized with $CH_3(CH_2)_4NH_2$ exposed to ST, 2 and 3: aptasensors exposed to *E. coli* and to *L. casei*, respectively, 4: glassy carbon electrode functionalized with $CH_3(CH_2)_4NH_2$, exposed to ST, 5: carboxylated SWCNT sensor without any functionalization, exposed to ST, 6: glassy carbon electrode after functionalization with the aptamer and exposed to ST.

Figure 3. a) Selectivity assays for the detection of *E. coli* CECT 675: EMF response of the biosensor functionalized with aptamer exposed to *S. enterica* (1), *L. casei* (2), *E. coli* CECT 4558; b) Potentiometric detection of microorganisms using aptasensors exposed to real samples (pre-treatment steps previously carried out to remove original matrix). Above: sample of milk containing 12 CFU of *E. coli* CECT 675 in 2 mL of sample. Below: sample of apple juice containing 26 CFU of *E. coli* CECT 675 in 1 mL of sample.

Figure 4. a) Change in EMF recorded as a function of time for different aptasensors when exposed to *S. aureus*. Inset, amplification of the curve after inoculation with $8x10^2$ CFU mL^{-1}; b) Potentiometric response as a function of concentration of bacteria in decade units (the circles represent the average responses of three different biosensors; error bars are standard deviation).

Figure 1

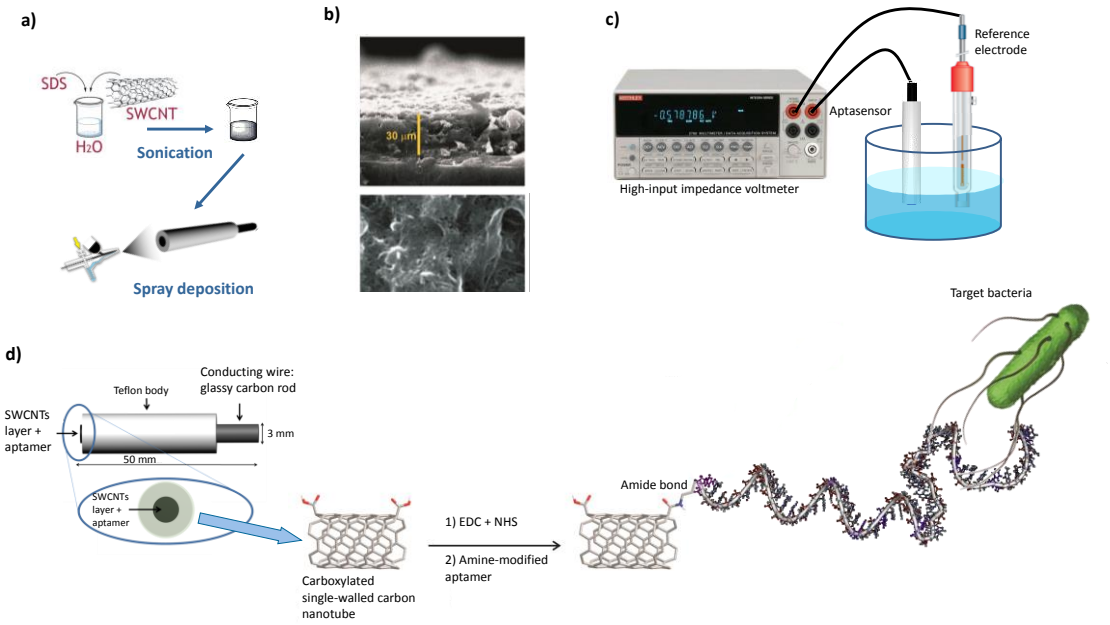

a)
SDS
SWCNT
H2O
Sonication
Spray deposition
b)
30 µm
c)
Reference electrode
Aptasensor
High-input impedance voltmeter
d)
Teflon body
Conducting wire: glassy carbon rod
SWCNTs layer + aptamer
3 mm
50 mm
SWCNTs layer + aptamer
Carboxylated single-walled carbon nanotube
1) EDC + NHS
2) Amine-modified aptamer
Amide bond
Target bacteria

Figure 2

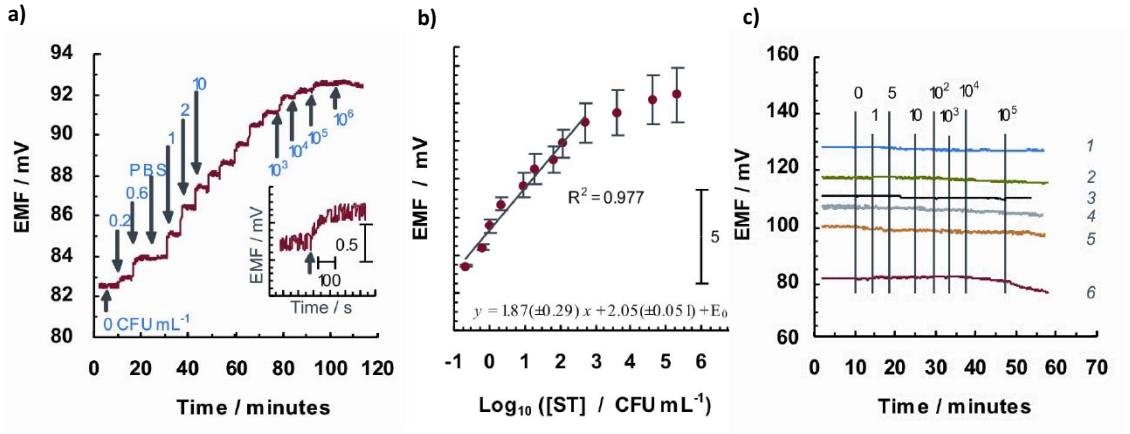

a)
b)
c)
EMF / mV
Time / minutes
PBS
0.2
0.6
1
2
10
0 CFU mL-1
Time / s
0.5
100
R2 = 0.977
5
y = 1.87(±0.29) x +2.05(±0.05 l) +E0
Log10 ([ST] / CFU mL-1)
0
5
1
10
1
2
3
4
5
6

Figure 3

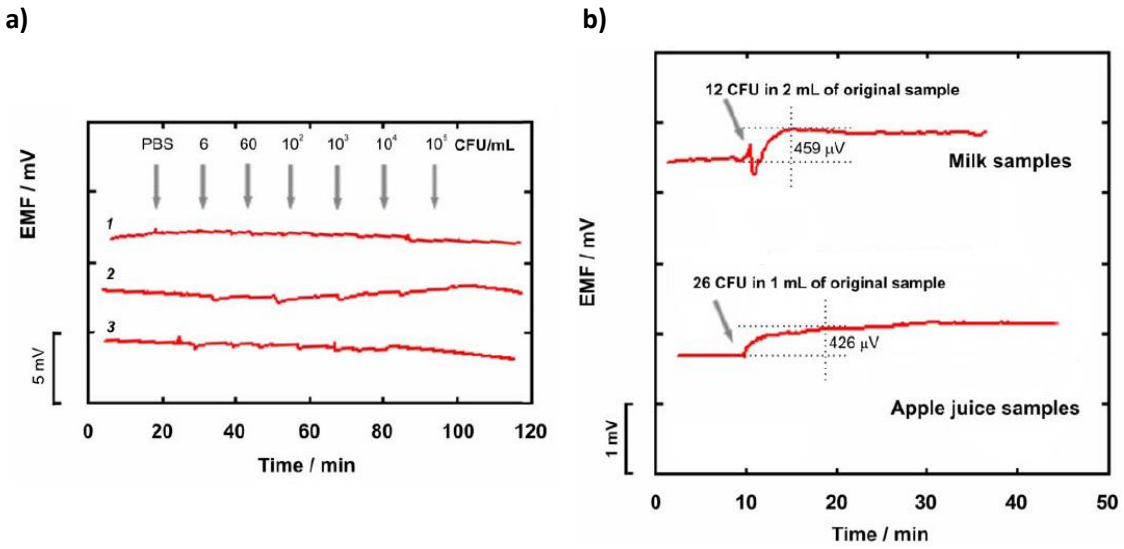
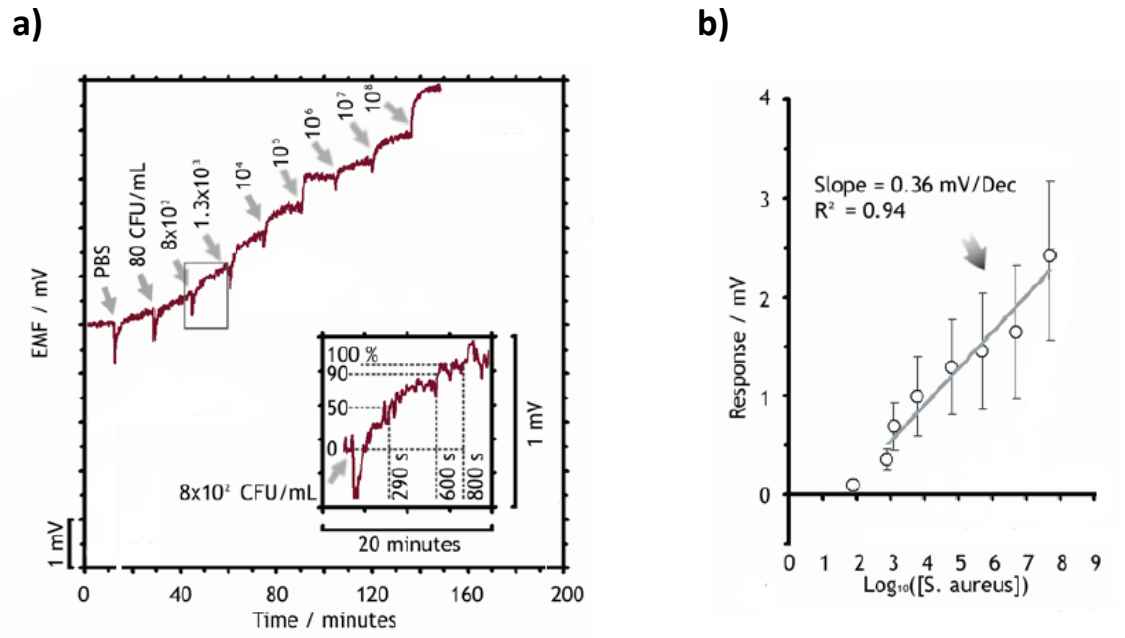

a)
b)
EMF / mV
PBS
6
60
10²
10³
10⁴
10⁵ CFU/mL
1
2
3
5 mV
0
20
40
60
80
100
120
Time / min
12 CFU in 2 mL of original sample
459 μV
Milk samples
26 CFU in 1 mL of original sample
426 μV
Apple juice samples
1 mV
0
10
20
30
40
50
Time / min

Figure 4


a)
b)
EMF / mV
PBS
80 CFU/mL
8x10²
1.3x10³
10⁴
10⁵
10⁶
10⁷
10⁸
100 %
90
50
0
290 s
600 s
800 s
1 mV
8x10² CFU/mL
20 minutes
1 mV
0
40
80
120
160
200
Time / minutes
Slope = 0.36 mV/Dec
R² = 0.94
Response / mV
Log₁₀([S. aureus])