Real-Time Potentiometric Detection of Bacteria in Complex Samples

Gustavo A. Zelada-Guillén, Suryakant V. Bhosale, Jordi Riu, F. Xavier Rius*

Departament de Química Analítica i Química Orgànica. Universitat Rovira i Virgili, 43007, Tarragona, Spain.

Authors' E-mail addresses: gustavoadolfo.zelada@urv.cat, suryakant.bhosale@estudiants.urv.cat, jordi.riu@urv.cat, fxavier.rius@urv.cat

To whom correspondence should be addressed. E-mail: jordi.riu@urv.cat. Telephone: +34 977558491

Detecting and identifying pathogen bacteria is essential to ensure quality at all stages of the food chain and to diagnose and control microbial infections. Traditional detection methods, including those based on cell culturing, are tedious and time-consuming and their further application in real-samples generally implies more complex pre-treatment steps. Even though state-of-the-art techniques for detecting microorganisms enable the quantification of very low concentrations of bacteria, to date it has been difficult to obtain successful results in real-samples in a simple, reliable and rapid manner. In this report, we demonstrate that the label free detection and identification of living bacteria in real samples can be carried out in a couple of minutes and in a direct, simple and selective way at concentration levels as low as 6 colony forming units/mL (CFU) in complex matrices such as milk or 26 CFU/mL in apple juice where the pre-treatment step of samples is extremely easy. We chose *Escherichia coli* (*E. coli*) CECT 675 cells as a model organism as a nonpathogenic surrogate for pathogenic *E. coli* O157:H7 to test the effectiveness of a potentiometric aptamer-based biosensor. This biosensor uses single-walled carbon nanotubes (SWCNT) as excellent ion-to-electron transducers and covalently immobilized aptamers as biorecognition elements. The selective aptamer-target interaction significantly changes the electrical potential, thus allowing for both inter-species and inter-strain selectivity and enabling the direct detection of the target. This technique is therefore a powerful tool for the immediate identification and detection of microorganisms. We demonstrate the highly selective detection of living bacteria with an immediate linear response of up to 10^4 CFU/mL. The biosensor can be easily built and used, is regenerated without difficulty, and can be used at least 5 times with no loss in the minimum amount of detected bacteria.

KEYWORDS: sensors, carbon nanotubes, aptamers, bacteria, real samples.

INTRODUCTION

Microbial diseases represent the main cause of death in many countries. For example, each year, foodborne diseases cause at least 76 million illnesses in the United States alone.[1] In sanitary terms, time of analysis and reliability in the identification of pathogens are both critical, especially during outbreaks. The detection method followed to detect bacteria is essential for an early successful diagnosis. Detection methods must be able not only to detect extremely low concentrations of pathogens but also to succeed in both deploying the techniques in real-samples and identifying the particular strain of the pathogen in order to provide precise information that could be vital for an accurate diagnosis, successful prevention and correct therapeutic treatment. The whole process, however, generally requires the use of cell culturing-based methods that can take up to several days to provide specific results. The same drawback arises in microbiological control of foods/beverages and other sample types. Rapid and sensitive detection and identification of a particular bacteria strain are therefore particularly important in medical diagnosis, biotechnology and food safety.[2,3]

The classic plate count technique has been the mainstay of pathogen detection in recent decades and it is a very cheap tool; however, it takes one to three days to provide results. Available state-of-the-art bacteria-detection techniques use a wide range of approaches. Successful detection methods include the use of polymerase chain reaction (PCR)[4,5], micromechanical oscillators[6], biofunctional magnetic nanoparticles[7,8], electrochemical-based methods (field-effect transistors[9], amperometry[10], square wave voltammetry[11]) and B-cell-based sensors[12], among others. Nevertheless, most of these techniques are expensive to implement, do not have adequate limits of detection or the analyzed samples always need to be pre-treated, what makes the overall procedure in some cases extremely long and complex to deploy in "real-life". Even though there have been some recent interesting attempts to reduce the complexity of the pre-processing steps[2], most of these lead to analysis times in the order of hours and none of the reported cases is able to attain a close-to-real-time detection of pathogens in complex matrices.

Nanomaterials are excellent for developing label-free, high-sensitivity sensors. Very recently, our group has demonstrated that it is possible to use single-walled carbon nanotubes (SWCNT) in ion selective electrodes (ISEs) as ion-to-electron transducers in potentiometric analysis [13,14]. Moreover, this approach can be used not only to detect ions but also to elicit a potentiometric response in the presence of proteins and bacteria by using label-free SWCNT/aptamer-hybrid material based biosensors[15,16]. The transducing properties of this SWCNT/aptamer-hybrid material originate from the extremely high surface-to-volume ratio of the nanotubes, the material's ability to support charge transfer between the ions that surround the cell wall of the target bacteria, the SWCNT/aptamer-functionalized layer, the remarkable SWCNT double-layer capacitance and the conformational changes in the linked aptamers during the target-recognition step[14-18]. These reasons combined with the simplicity and portability that potentiometry offers to chemical/biological analysis in general[19] are precisely what makes SWCNT/aptamer a perfect material for detecting microorganisms[20]; that is, SWCNT/aptamer makes the detection procedure a simple task that does not require high technical skills. Nonetheless, until now, two main drawbacks remain unsolved. First, discriminating between different strains of a same pathogen has been an elusive task up to date and second, it has not been possible to successfully apply this material to identifying and detecting microorganisms in real samples because the electroactive species present in environmental and food/beverage sample solutions tend to produce either false positives or hinder the potentiometric response generated by the ionic screening of the target. In this study, it is demonstrated for the first time that a particular strain of a microorganism can be detected and identified in real samples with the minimum number of pretreatment steps, in close to-real-time and almost zero-tolerance conditions. This was achieved by developing a biosensor based on aptamers (also called aptasensor) chemically linked into a layer of previously carboxylated SWCNTs which was able to selectively detect *E. coli* CECT 675 as a nonpathogenic surrogate for pathogenic *E. coli* O157:H7 in complex liquid samples such as milk and fruit juice.

Escherichia coli (*E. coli*) is one of the most common bacteria types and is a normal inhabitant of the large intestine of warm-blooded animals. Some of the strains of *E. coli* are particularly virulent and, as is the case with *E. coli* O157:H7, cause a wide spectrum of human diseases, ranging from some types of hemorrhagic and non-hemorrhagic diarrhea, to occasional kidney failure or hemolytic uremic syndrome and death due to ingestion of contaminated food [21,22]. To demonstrate the plausibility of identifying targets using our biosensor, we assessed its selectivity against different microorganisms such as *Salmonella enterica*, *Lactobacillus casei* and a different strain of *Escherichia coli* (CECT 4558), and found that none of them gave a detectable potentiometric signal. Furthermore, in all the samples contaminated with the target microorganism and subsequently analyzed with our aptasensor, the minimum amount of bacteria detected was far below the lower limits allowed by the European

Regulations 2073/2005 and 1441/2007, which demonstrates that the biosensor can be successfully used to analyze real samples with minimum pre-treatment steps, and that the change in the potentiometric response can be achieved in almost real-time.

EXPERIMENTAL SECTION

Chemicals, aptamer and culturing media

Water used to prepare the solutions was purified through a Milli-Q system (Millipore, Madrid, Spain) and in all cases the resistivity level of purified water was 18.2 MΩ cm. The reagents sodium dodecyl sulphate (SDS), N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), 2-(N-morpholino) ethanesulfonic acid (MES) and cetyltrimethylammonium bromide (CTAB), KH_2PO_4 and Na_2HPO_4 were purchased from Sigma-Aldrich (Tres Cantos, Spain). All the substances were used as received. Phosphate buffer solution (PBS) 1.7 mM pH 7.4 was prepared using a 1:100 dilution of a 0.17 M stock solution of corresponding amounts of KH_2PO_4 and Na_2HPO_4, and the pH was adjusted as required.

Tryptic soy agar (TSA) / broth (TSB), Lactobacilli MRS (deMan, Rogosa and Sharpe) agar/broth, XLD (xylose-lysine-deoxycholate) agar, MacConkey agar/broth and Luria-Bertani (LB) agar/broth were purchased from Becton, Dickinson and Company (Sparks, U.S.A.). The *E. coli* CECT 675 binding aptamer of 81-mer with the sequence[9] 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', and $-(CH_2)_5NH_2$ modified in the 3' end was purchased from Eurogentec (London, UK). The aptamer was resuspended in MilliQ water and stored at -80 °C. Quality control of the aptamer was carried out by MALDI-TOF analysis (Voyager-DE STR, Applied Biosystems, Carlsbad CA, USA) to ensure that the aptamer had not degraded before the biosensors were prepared.

The SWCNTs were purchased in bulk form from HeJi (Zengcheng, China) and had > 90 % purity, an average length of 150 μm and an average diameter of 1.4-1.5 nm. The SWCNTs were oxidized in a silica furnace chamber (365 ºC, synthetic air flow-rate of 100 cm^3 min^{-1} and 90 minutes) 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[23] . 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 acid completely. The filtered SWCNTs were dried overnight at 80 ºC.

Instrumentation and materials

A FEI Quanta model 600 environmental scanning electron microscope (ESEM) (FEI Company, Inc., Hillsboro, USA) was used for the microscopic analysis. Potentiometric measurements were taken in all cases with a Keithley high-input impedance voltmeter M6514 (London, U.K.) using an Ag/AgCl/KCl (3 M) double junction reference electrode containing a 1 M LiAcO electrolyte bridge (type 6.0729.100, Metrohm AG, Herisau, Switzerland) and the SWCNT-based biosensor as the working electrode. EMF was automatically recorded to a Microsoft Excel sheet by means of an input/output add-in tool provided also by Keithley. Sterile cellulose acetate filters (13 mm Ø and 0.45 μm pore size) were purchased from GE (Brussels, Belgium).

Preparation of the aptasensor

The solid contact biosensor was made of a 50 mm-long 3 mm Ø glassy carbon cylindrical rod (HTW Hochtemperatur-Werkstoffe GmbH, Thierhaupten, Germany) covered by a Teflon jacket of 7 mm Ø. The surface of the glassy carbon was successively polished using 25 and 1 μm grain size polishing alumina (Buehler, Lake Bluff, USA) before the SWCNT layer was deposited by spraying the SWCNT on the same surface. For the spraying process, we previously homogenized the SWCNT dispersion with a tip-sonicator (amplitude 60 %, 0.5 s^{-1}, Ultraschallprocessor UP200S, Dr. Hielscher, Teltow, Germany) for 30 minutes to prepare a solution of 25 mg of purified SWCNT dispersed in 100 mL of MilliQ water containing 100 mg of SDS. 10 mL of the SWCNT/SDS/H_2O solution was sprayed onto the exposed glassy carbon surface under a high temperature (200 ºC) air blow, and washed with MilliQ water to progressively remove the SDS. A 30 μm homogeneous layer of SWCNT (measured with ESEM) was deposited after the spraying process. The carboxylic groups on the sidewalls of the deposited SWCNTs were activated using a solution containing 100 nmol of EDC and 25 nmol of NHS in a 50 mM MES buffer pH 5 for 30 minutes[24]. Subsequently, the electrodes were soaked overnight in 500 μL of a 1 μM aptamer solution, which also consisted of PBS pH 7.4 (1 mM) and CTAB (0.2 mM). This well-known carbodiimide mediated chemistry was followed in order 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.

Microorganisms and culturing conditions

All the microbiological manipulations were performed using sterile material and in a microbiological safety cabinet, model BIO II A (Telstar Industrials, Terrassa, Spain). Lyophilized strains of *Escherichia coli* (CECT 675), *Salmonella enterica* (CECT 409) and *Lactobacillus casei* (CECT 4180) were purchased from Colección Española de Cultivos Tipo (Valencia, Spain). *Escherichia coli* (CECT 4558) was kindly donated by the Biotechnology Department of the Universitat Rovira i Virgili (Tarragona, Spain). All bacteria strains were cultivated under the same incubation time and temperature conditions, but in different growth media: *E. coli* was cultivated in LB broth, *L. casei* in Lactobacilli MRS broth, and *S. enterica* in TSB. The bacteria samples were stored at -20 °C in glycerol / broth medium (10% v/v) and reactivated by incubating the bacteria in 10 mL of sterile broth medium at 37 °C for 24 h. The bacteria samples were then centrifuged at 6000 rpm for 15 minutes and the supernatant was discarded.

The precipitate was washed with PBS 1.67 mM, centrifuged again and the supernatant discarded. The pellet was then resuspended in 10 mL of sterile PBS 1.67 mM, and the resulting solution was eightfold 1:10 diluted to give a series of 10^{-1} to 10^{-8} stock solutions of bacteria. The stock solutions were quantified in quintuplicate using the standard plate count method[25] and in an appropriate culturing agar medium (TSA for *S. enterica*, LB for *E. coli* and MRS for *L. casei*), and the same procedure was also followed to standardize the variable aliquots of stock solutions that were used to inoculate the samples to be analyzed.

Analytical procedure

All the electromotive force (EMF) measurements were taken in low ionic strength buffer (1.7 mM PBS) at pH = 7.4, stirred at 300 rpm and contained in an isothermal vessel at 22±0.5 °C using 5 mL of sterile PBS before any bacteria were inoculated. The changes on the electromotive force were automatically measured at periods of 10 seconds by adding concentrations of bacteria in a stepwise mode and following changes on the recorded values. We used real samples contaminated with *E. coli* (CECT 675), control samples that did not contain any microorganism, and selectivity control samples which were contaminated with *E. coli* (CECT 4558), *L. casei* and *S. enterica* in either PBS or real-samples. The contaminated and control samples were stored at 4 °C before use, and were previously inoculated with the appropriate microorganism as needed. The real samples used were semi-skimmed milk and apple juice purchased in a standard supermarket. Each analyzed sample (either stock solutions or real samples) was simultaneously evaluated following the standard plate count method in quintuplicate using the appropriate culturing agar in order to validate the method reported herein. For *E. coli*-containing samples, further confirmatory tests were carried out using the conventional method based on the MacConkey agar test[11,25]. The dilution was corrected for all the stepwise concentration experiments. Consecutive filtering of the matrix (the volume depended on the sample analyzed, but typically ranged from 1 mL to 2 mL) and washing with PBS (minimum 10 mL) was done with an on-line filtration system in order to remove the charged species that are usually present in real samples and which may interact with the carbon nanotubes, whilst also keeping the total amount of microorganism cells on one side of the filter. Further elution of the retained cells was easily done by passing 6-8 mL of PBS in the opposite direction of the initial filtering, and the outcoming solution of eluted bacteria in PBS was injected directly into the measuring vessel in order to monitor the EMF response provided by the potentiometric system biosensor-reference electrode (see ***Figure 1***). 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. After each set of inoculations and measurements, the biosensors were regenerated by dissociating the aptamers from the bacteria in 2 M NaCl for 30 min and then reconstituted by conditioning in PBS, thus leaving the aptasensor ready to record new measurements.

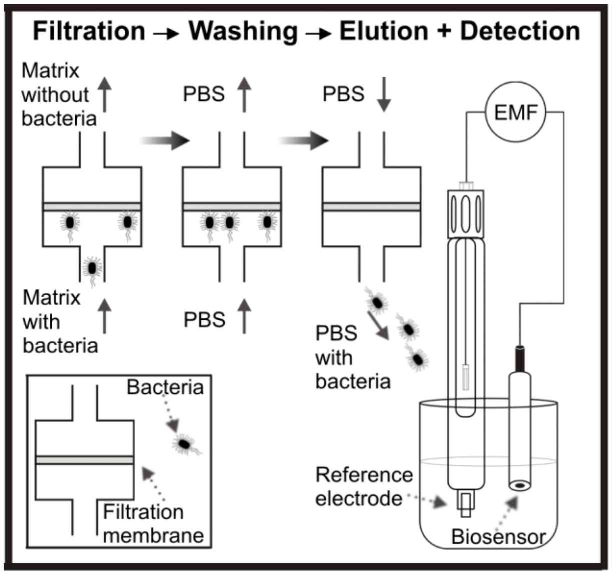

Figure 1. Experimental setup for the pre-treatment steps required to remove the matrix in real samples and to detect microorganisms contained therein using the potentiometric biosensor. Starting from left to right: first step, filtration of sample and matrix removal; second step, washing with PBS; third step, elution with PBS and potentiometric detection of bacteria recovered in eluate.

RESULTS AND DISCUSION

Biosensors were exposed to stepwise increasing concentrations of *E. coli* CECT 675 in PBS and the EMF response was recorded as a function of time in order to assess the performance of each biosensor (sensitivity, precision, limit of detection, response time and stability) before the final deployment in real-samples (***Figure 2***). Under the experimental conditions, instrumental detection response (also known as critical value), measured as the average plus 3 standard deviations of the instrumental noise[26], was 63.4 μV for all the sensors tested, so higher changes in the EMF response should be easily resolved. There was an immediate and evident change in the signal at all concentrations of the target bacteria when this was added. A quick increase in EMF was observed within the whole dynamic range as 50% of the potentiometric response was attained in the order of seconds when any concentration of *E. coli* CECT 675 was added, starting from as low as 4 CFU/mL (***Figure 2a***). Stabilization in EMF response strongly depended on the concentration. This is demonstrated by the fact that the EMF response could only be stabilized for the lowest concentration of bacteria detected (4 CFU/mL) after 120 seconds of the sample addition, whereas for higher values (e.g. 10^4 CFU/mL), the stabilization of the EMF response was rather slower and remained in the order of 10-20 minutes. Once the EMF response was reached, it was stable for at least one and a half hours at concentrations below 10^4 CFU/mL, which is enough time to perform any further analysis of bacteria in either PBS or real samples. Nonetheless, stability at concentrations above 10^4 CFU/mL dramatically decreased after inoculation because the response drifted once the maximum signal had been reached. This was possibly caused by stochastic charge transfer processes between the surface of the biosensor and the excessive amount of bacteria that is not tethered to the biosensor by means of the aptamers. This occurs when the available binding sites on the biosensor surface become over-saturated in high concentrations of the target microorganism. In any case, analysis of samples with concentrations of bacteria higher than 10^4 CFU/mL can be made but the exact amount of bacteria cannot be found**.** ***Figure 2b*** shows the calibration curve for the range of 4 to ~10^4 CFU/mL.

Electromotive force as a function of decade for the bacteria concentration can be approximated to a straight line model over the same calibration range and shows a dynamic range of 5 orders of magnitude. The average slope was 2.0 mV/Decade (Standard deviation = 0.8 mV/Decade) (the average value was obtained by testing three different biosensors), and the R^2 value was 0.96.

It is interesting to note that at lower bacteria levels (see ***Figure 2b*** at the concentration range below 2.2×10^2 CFU/mL) the biosensor offers a higher sensitivity and a slightly better coefficient of determination than at the whole concentration range indicated in the graph. This could be attributed to the fact that at low concentrations of bacteria there are evidently more available binding sites on the biosensor surface than at higher concentrations and thus, a higher efficiency on the binding event may be expected. However, both the linearity observed for the range 4 – 2.4×10^4 CFU/mL and the standard deviation of the slope are still acceptable for the analytical purposes of the biosensor when the main concern is deciding whether the pathogen is present or absent in the sample analyzed, since the minimum amount of bacteria able to be detected remains unaffected.

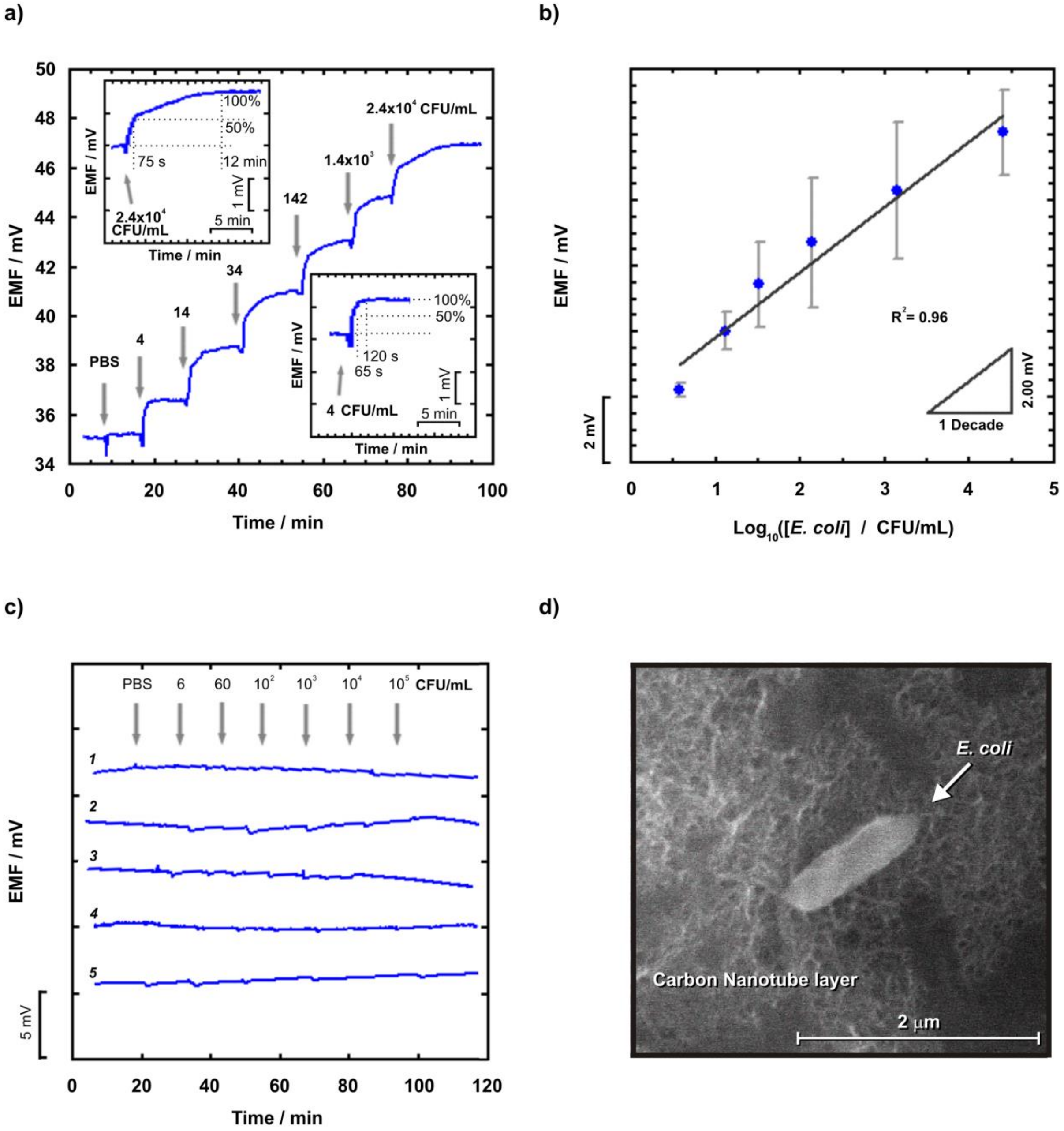

Figure 2. **a)** Performance of SWCNT biosensors functionalized with anti-*E. coli* CECT 675 RNA aptamer: EMF response exposed to stepwise increasing concentrations of *E. coli* CECT 675 as a function of time; insets show the time required to achieve 50% and 100% of the EMF response after inoculation with 4 CFU/mL (right-down) and 2.4x10^4 CFU/mL (left-up) **b)** EMF response as a function of the logarithm of the *E. coli* CECT 675 concentration. The solid circles are the average potentiometric

response of three different biosensors exposed to the same concentration values, the solid line is the linear regression fit, and the triangle inside represents the sensitivity of the method. Error bars indicate the minimum and maximum response values obtained with three different biosensors. **c)** Selectivity assays and blanks: EMF response of the biosensor functionalized with aptamer exposed to *S. enterica* (*1*), *L. casei* (*2*), *E. coli* CECT 4558 (*3*); EMF response of the SWCNT electrodes exposed to *E. coli* CECT 675 (*4*) and SWCNT electrodes incubated with aptamer overnight (without functionalization) exposed to *E. coli* CECT 675 (*5*). **d)** ESEM image of an *E. coli* CECT 675 cell captured on the SWCNT/aptamer.

The sensor was regenerated after the assays by using an aqueous solution of 2 M NaCl to release the bacteria bound to the aptamers and by further washing with MilliQ water for 10 minutes. After the last step, the sensor was stored in PBS solution. Regenerated sensors remained practically unchanged in terms of sensitivity and limit of detection and we observed that they may be exposed to at least 5 regeneration cycles before the limit of detection and sensitivity are affected. The EMF noise of the regenerated sensors in all the cases remains, however, unaltered and does not depend on how many regeneration cycles the sensor has been exposed to.

Selectivity was tested by exposing the aptasensor to stepwise concentrations of *E. coli* CECT 4558, *L. casei* and *S. enterica*, in the same range of concentrations than the ones used for *E. coli* CECT 675 in PBS (***Figure 2c*** curves *1* - *3*). The results demonstrated that there is no cross-reactivity in either inter-species or inter-strain tests, which means that the biosensor is highly selective. Therefore, strain-specific tests for identifying bacteria could be created if the system is used to analyze real-samples. Control tests were also carried out to determine whether the potentiometric signal was due exclusively to the biosensor's bacterial recognition event and not to either unspecific interactions between the SWCNTs and the target bacteria or the leakage of adsorbed aptamers remaining from the functionalization step when the biosensors were prepared. To evaluate whether unspecific interactions led to a potentiometric response, a SWCNT electrode that had not been functionalized with the aptamer was exposed to stepwise increasing concentrations of target bacteria (*E. coli* CECT 675), starting from 0 CFU/mL up to 10^5 CFU/mL, but no response was observed (***Figure 2c*** curve *4*). In the same way, another control experiment assessed the possibility that the potentiometric response was generated by the release of those aptamer molecules that may be physically adsorbed onto the SWCNT walls. This test was carried out by preparing an SWCNT electrode that had previously been incubated directly in 500 μL of a 1 μM aptamer solution for 24 hours to allow the aptamer molecules to adsorb onto the SWCNT layer,[27] although the process did not include the chemical step of covalent bond formation between the carboxylic moieties of the SWCNT and the aptamer. This electrode was also exposed to increasing amounts of target bacteria and the potentiometric response was recorded in real time, but no EMF response was obtained (***Figure 2c*** curve *5*). The comparison of the EMF response of the biosensor when the aptamers are covalently immobilized over the SWCNTs (***Figure 2a***) with the lack of EMF response when the aptamers are incubated overnight without linking to the SWCNTs (***Figure 2c*** curve *5*) clearly shows that the covalent immobilization of the aptamers on the SWCNTs assures the generation of the proper signal in our biosensor. Regeneration results presented previously in the manuscript also support this fact. According to ***Figure 2c*** (curves *4* and *5*), both control experiments confirmed that the responses observed are caused exclusively by the binding event between the target bacteria and the aptasensor, because the EMF did not change when any of the control electrodes were exposed to target bacteria.

As in all potentiometric measurements, using the SWCNT/aptamer based biosensor to detect microorganisms in liquid samples requires control of the ionic strength of the sample, otherwise the different charged species contained in the samples may lead to a potentiometric response and false-positive results would be produced. For example, the potentiometric response for milk and apple juice samples leads to a high jump in the potentiometric measurement if no pre-processing steps are carried out in a sample without target bacteria, as is demonstrated in ***Figure 3a*** and ***b*** (insets). For this purpose, we filtered the complex matrices that constitute the real samples containing microorganisms in order to separate the microorganisms from the real sample matrix. The microorganisms are further eluted in a controlled PBS medium prior to potentiometric measurement with the biosensor. The whole process of filtration, washing and elution each sample was completed within just a few minutes, as is explained in the analytical procedure section. Control tests demonstrated that the pre-processing steps eliminate the possibilities of false-positives if applied to real samples (***Figure 3a*** and ***3b,*** see insets and curve ***2*** in both figures). This procedure is also compatible with the selectivity of our biosensor. When this procedure is applied to real samples containing microorganisms, it is possible to differentiate between samples contaminated with *E. coli* CECT 675 and samples contaminated with other types of pathogen and, moreover, between different strains of the same pathogen type (see curve ***1*** in both ***Figures 3a*** and ***3b***, and compare with ***Figure 3c***). The lowest amount of bacteria detected in complex matrices was 12 CFU in 2 mL of milk (6 CFU/mL) and 26 CFU in 1 mL of apple juice, which means that the presence of target bacteria detected in real-samples can be assessed in almost zero-tolerance conditions and with close to real-time responses. According to the European Regulations 2073/2005 and 1441/2007, the concentration of *E. coli* allowed to be present in beverages must be below 1000 CFU/mL in the case of apple juice samples, and below 5 CFU/mL for milk samples.

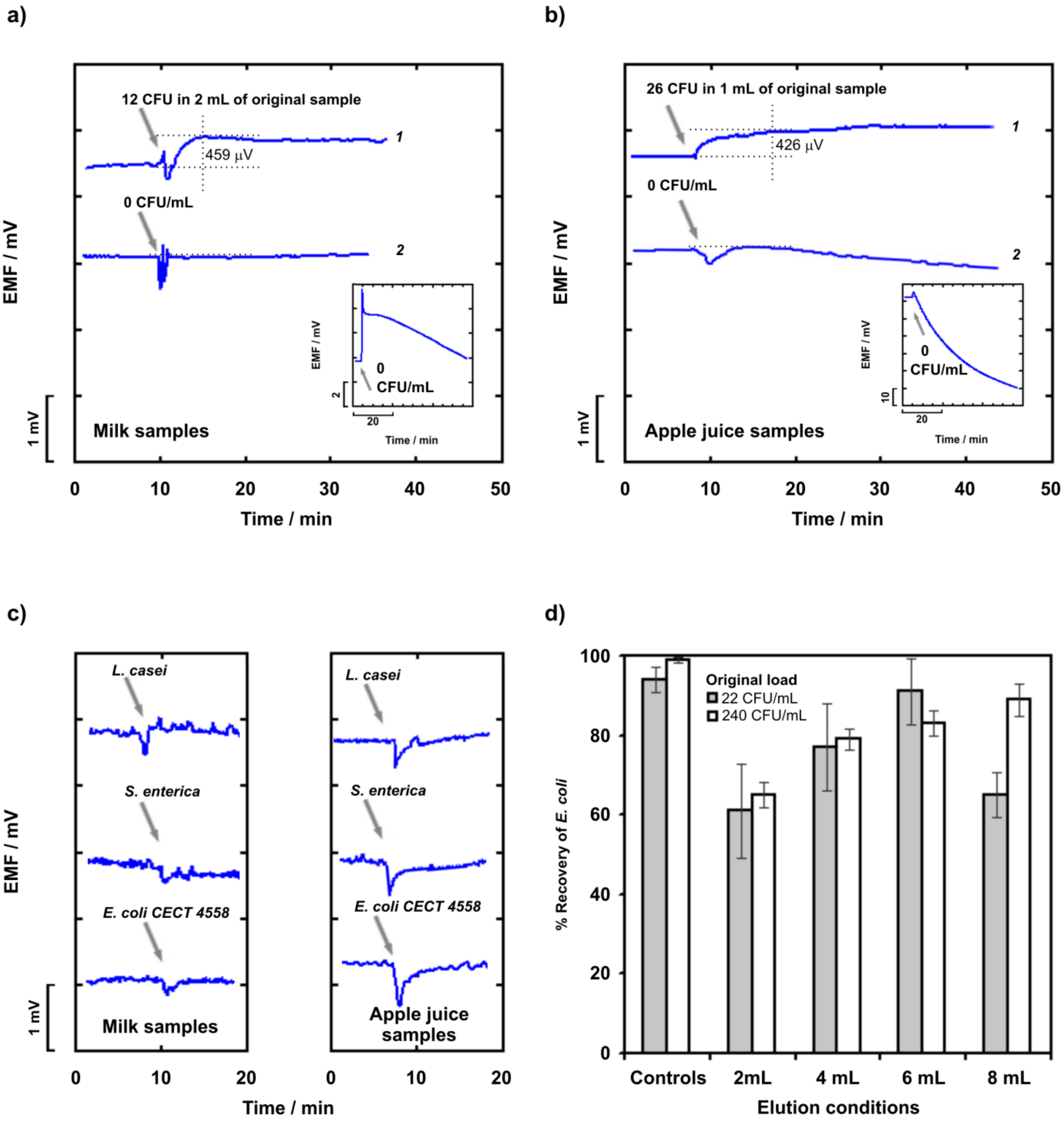

Figure 3. Potentiometric detection of microorganisms using aptasensors exposed to real samples (pre-treatment steps previously carried out to remove original matrix). **a**) Sample of milk: original matrix containing inoculated *E. coli* CECT 675 12 CFU in 2 mL of sample (*1*) and without inoculated bacteria

(*2*). **b**) Sample of apple juice: original matrix containing inoculated *E. coli* CECT 675 26 CFU in 1 mL of sample (*1*) and without inoculated bacteria (*2*). Insets in **a)** and **b)** respectively show the potentiometric response for milk and apple juice samples if no pre-processing steps are carried out in a sample without target bacteria. **c**) Selectivity assays in real samples (milk in left hand chart, and apple juice in right hand chart): original matrix containing 10^3 CFU/mL of the following bacteria: *L. casei* (*1*), *S. enterica* (*2*), *E. coli* CECT 4558 (*3*). **d**) Recovery of *E. coli* CECT 675 loaded in PBS after pre-treatment steps with different elution conditions and in two different concentrations of bacteria (22 and 240 CFU/mL): Controls correspond to the bacteria detected after following the pre-treatment procedure when the filter membrane is removed from the system so as to assess the loss in recovery due to the irreversible attachment of bacteria onto the inner channels of the system. The different volumes (2, 4, 6 and 8 mL) indicated on the *x* axis represent the quantity of PBS used the in elution step of the pre-treatment process. The *y* axis represents the average % recovery performed in triplicate, compared to the initial amount of bacteria loaded. The error bars are the standard deviation (N=3). The bacteria were counted using the standard plate count method in triplicate.

The previously estimated detection limit values show that our system could be successfully used to detect bacteria in complex samples at concentrations around the limit stated in the regulations. Even though the lowest amount of bacteria detected in the case of milk is slightly above the limits established in the regulations, this limit could be lowered either by using aptamers with higher affinity towards the target bacteria or by improving the pre-processing protocols. In a previous study we demonstrated that *Salmonella* could be potentiometrically detected in buffer solutions with an SWCNT/aptamer biosensor[16] based on anti-*Salmonella* Typhi Pili IVB aptamers[28] and that the corresponding limit of detection was 1 CFU/mL because of the high availability of pili on the bacteria's surface. Therefore, another approach that could increase performance at lower limits of detection would be to use aptamers synthesized against bacterial surface structures that are present in high numbers throughout the entire cell wall, for example, fimbriae, flagella or pili. It is interesting that the aptamer used in the development of our biosensor was initially isolated with the aim of detecting whole cells of *E. coli*, rather than a particular target situated on the external part of the cell wall (as in the case of anti-*Salmonella* Typhi pili aptamers, mentioned above); hence, the corresponding epitope in *E. coli* might be any surface structure that is present in low numbers or less exposed to tethering by aptamers (e.g. S-layer proteins)[29], which in turn might reduce the possibility of reaching ultra-low detection limits and be the reason why such a long stabilization time is required to achieve a 100% EMF signal (***Figure 2a***) for high concentrations of bacteria.

In order to evaluate whether the real sample-filtering/elution-of-microorganisms steps are appropriate to our purposes, we also assessed the recovery of the target microorganism. We used the standard plate count method to count the number of CFUs in equivalent filtered volumes of the same samples that we used in the potentiometric detection experiments. We observed that under these experimental conditions, the pre-processing procedure allows a recovery of 80-90% (***Figure 3d***). We also observed that an eluent volume of 6 mL PBS enables a maximum recovery at low concentrations of bacteria (22 CFU/mL). Even though higher recovery rates can be obtained when the elution step is carried out with a higher volume of eluent PBS (8 mL) in samples containing higher concentrations of bacteria (240 CFU/mL), the procedure works better in real-samples when the elution volume is kept as low as possible so as not to dilute the possible low bacteria content of many unknown samples. The same pre-treatment conditions were applied during both control and real samples.

The results obtained in the present work overcome many difficulties shown by other reported methods for the detection of pathogens. Available PCR-based detection methods have achieved detection limits as low as 5 CFU in a 20 minutes assay[4] and 6 CFU in 5 hours[5]. Other methods that are based on physical separation of pathogen cells using biofunctional magnetic nanoparticles and further optical counting[7] or traditional culturing[8] allow also similar limits of detection (1 to 10 CFU) in times of analysis of many hours. Electrochemical-based methods consisting of field-effect transistors[9], amperometric[10] and square wave voltammetric biosensors[11] have also reached low detection limits (10 to 10^3 CFU/mL) within analysis times in the order of half an hour to many hours. Even though the method reported in the present work allows limits of detection comparable to those mentioned above, our results have overcome some operational and performance parameters such as simplicity, time of analysis and selectivity. Thus, the main advantages of the method reported herein are: (i) a close to real-time analysis of real samples allowed by the fast electrochemical response observed when the bio-recognition event takes place and the very simple pre-processing steps involved in the sample pre-treatment; (ii) the ease of use and low cost of the potentiometric devices implicated in the electrochemical detection as well as the simplicity involved in the construction of the biosensor (iii) the inter-species and inter-strain selectivity observed so far are very important because they demonstrate that the identification of a particular pathogen strain could be carried out in a direct way and thus, the need of serological confirmatory tests that often require several hours or days to be accomplished might be eliminated or, in the worst case scenario, reduced.

CONCLUSION

In this paper we have developed and deployed for the first time a potentiometric biosensor that uses carbon nanotubes chemically linked to aptamers as probes to selectively detect and identify a particular strain of *Escherichia coli* in real complex samples in a few minutes. A simple potentiometer, an on-line filtration system and a customizable aptasensor, which does not show both inter-species or inter-strain cross-reactivity, can be used in close to real-time conditions for the simple and rapid elimination of the matrix in real samples, to recover the microorganisms and to perform the on-site highly selective detection/quantitation of the target bacteria. The system displays a moderate sensitivity but the responses are clearly distinguishable from the noise. Our research demonstrates the feasibility of using potentiometry to identify microorganisms in complex matrices, because the whole system may be customized according to the pathogen to be detected, both the aptamer and the pre-treatment procedure can be selected depending on the type of bacteria and the complexity of the matrix. This opens the door to using simple potentiometry for the immediate detection of bacteria in real samples thus simplifying the traditionally complex procedures for detecting and identifying pathogens and providing a detection technique that is easily available to almost every bio/chemical laboratory. It is necessary, however, to have the appropriate aptamer. It would be very interesting from the point of view of applying the technique to different samples to have aptamers with different selectivities in order to distinguish not only among serovars but also among different species or even families.

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