Immediate Detection of Living Bacteria at Ultra-Low Concentrations Using a Carbon Nanotube-Based Potentiometric Aptasensor**

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The control of diseases has been one of the most important public health concerns of our society for decades. Typical standard methods that are used to assess the presence of microbiological threats consist of specific enrichment media to separate, identify and count bacterial cells. This process takes at least two days after the test sample has been obtained. In recent years, several research groups have tried to attain zero tolerance detection systems within much shorter overall response times.[1] Currently available ultrafast polymerase chain reaction (PCR) detection methods are able to sense 5 CFU (colony-forming units) in an assay time of 20 minutes,[2] which is a major achievement, as is the detection of biowarfare pathogen genes with a DNA-based nanobarcode using a one-minute test.[3] However, these methods require pre-treatment steps to condition the test samples and to perform cell lysis in order to extract the suitable target DNA, a process that significantly complicates these assays. To overcome these drawbacks, there has been a continuing search for methods that allow the direct detection of whole microorganisms. The detection of one cell perched on the tip of a micromechanical oscillator[4] was an important approach for detecting single cells, although the assay was performed at high concentrations of heat-killed bacteria (10^5 CFU/mL) and without a sample matrix. Moreover, the instrumental complexity of this method is high enough to prevent its widespread use. Further progress was made when scanning electron and fluorescence microscopy techniques were used to detect biofunctional magnetic nanoparticles during the extraction and counting of 4 to 10 CFU.[5] Nevertheless, the special care needed when examining the samples by microscopy and the time invested in each observation barely permit a reasonable sample throughput. A

fast and versatile method was reported by Rider et al. in 2003 when they detected 500 CFU/g in only 5 minutes using a B cell-based sensor modified to act as a photo-emitter.[6] However, the main shortcomings in this case are the expensive and time-consuming processes involved in fabricating the device. Therefore, there is still a demand for a fast, sensitive, selective, inexpensive and easy-to-use method for detecting and quantifying pathogenic bacterial cells.

Electrochemical detection techniques have a series of advantages such as rapid response, ease of use, low-cost and small sized commercial detectors. Among the electrochemical techniques, the simplest, most widespread and field-portable methodologies are based on potentiometry. The new wave of potentiometric solid state electrodes represents an attractive tool for real-time bioanalysis in liquid samples.[7] However, to date, it has been difficult to carry out the specific and direct electrochemical detection at ultra low levels of whole living bacterial cells without chemical labeling because the interaction receptor-bacteria does not provide a measurable electrochemical signal.

Recently, Crespo et al.[8] 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[9] together with their remarkable double layer capacitance[10] explain their transducing behavior. Moreover, they are easily deposited over many surfaces making them ideal for solid contact electrode design.[11] However, in order to selectively detect a particular target, SWCNT must be coupled to the suitable receptor. Aptamers are highly suitable receptors for the selective and high proficiency detection of a wide range of molecular targets, including bacteria.[12-14] Moreover, aptamers can self-assemble to carbon nanotubes via π-π stacking interaction

between the nucleic acid bases and the carbon nanotubes walls,[15] thus constituting a hybrid material that has been applied to nanobiosensors.[16-17] Also, Pan et al. recently obtained a high-affinity RNA aptamer that specifically binds to type IVB pili of *Salmonella* Typhi (ST).[18]

With these developments in mind, we report a potentiometric biosensor for selectively detecting one single CFU of ST close to real time. This aptamer was modified with a five carbon spacer and an amine group ($-(CH_2)_5NH_2$) at the 3' end and was covalently immobilized into a layer of previously carboxylated SWCNT.[19] This step used a well-known carbodiimide mediated wet-chemistry to form amide bonds between the amine spacer and the carboxylic moieties on the sidewalls of the nanotubes.[20-21] Before linking the aptamers to the carboxylated SWCNT, a 30 μm thick layer of nanotubes were sprayed onto the polished surface of a glassy carbon (GC) rod that was electrically contacted to a potentiometer.[10] We used an Ag/AgCl double junction electrode as reference for the electromotive force (EMF) measurements. Further information about materials and methods is available in the Supporting Information.

The hybrid material aptamer-SWCNT acts as both the sensing and the transducing layer of the biosensor. In the absence of the target analyte (Fig. 1a), the aptamers are self-assembled to carbon nanotubes via π-π stacking interaction between the puric and pyrimidic bases and the carbon nanotubes walls.[22] The presence of the target bacteria promotes a conformational change in the aptamer that separates the phosphate groups, largely ionized at pH 7.4, from the SWCNT sidewalls, inducing a charge change to the

SWCNT and the subsequent change of the recorded potential (Fig. 1b). The bacteria linked to the aptamer could also lean towards the carbon nanotubes establishing a charge transfer between the highly concentrated H^+ ions that surround the cell wall[23] and the carbon nanotubes. However, both mechanisms could occur simultaneously and are currently being investigated.

To explore the response of the biosensor to stepwise additions of living ST in phosphate buffer solution (PBS, 1.7 mM, pH = 7.4), we performed consecutive inoculations along time. All the electromotive force measurements were performed at low ionic strength (1.7 mM PBS) and in neutral pH with a Keithley high-input impedance voltmeter M6514 (London, U.K.) in an isothermal vessel at 22 ± 0.5°C, using 5 mL of sterile and pure PBS before any inoculation of bacteria. The amount of bacteria that was contained in each aliquot was simultaneously standardized in quintuplicate using the agar plate count technique. Stock solutions of bacteria consisted in consecutive 1:10 dilutions in sterile PBS (the same matrix that is used for EMF measurements) of a suspension of bacteria cultured for 12-24 hours, that had been previously washed, precipitated and reconstituted in PBS. The initial EMF values (E_0) for each of the biosensors were in the interval of 80-130 mV; however it was seen that this value does not exert any influence on the final response for either ST or any other type of bacteria. Figure 2a shows an environmental scanning electron microscopy (ESEM) image of a single ST cell placed on the SWCNT-aptamer layer. The potentiometric response of our biosensor was found to be immediate after each inoculation, ranging from 0.2 CFU mL^{-1} (1 CFU in 5 mL PBS) to 10^6 CFU mL^{-1}. Figure 2b shows that the response time is shorter than 60 s indicating a fast affinity equilibrium between the aptamers and ST. The recorded potential does not decrease after the solution is diluted,

indicating that the equilibrium is not easily reversed. In all the tested sets of inoculations for all the tested sensors (5 sensors), a linear relationship existed between the EMF response and the logarithm of the bacteria concentration up to 10^3 CFU mL^{-1} (Fig. 2c). A sensitivity of 1.87 mV/decade (SD=0.29 mV, N=5) has been obtained for this concentration range. However, the slope decreases considerably at higher levels, reaching a plateau at concentrations above 10^6 CFU mL^{-1}. This behavior can be explained by the progressive saturation of the available binding sites (Fig. 2b and 2c). After each set of inoculations, the sensors were easily regenerated by dissociating the aptamers from the bacteria in NaCl 2 M for 30 minutes, and then reconstituted by conditioning in PBS, thus leaving the biosensor ready to take new measurements. Even though the saturation level of the electrodes decreased after 10 regeneration cycles, all the electrodes were able to detect the minimum bacteria concentration for at least 3 months.

Our biosensor also shows a high degree of selectivity. No response was shown for parallel experiments using either *Escherichia coli* as a Gram-negative foodborne agent or *Lactobacillus casei* as a non-toxic Gram-positive microorganism. Moreover, control experiments confirmed that the responses are caused exclusively by the binding event between ST and the aptamer, and the subsequent transduction of the SWCNT layer. Several modified solid-contact sensors were tested in order to discard the possibility that the electric potential was originated by unspecific adsorptions. We tested carbon nanotube based electrodes functionalized with the 1-pentylamine molecule ($CH_3(CH_2)_4NH_2$) that represents the five-carbon spacer between the carbon nanotube and the aptamer, and carbon nanotube based electrodes without any other chemical modification. We also examined the potentiometric response using only the original glassy carbon support as the sensor, which

had been either aptamer-functionalized or 1-pentylamine-modified. There was no potentiometric response under any of these conditions, showing that the EMF change is only generated when aptamers attached to SWCNTs interact with ST (Fig. 2d).

This communication demonstrates that easy-to-build aptamer-based SWCNT potentiometric sensors are highly selective and can be successfully used to detect living microorganisms in a close to real-time assay, thus making the detection of pathogens as easy as measuring the pH. As demonstrated here, a highly accurate linear response can be obtained with good reproducibility and without any kind of pretreatment, starting at ultra-low concentrations of bacteria and a dynamic range of 4 logarithmic units ($0.2\text{-}10^3$ CFU mL^{-1}), and progressing in just a few seconds to concentrations far below those reported previously. Higher concentrations of bacteria can also be detected but in a semiquantitative way. However, the most important strength of this biosensor is that simple positive/negative tests can be carried out in real zero-tolerance conditions and without cross reaction with other types of bacteria. The ease with which measurements are taken in potentiometric analysis opens the door to greater simplicity in microbiological analysis.

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Figure 1. a) Possible conformations of the aptamers that are self-assembled to carbon nanotubes. b) Schematic representation of the interaction between the target bacteria and the hybrid system aptamer-SWCNT.

Figure 2. a) Environmental scanning microscope image obtained from an aptamer-functionalized SWCNT electrode after exposure to ST. b) Aptamer-functionalized SWCNT electrode exposed to stepwise concentrations of ST and the corresponding potentiometric response; arrows represent the inoculations with ST; values are the final concentration of bacteria. Inset is amplification for the inoculation step at 0.2 CFU mL^{-1} to show the fast response; time is in seconds. The signal provided by the first aliquot containing 1 bacteria (0.2 CFU mL^{-1}) is high enough to be resolved from the instrumental limit of detection,[24] delimited by 3 x SD_{noise} [standard deviation of noise = ± 0.08 mV]. c) EMF response versus decade of concentration of ST. At higher amounts of bacteria, every EMF increase was less prominent, demonstrating a progressive saturation of the available binding sites. The solid line is the linear regression fit and the equation below was obtained for that range (E_0 is the corresponding sensor potential before any inoculation, and it is particular for each sensor). Error bars are SD of the response obtained at same concentrations for 5 different sensors. Error values in parenthesis are SD for the different regression equations obtained for 5 different sensors. d) Controls and selectivity assays. EMF response versus recorded time, for stepwise concentrations of bacteria. Solid vertical lines represent inoculation with increasing amounts of bacteria. From top to down: *1*, carbon nanotube sensor without aptamer, but functionalized with $CH_3(CH_2)_4NH_2$ using same procedure for amide bonding, exposed to ST; *2* and *3* SWCNT-Aptamer biosensors 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 1

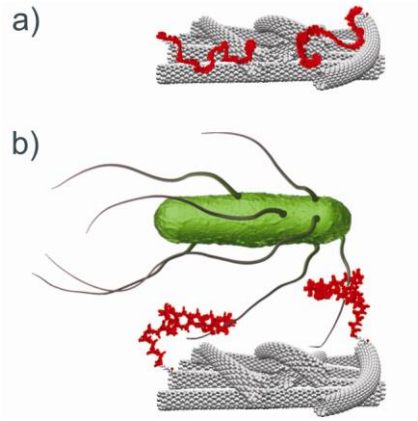
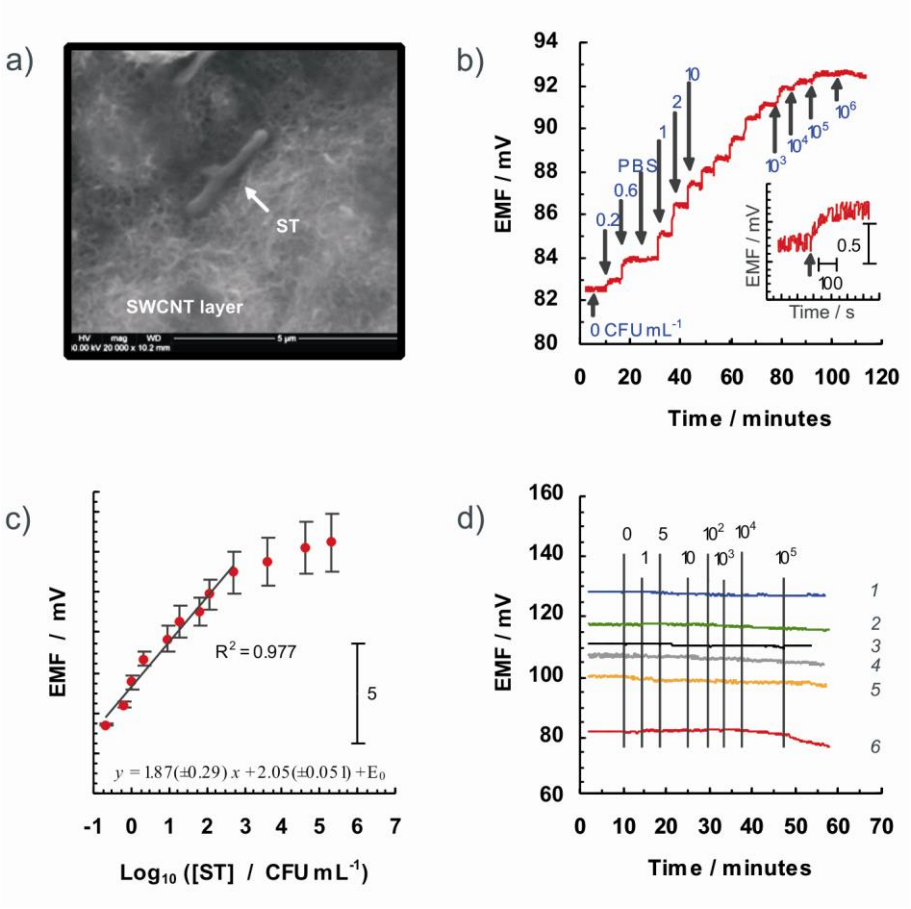


Figure 2


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