TITLE:

Label-free detection of *Staphylococcus aureus* in skin using real-time potentiometric biosensors based on carbon nanotubes and aptamers.

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ABSTRACT:

In this paper we report the first biosensor that is able to detect *Staphylococcus aureus* in real-time. A network of single-walled carbon nanotubes (SWCNTs) acts as an ion-to-electron potentiometric transducer and anti-*S. aureus* aptamers are the recognition element. Carbon nanotubes were functionalized with aptamers using two different approaches: 1) non-covalent adsorption of drop-casted pyrenil-modified aptamers onto the external walls of the SWCNTs; and 2) covalent bond formation between amine-modified aptamers and carboxylic groups previously introduced by oxidation at the ends of the SWCNTs. Both of these approaches yielded functional biosensors but there were large differences in the minimum detectable bacteria concentration and sensitivity values. With covalent functionalization, the minimum concentration detected was 8 x 10^2 Colony-Forming Units (CFU)/mL and the sensitivity was 0.36 mV/Decade. With the non-covalent approach, the sensitivity was higher (1.52 mV/Decade) but the minimum concentration detected was greatly affected (10^7 CFU/mL). In both cases, potential as a function of Decade of bacteria concentration was linear. Functional biosensors were used to test real samples from freshly excised pig skin, contaminated with the target microorganism, as a surrogate for human skin.

KEYWORDS:

Staphylococcus aureus, real-time, carbon nanotubes, aptamers, label-free, potentiometry.

MAIN TEXT:

1. Introduction

Staphylococcus aureus (*S. aureus*) is a common Gram-positive pathogen that can be present on skin and mucous membranes of healthy humans or in inadequately treated food. In uncontrolled conditions, *S. aureus* can cause a wide range of diseases including several types of dermatitis and gastrointestinal tract infections and is widely involved in many cases of enterotoxin-related food poisoning worldwide. However, the microorganism plays a major role in many other life-threatening infections such as pneumonia, septicemia, osteomyelitis, toxic shock syndrome and about a third of the total cases of endocarditis worldwide. Nowadays, antibiotic-resistant strains of *S. aureus* are seriously challenging global monitoring platforms not only in clinical settings such as in hospitals, but in whole communities. Both the fatal consequences and the alarming increase observed in the current number of antibiotic-resistant *S. aureus*-related infection cases have in some regions even surpassed the control capacity of local healthcare organizations. Consequently, there is currently a huge demand for rapid methods to detect *S. aureus* with the potential to be incorporated into point-of-care diagnostic systems (Chambers and DeLeo, 2009; David and Daum, 2010; Frank et al., 1999; Hidron et al., 2008; Lowy, 1998). *S. aureus* is traditionally detected using the standard plate count method after selective enrichment in broth (Gerhardt et al., 1981). However, the time needed to produce a confirmatory result typically ranges from 2 to 4 days. Moreover, the biochemical tests usually needed for corroboration convert the existing detection protocols into a complicated task which requires skilled staff and expensive laboratory facilities.

Currently available biosensors aimed at the rapid detection of *S. aureus* are mainly focused on the indirect detection of related nucleic-acid sequences (Farabullini et al., 2007; Niemz et al., 2011; Pividori et al., 2001; Tombelli et al., 2006) or toxins and other biomolecules excreted by the pathogen under certain conditions (Banerjee and Bhunia, 2010; Liu et al., 2007; Yang et al., 2010). However, biosensing methods based on the indirect detection of the target pathogen are usually limited by many factors given that their results are not always correlated to the presence of the microorganism in the sample analyzed. State-of-the-art direct biosensing techniques such as surface plasmon resonance biosensors based on antibodies (Subramanian et al., 2006) and phages (Balasubramanian et al., 2007) –able to detect 10^5 Colony-forming units (CFU)/mL in 2 h or 10^4 CFU/mL in about 20 minutes, respectively– or fluorescence-based immunoassays –able to detect 10^4 CFU/mL in 15 minutes (Su et al., 2007)– represent a huge advance in the fast detection of this pathogen. In this context, biosensors based on electroanalytical methods are preferred because electrochemical devices are cheap, portable, easy to use and easy to miniaturize, afford lower detection limits than other techniques and the response may be monitored in close to real-time conditions (Palchetti and Mascini, 2008). However, 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 such as antibodies (McKenney et al., 1999; Kropec et al., 2005), seriously limit in the development of biosensors for direct electrochemical detection. Consequently, only a few electrochemical biosensors for *S. aureus* detection have been developed to date, with detection limits ranging between 10^4 and 10^5 CFU/mL in the best case scenario (Escamilla-Gómez et al., 2007; Mirhabibollahi et al., 1990). Recent advances using amperometric detection techniques have enabled much lower detection limits of about 10^3 CFU/mL in a total analysis time of 50 minutes (Escamilla-Gómez et al., 2008), or $6.5x10^2$ CFU/mL in 3 hours (Morales et al., 2007) when selective enrichment steps are introduced. Unfortunately, as in all the previous examples, the existing electroanalytical biosensing platforms have faced important and not yet solved challenges that limited the detection of *S. aureus* only to either label-mediated biosensing or to pre-enriched sample analysis.

Very recently, a new generation of DNA aptamers –tailored DNA or RNA segments acting as artificial recognition elements (de-los-Santos-Álvarez et al., 2008)– able to recognize conserved epitopes on the surface of *S. aureus* (Cao et al., 2009) has provided promising solutions for this important issue. But the deployment of the above-reported *S. aureus*-biosensing platforms into clinical settings for the rapid detection of this life threatening pathogen in clinical samples such as infected wounds or skin biopsy specimens has remained unsolved for years. Therefore, to address this challenge, in this paper it is demonstrated for the first time that *S. aureus* can be detected and identified by potentiometry with a minimum number of pretreatment steps, in real-time conditions and without the need for labels, and achieving better limits of detection than the methods reported so far. We used a hybrid transducing/biosensing material (Zelada-Guillén et al., 2009) consisting of single-walled carbon nanotubes (SWCNT) as ion-to-electron potentiometric transducers and aptamers as biorecognition molecules. The transducing properties of this SWCNT/aptamer hybrid material derive from the remarkable SWCNT double-layer capacitance, the great ability to support charge transfer between the SWCNT/solution interface and the ions that surround the cell wall of the target bacteria, and the extremely high surface-to-volume ratio of the nanotubes that are also able to sense conformational changes in the linked aptamers during the target-recognition event that switches the surface charge on the SWCNT layer (Crespo et al., 2009; Düzgün et al., 2011; Yañez-Sedeño et al., 2010). To explore alternative methods for the functionalization of nanotubes with aptamers and thus open the door to easier functionalization procedures, we attached the

aptamers to a homogeneous layer of SWCNT using two functionalization strategies –a covalent approach and a non-covalent approach– and analyzed the effects on the biosensors' performance parameters. The covalent approach consisted of linking the aptamers to the nanotubes chemically by amide bonds formed between the –COOH groups of previously carboxylated SWCNT and an amine moiety introduced at the 3' end of the aptamer by well-known carbodiimide mediated chemistry (Jung et al., 2004; Wong et al., 1998). The non-covalent approach was performed by direct physisorption onto the SWCNT sidewalls of pyrenil moieties previously introduced to the 3' end of the aptamer. Pyrenil groups strongly interact with the sidewalls of the nanotubes by π-stacking, and this property is commonly used to fix pyrenil-modified biomolecules to carbon nanotubes by drop casting (Chen et al., 2001). By comparing the performances of the biosensors prepared using these two strategies, we aimed to determine the best approach in terms of analytical performance and biosensor construction simplicity. Finally, we assessed the functional biosensors as potential tools for detecting *S. aureus* in human skin using, as a surrogate for human skin (based on the morphological similarity of the two skin types), freshly excised dorsal skin of the domestic pig (Meyer et al., 1978). We assessed their selectivity against different microorganism usually found in this kind of samples such as *Escherichia coli* and *Staphylococcus epidermidis* and found that none of them gave a detectable electrochemical signal. In this way, we opened the possibility for the immediate detection of *S. aureus* in close to clinical conditions.

2. Materials and methods

2.1. Chemicals and aptamers

Solutions were prepared using deionized water purified through a Milli-Q system (Millipore, Madrid, Spain) with a resistivity level of 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 substances were used as received. Phosphate buffer solution (PBS) 1.7 mM pH 7.4 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, and the pH was adjusted as required. The 88-mer *S. aureus*-binding DNA aptamers with similar affinities (Cao et al., 2009) and the sequences 5'-GCAAT-GGTAC-GGTAC-TTCCT-CCCAC-GATCT-CATTA-GTCTG-TGGATAAGCG-TGGGA-CGTCT-ATGAC-AAAAG-TGCAC-GCTAC-TTTGC-TAA-3'-$(CH_2)_6NH_2$ (NH_2-Aptamer) and 5'-GCAAT-GGTAC-GGTAC-TTCCG-CGCCC-TCTCACGTGG-CACTC-AGAGT-GCCGG-AAGTT-CTGCGTTATC-AAAAG-TGCAC-GCTAC-TTTGC-TAA-3'-C3-Pyr (Pyr-Aptamer, C3-Pyr = pyrenil moiety with a phosphoramidite spacer) were purchased from Eurogentec (London, UK). The aptamers were resuspended in deionized water and stored at -80 °C. All the materials and solutions were adequately sterilized and manipulated under sterile conditions.

2.2. Microorganism culturing

The manipulation of microorganisms was carried out in a biosafety level II cabinet, model BIO II A (Telstar Industrials, Terrassa, Spain). Information about culturing media, bacteria strains and culturing procedures can be found in the Supplementary Online Material.

2.3 Instrumentation and materials

Electromotive force (EMF) measurements were automatically recorded with a high-input impedance voltmeter model EMF-16 (Lawson Laboratories, Inc., Malvern, PA, U.S.A.) 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 biosensor or SWCNT-based electrode as the working electrode.

2.4. Development of the biosensors

The development of the biosensors prepared by both procedures, the covalent functionalization approach and the non-covalent functionalization method, as well as the preliminary viability tests performed with the biosensors are described in the Supplementary Online Material.

2.5. Analytical procedure

Constant stirring (300 rpm) was applied during all the potentiometric measurements in an isothermal vessel at 22±0.5 °C containing 5 mL of sterile PBS working solution (1.7 mM and pH 7.4) before addition of the sample. The electrochemical cells were inoculated in a stepwise mode with the stock solutions of serially diluted bacteria (10^{-8} to 10^{-1} solutions prepared as mentioned in the Supplementary Online Material) in order to progressively increase the bacteria concentration within the electrochemical cell and simultaneously monitor the EMF changes with the biosensors at periods of 10 s. The dilution was corrected for all the stepwise concentration experiments. The potentiometric response of the biosensors was evaluated following the EMF changes after stepwise inoculations with increasing concentrations of *S. aureus*. Selectivity assays were carried out by testing the biosensors against stepwise increasing concentrations of *Escherichia coli* (*E. coli*) and *Staphylococcus epidermidis* (*S. epidermidis*). Parallel control assays were performed by monitoring the potentiometric response of SWCNT sensors without aptamers against increasing concentrations of *S. aureus*. After each set of potentiometric measurements, biosensors were regenerated in a 2 M NaCl aqueous solution for 1 hour in order to dissociate the bacteria from the aptamers. The regenerated biosensors were then washed for 15 minutes in sterile deionized water and stored in PBS working solution to prepare them for new measurements.

2.6. Analysis of skin samples

For the detection of *S. aureus* in a surrogate for human skin, 2 cm x 2 cm segments of freshly excised dorsal pig skin (purchased from a local butcher's) were successively washed with water and soap, rinsed with water, dried, further sterilized at the surface with 70% v/v ethanol/water, and finally dried for 10 minutes. The surface was then inoculated with 50 μL of a 10^{8} CFU mL^{-1} *S. aureus* stock solution in PBS, distributed over the surface with a sterile microbiological loop, and dried for 30 minutes. Control pig skin without *S. aureus* inoculated was prepared following the same steps but substituting the bacteria-containing solution with sterile PBS. Finally, the inoculated pig skin was rubbed with two sterile cotton swabs moistened with sterile PBS working solution. One of the swabs was used for growth control by smearing the adsorbed bacteria onto the solidified surface of Mannitol Salt Agar (MSA) contained in a Petri dish that was then incubated for 48 h at 37 °C. MSA is a selective solid medium for confirming the growth of *S. aureus* and the presence of the microorganism is evidenced when pale-yellow colonies surrounded by a yellow halo are found (Gerhardt et al., 1981). The other swab was introduced in a tube containing 2 mL of sterile PBS working solution in order to transfer the recovered microorganisms to the PBS by intense shaking and later detect the *S. aureus* present in an aliquot of this solution using the developed biosensors. The tube was shaken intensely and 500 μL of the solution was then transferred into an electrochemical cell containing

the tested biosensor (Fig. 1). Three functional biosensors for each type of functionalization were used in this experiment. The amount of *S. aureus* recovered from pig skin by this latter procedure was assessed by parallel plate counting in TSA in triplicate. Control pig skin without *S. aureus* (prepared as described previously) was used as a blank in both control quantification assays and control potentiometric assays of the tested biosensors following the latter swab-smearing based procedure. Finally, selectivity assays were performed by substituting *S. aureus* for *E. coli* and *S. epidermidis* in this same protocol. The biosensors were then tested in the same way to evaluate them with different microorganisms that could be present in real skin samples.

(XXXXHere_Fig._1XXXX)

3. Results and discussion

Recognition of *S. aureus* by the biosensors following the covalent functionalization approach was evidenced when the biosensors were incubated for 30 minutes in a 10^8 CFU/mL solution of *S. aureus*, which was then thoroughly washed and smeared onto the solid surface of agar to yield positive growth after 48 h at 37 °C. Control tests with SWCNT sensors without aptamer did not produce colonies under these conditions. Non-covalently functionalized biosensors prepared by drop casting the Pyr-Aptamer solution also yielded positive growth on agar. This was confirmed by triplicate experiments of the previous assays. The instrumental limit of detection (3 x standard deviation of the noise) determined from electromotive force (EMF) measurements recorded using both biosensor types was 96 μV and 42 μV for the covalently prepared biosensors and non-covalently prepared biosensors, respectively. EMF variations above these values should be easily detected by the corresponding biosensor type.

In the case of non-covalently functionalized biosensors prepared by drop casting Pyr-Aptamer solution, the EMF response to *S. aureus* began at the relatively high concentration of 10^7 CFU/mL and lower concentrations did not change the EMF values (Fig. 2a). The EMF response was linearly dependent on the bacterial concentration in Decade units with a slope of 1.52 mV/Decade in the very limited range of 1 order of magnitude between 10^7 CFU/mL and 10^8 CFU/mL (Fig. 2b). The biosensor response time was about 120-200 seconds, but an immediate change was observed after the addition of *S. aureus* at concentrations above 10^7 CFU/mL. The EMF response was quite stable for at least 10 minutes after bacteria inoculation during the range explored. However, when exposed to concentrations above 10^8 CFU/mL, signal drift was observed and a progressively more pronounced decay on signal stability appeared as the concentration of bacteria increased. This effect may be attributed to colloidal agglomeration of the highly concentrated non-bound bacteria onto the surface of the biosensor. This behavior would limit the applicability of biosensors at higher concentrations. Sample dilution would not be an option for reducing such an effect in samples with a high bacterial load, since, as explained earlier, the working range of this non-covalently prepared biosensor is very limited. The regeneration of this type of biosensors with 2M NaCl, subsequent washing with deionized water and further preconditioning with PBS demonstrated that non-covalently functionalized biosensors remained functional only for two to three bacteria-detection assays in the best case scenario. After further regeneration cycles, the performance of the biosensors declined dramatically and no potentiometric response was observed when the biosensors were exposed to the same working range described previously. Biosensors prepared by this method and exposed to increasing concentrations of *E. coli* and *S. epidermidis* at the concentration range of 0 – 10^8 CFU/mL did not lead to a clear change in

EMF. Simultaneous control assays with non-functionalized SWCNT sensors also resulted in the same behavior.

Covalently prepared biosensors afforded much lower detectable concentrations when exposed to *S. aureus* since the first clear potentiometric change was observed at a concentration of 8 x 10^2 CFU/mL (Fig. 2a). At concentrations above this value, EMF as a function of the concentration of bacteria in Decade units could be approximated to a straight line model over a dynamic range of 6 orders of magnitude (Fig. 2b). The average slope –sensitivity– was 0.36 mV/Decade (standard deviation = 0.25 mV/Decade), and the R^2 value was 0.94. It is interesting to note that the relatively large standard deviation, especially at higher concentration of bacteria, may be attributed to experimental errors originated when solutions containing *S. aureus* were prepared, given the facility for the microorganism to form stable agglomerates in aqueous solutions. It is also important to note that high concentrations of bacteria usually do not require a result with a very high precision rather than a good estimation of the presence of the pathogen without false positives. The signal was also stable in the concentration range analyzed. A response time of 6 to 11 minutes (90% of the total response, see amplification in Fig. 2a) was observed for the concentration range 8x10^2 CFU/mL to 10^8 CFU/mL 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 biosensor. However, after inoculation with bacteria at concentrations above 10^8 CFU/mL stability dramatically decreased and EMF response remained stable for about 20 minutes before negative drift appeared. As mentioned previously, this effect was probably caused by stochastic charge transfer processes between the surface of the biosensor and the highly concentrated *S. aureus* not tethered to the biosensor by the aptamers. Control experiments conducted in parallel with non-functionalized SWCNT sensors did not lead to a clear change in EMF, which confirms that the potentiometric response is driven by the recognition of the target microorganism by the biosensors. .The biosensors were easily regenerated by incubation in 2M NaCl, followed by a washing step in deionized water and further precondition in PBS. No performance loss was observed after three to six regeneration cycles in the best case scenario. However, in some cases, additional washing was needed before the preconditioning step in order to keep the biosensor functional, which was probably originated by an accumulation of debris on the biosensor surface. When selectivity assays were carried out with functionalized biosensors exposed to stepwise increasing concentrations of *E. coli* and *S. epidermidis* between 0 and 10^8 CFU/mL, no change was observed in EMF (Fig. 2c). However, at concentrations above 10^7 CFU/mL, progressive drift was observed on the baseline after addition of the sample.

(XXXX_Here_Fig._2_XXXX)

The different performances between of the two types of biosensor in terms of the minimum concentration detected and sensitivity are explained by the differences in the bacterial-adsorption profiles due to the functionalization procedure followed. This was demonstrated when the *S. aureus* linked to the biosensor surfaces of each biosensor type at a concentration of 10^8 CFU/mL was grown in TSA according to the standard plate count method. Using this procedure, differences in bacterial adsorption were observed for each biosensor type. While the biosensors prepared by the covalent method yielded a surface bacterial concentration of 9.1x10^6 CFU (standard deviation, SD=5x10^6, with three biosensors tested), the biosensors prepared by the non-covalent approach only yielded a surface bacterial concentration of 2.4x10^3 CFU (SD=8x10^3, three biosensors tested). The observed difference suggests that the bacteria remain more easily tethered to covalently functionalized biosensors by a difference of almost 4 orders of magnitude if

compared with non-covalently functionalized biosensors. The surface bacterial concentration evidently depends on the number of binding sites available for pathogen recognition. However, the aptamer-bacteria recognition event depends on the conjunction of many other factors such as an appropriate spatial orientation of the recognition molecules at the sensor's surface or the stability of aptamer molecules once deposited on the surface of the sensor. Therefore, several reasons may be behind the lower affinity of the biosensors prepared by non-covalent functionalization. An excess of Pyr-Aptamer molecules closely adsorbed during the drop casting procedure probably resulted in random molecular overlapping and so the recognition of the target bacteria by the biosensor was compromised by self-entanglement of Pyr-Aptamer molecules (which could clearly reduce the availability of aptamers that are able to recognize their target). Another possibility is the progressive leaching of the aptamer+bacteria complex, which was probably caused by an excessive accumulation of aptamer, which may have reduced the fixation strength of outer aptamer layers to the nanotube sidewalls by inner layers of more strongly adsorbed aptamers. The differences observed between the regeneration results in both types of biosensors presented previously in the manuscript also support this fact. Both possibilities are performance limiting factors for biosensor prepared by this procedure, and further theoretical/experimental research is needed in order to unravel this trend.

Many strategies may be exploited to improve on the detection limit in both types of biosensors in order to also expand their applications portfolio into different analytical categories. As an example, the compatibility of the biosensors with foodstuff analysis may be achieved by incorporating sample pretreatment steps into the analytical process. The prior removal of the matrix and the further preconcentration of *S. aureus* are interesting options in food sample pretreatment. However, to assess the potential use of our biosensors in the detection of *S. aureus* in human skin, we used in this work segments of freshly excised dorsal pig skin as a surrogate. These segments were inoculated with 50 μL of a 10^8 CFU/mL solution (corresponding to $5x10^6$ CFU). Aliquots of PBS containing the *S. aureus* recovered from pig skin segments using sterile cotton swabs were analyzed using functional biosensors prepared by both functionalization methods (Fig. 3). The biosensors prepared covalently showed a clear change in EMF of 390±16 μV (N=3) when 500 μL of this solution was inoculated into the electrochemical cell (Fig. 3) but no evident change was observed when 500 μL of the same solution was added to the cell containing the sensors functionalized using the non-covalent approach. Control SWCNT sensors did not show any potentiometric response at these conditions either. The standard plate count method was used in triplicate to quantify the bacteria recovered in PBS solution by this swab-based technique. The amount of bacteria recovered using the swab-based protocol was $7.3x10^5$ CFU with a standard deviation of $5.1x10^5$ CFU (5 skin samples), which represents an average recovery rate of 15% with a very high standard deviation, because the amount of recovered *S. aureus* ranged from 1.6% to 29.6% the original load. Therefore, to correlate the EMF response with the concentration of target microorganism in the PBS solution recovered from skin and introduced into the electrochemical cell, we simultaneously quantified the bacteria within that solution by plate counting. Results obtained after culturing the PBS solution with the swab-recovered bacteria demonstrated that both covalently and non-covalently prepared biosensors were exposed to *S. aureus* concentrations ranging from $2.4x10^3$ CFU/mL to $2.0x10^4$ CFU/mL in the electrochemical cell. This range is in accordance with the working range of the biosensors prepared by the covalent approach and the positive change in EMF confirmed the compatibility of the sample recovery protocol with the potentiometric analysis using this type of covalently functionalized biosensors. Also, these values were far below the limit of detection of the biosensors prepared by the non-covalent method, which explains why no potentiometric response was observed in

this type of biosensor. We also carried out selectivity assays by inoculating pig skin with either *E. coli* as a gram-negative pathogen or *S. epidermidis* as a non-pathogenic gram-positive microorganism that may also be present in real skin samples. The load of bacteria inoculated into the pig skin segments and swab-based sample recovery procedure remained constant for samples with *S. aureus*, *E. coli* and *S. epidermidis*. Neither the *E. coli* nor the *S. epidermidis* showed a change in EMF when the samples were analyzed with both biosensor types. The concentration of bacteria within the electrochemical cells after inoculation with 500 μL of the solution containing the recovered microorganisms ranged between $5.6x10^2$ CFU/mL and $9.1x10^4$ CFU/mL for *E. coli* and between $3.8x10^3$ CFU/mL and $3.1x10^4$ CFU/mL for *S. epidermidis*. The absence of a response in the biosensor covalently functionalized to these two microorganisms in pig skin suggests that biosensors functionalized by the covalent approach perform well in the identification of *S. aureus* in either skin or aqueous samples. The whole procedure, including sample recovery with swabs, resuspension in buffer and inoculation into the cell takes about 2 minutes while the EMF response value achieved after inoculating with the aliquot takes less than 6 minutes. However, an increase in EMF was immediately observed after the sample was added to the electrochemical cell, so a response was achieved in real-time.

(XXXX_Here_Fig._3_XXXX)

4. Conclusions

In this paper we have demonstrated that potentiometric biosensors based on single-walled carbon nanotubes as ion-to-electron transducers and aptamers as recognition elements are excellent biosensors for real-time and label-free detection of *S. aureus* as they improve on all the current detection methods for this pathogen. The biosensor performance parameters depended on the functionalization approach employed during the biosensor construction. The biosensors prepared by covalently linking the aptamers to the SWCNTs detected *S. aureus* concentrations five orders of magnitude below that of the biosensors prepared by the non-covalent approach. Non-covalently functionalized biosensors showed more limited working ranges with higher sensitivity values than their covalently functionalized counterparts. Biosensors prepared by the covalent method also showed a higher stability. Both biosensor types demonstrated great versatility in selectivity assays, which suggests the applicability of SWCNT/aptamer-based potentiometric biosensors in the highly selective identification of *S. aureus*. However, since the performance parameters of non-covalently functionalized biosensors are more limited, covalently functionalized biosensors are the best option in *S. aureus* detection in real samples. Finally, biosensors prepared covalently also showed many more advantages in terms of simplicity, analysis time and detection limit compared with both the standard detection methods and current state-of-the-art biosensing platforms for *S. aureus* detection. This was additionally demonstrated with the highly selective detection of *S. aureus* in human skin surrogates by a simple real-time assay that did not need require highly trained staff.

ACKNOWLEDGEMENTS:

We thank S. Moreno (Department of Biochemistry, URV), S. Macho and T. Codera (Department of Analytical and Organic Chemistry, URV) for providing essential equipment and materials and for their unconditional support. This study has been supported by the Spanish Ministry of Science and Innovation (MICINN)

through project grant CTQ2010-18717. G.A.Z.-G. thanks the Generalitat de Catalunya (Catalan Government) and the European Social Fund for awarding the FI-DGR fellowship.

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FIGURES:

Fig._1:

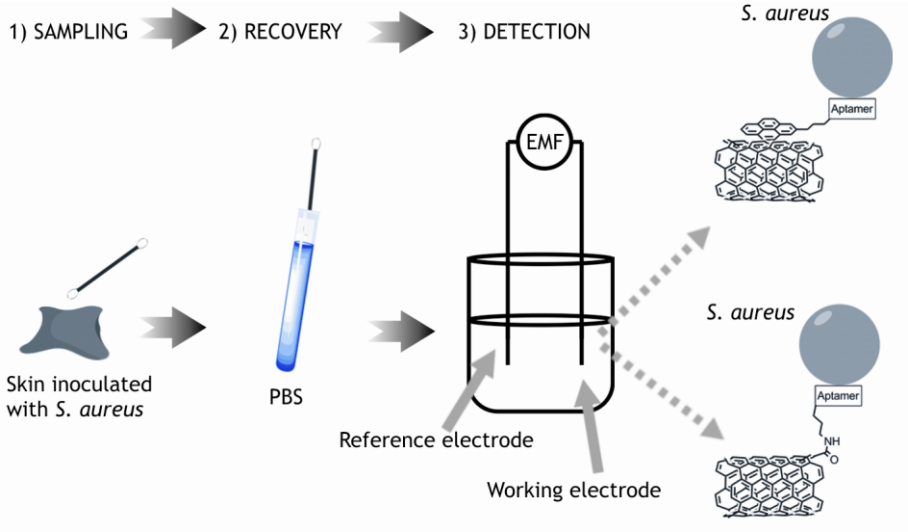

1) SAMPLING
2) RECOVERY
3) DETECTION
S. aureus
Aptamer
EMF
Skin inoculated with S. aureus
PBS
S. aureus
Aptamer
NH
O
Reference electrode
Working electrode

Fig._2:

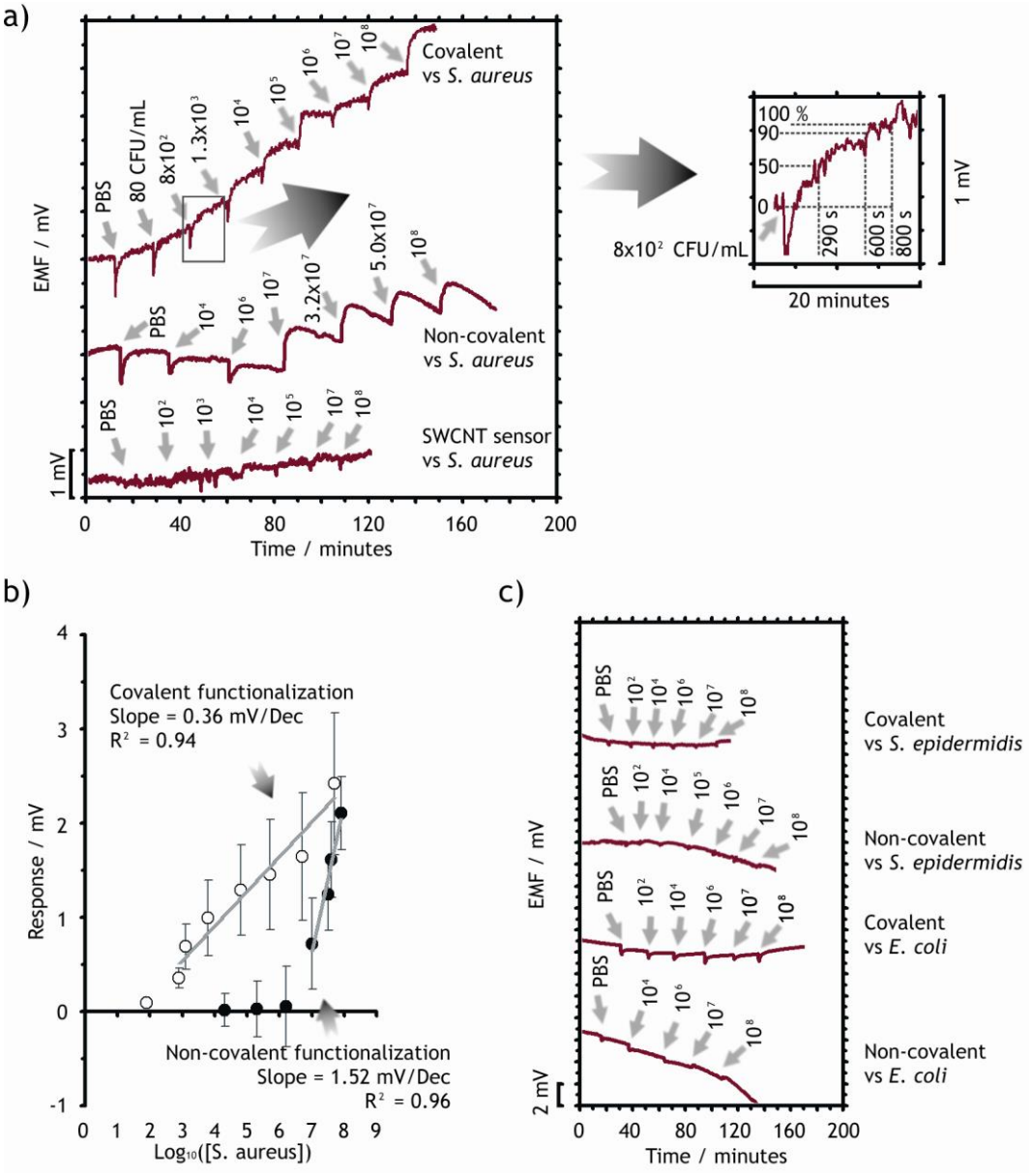
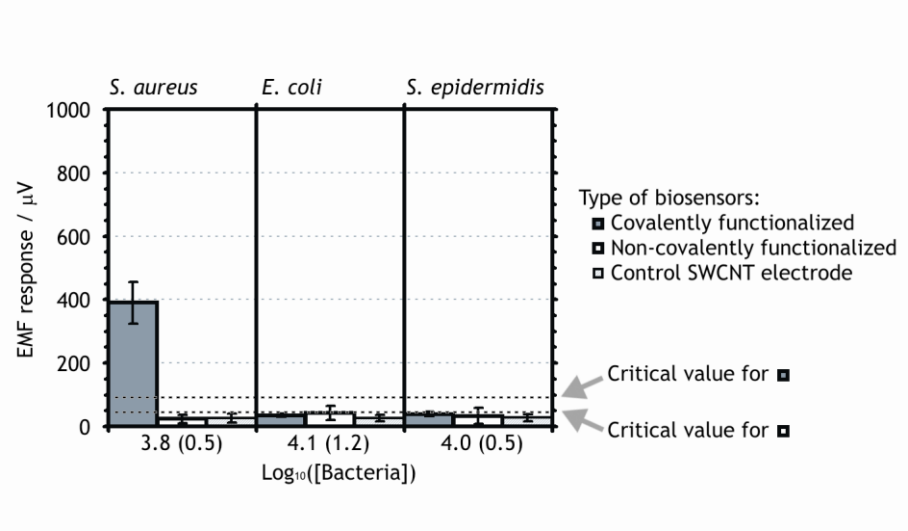

a)
EMF / mV
PBS
80 CFU/mL
8x10²
1.3x10³
10⁴
10⁵
10⁶
10⁷
10⁸
Covalent
vs S. aureus
PBS
10⁴
10⁶
10⁷
3.2x10⁷
5.0x10⁷
10⁸
Non-covalent
vs S. aureus
PBS
10²
10³
10⁴
10⁵
10⁷
10⁸
SWCNT sensor
vs S. aureus
1 mV
0
40
80
120
160
200
Time / minutes
8x10² CFU/mL
100 %
90
50
0
290 s
600 s
800 s
1 mV
20 minutes
b)
Response / mV
Covalent functionalization
Slope = 0.36 mV/Dec
R² = 0.94
Non-covalent functionalization
Slope = 1.52 mV/Dec
R² = 0.96
Log₁₀([S. aureus])
c)
EMF / mV
PBS
10²
10⁴
10⁶
10⁷
10⁸
Covalent
vs S. epidermidis
PBS
10²
10⁴
10⁵
10⁶
10⁷
10⁸
Non-covalent
vs S. epidermidis
PBS
10²
10⁴
10⁶
10⁷
10⁸
Covalent
vs E. coli
PBS
10⁴
10⁶
10⁷
10⁸
Non-covalent
vs E. coli
2 mV
Time / minutes

Fig._3:



LEGENDS:

Fig. 1. Experimental setup for the sample recovery steps required to analyze human skin surrogates using the potentiometric biosensor. 1) sampling from skin segments using a sterile swab moistened with PBS; 2) recovery by resuspension of the collected microorganisms in a tube containing PBS; 3) potentiometric detection of bacteria in aliquots of the recovery solution. Biosensors functionalized by both methods and the interaction with *S. aureus* are schematized on the right.

Fig. 2. Performance of biosensors prepared by covalent functionalization with NH_2-Aptamer and non-covalent functionalization with Pyr-Aptamer. a) Change in EMF recorded as a function of time for different biosensors when exposed to *S. aureus* (right, amplification of the curve after inoculation with $8x10^2$ CFU/mL). 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). c) Change in EMF recorded as a function of time, when biosensors were exposed to stepwise increasing concentrations of different microorganisms (values are in CFU/mL), *S. aureus*, *E. coli*, *S. epidermidis* and a SWCNT sensor without aptamer to *S. aureus*.

Fig. 3. Average potentiometric response observed when biosensors were exposed to samples containing different microorganisms recovered from pig skin segments. All the skin samples were initially inoculated with $5x10^6$ CFU of the microorganism tested. Error bars are standard deviation for three different experiments. The average concentration (CFU/mL) in the electrochemical cell is given below the chart in logarithmic units (the values in parenthesis are SD, N=3).