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Novel integrated and portable endotoxin detection system based on an electrochemical biosensor

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This paper describes the design, implementation and validation of a sensitive and integral technology solution for endotoxin detection. The unified and portable platform is based on the electrochemical detection of endotoxins using a synthetic peptide immobilized on a thin-film biosensor. The work covers the fabrication of an optimized sensor, the biofunctionalization protocol and the design and implementation of the measuring and signalling elements (a microfluidic chamber and a portable potentiostat/galvanostat), framed ad-hoc for this specific application. The use of thin-film technologies to fabricate the biosensing device and the application of simple immobilization and detection methods enable a rapid, easy and sensitive technique for in-situ and real time LPS detection.

Introduction

Sepsis is a severe pathophysiological syndrome responsible for 750.000 fatalities per year only in the United States.[1] This medical condition is characterized by a systemic inflammatory response of the organism to an infection and it can evolve into a more severe syndrome called septic shock.[2,3] In Gram negative bacteria endotoxin or lipopolysaccharides (LPS) are the main trigger of the process and minimum amounts of this glycolipid can cause serious effects in the organism such as vascular blood clotting and multi-organ failure, since the human body is extremely sensitive to them.[4]

Endotoxins are, therefore, pathogen molecules whose ubiquity poses a serious problem for the pharmaceutical industry and biomedical environments. Nowadays, more than 500 products (including drugs, devices, and biological products) must be tested to ensure that they do not contain endotoxins above certain limits, namely 0.2-5 endotoxin units (EU)/kg body·hour for intravenous injection and 2.15-20 EU for medical devices (being 1 EU = 100 pg of Escherichia coli LPS), as established by the European and American Pharmacopoeias and the Food and Drug Administration (FDA).[5–7] These control requirements have prompted a growing demand for reliable, sensitive and specific endotoxin detection and quantification techniques from industrial and health sectors.[8]

Currently, there are four main companies that manufacture and commercialize endotoxin detection tests (Charles River, Lonza, Seikagaku and Biomerieux). Three of them base their devices on the test LAL (Limulus amebocyte lysate test). This test is based on the coagulation cascade that occurs when the LPS interacts with the blood cells of the horseshoe crab, Limulus polyphemus.[9,10] PYROGENT™ (Lonza) and Endosafe® (Charles River) are two commercial kits for LPS detection for instance. Besides this, there are other kits (not approved by the FDA) that are being marketed like EndoLISA® and PyroGene®. In both kits the endotoxin content is quantified by means of the recombinant Factor C (rFC) and a fluorescent substrate.[11] All these techniques are based on colorimetric measurements and, in spite of being sensitive; they are long and expensive to carry out and most of them are subjected to numerous interferences.

To overcome these drawbacks, research groups all over the world are focusing their studies on the implementation of electrochemical biosensing techniques for endotoxin detection. This detection can be achieved through the immobilization of molecules with high affinity to LPS like aptamers, enzymes and peptides.[12–16] Electrochemical techniques, and more specifically cyclic voltammetry (CV), have been widely used in the implementation of biological detection systems and applied to characterize many different electrode reactions and bioreceptor-analyte interactions.[17] However, all the methods proposed are based on macroelectrodes that hamper the integration and portability of the devices. Integrated microbiosensors seem to be a suitable alternative to meet the demands for the more compact and robust LPS detection system easily applied to diagnosis and quality systems of industrial plants the market is asking for.

As can be inferred from the working principle of biosensors, the biofunctionalization of the sensors is of crucial importance within the development and implementation of new biosensing devices. This functionalization includes the selection of an appropriate bioreceptor and its immobilization. The method chosen here for the covalently bonding of the ligand on the surface of the working electrode are self-assembled monolayers (SAMs).[18] SAMs are organized structures of organic molecules that bind to the electrode surface through their terminal thiol group and allow the efficient and simple immobilization of different compounds used for biological detection.[19,20] In this work particularly, we have used mixed SAMs. These mixed SAMs, composed of two alkanethiols with different length and functional group, are known to minimize the unspecific binding of the analytes on the sensor surface and to increase the stability of the immobilized ligand.[21,22] Regarding the nature of the bioreceptor, the increasing number of bacteria that are resistant to antibiotics is prompting an intense research on alternative therapies, like those based on antimicrobial peptides.[23–25] The peptides derived from the Limulus anti-LPS factor (LALF) have shown a high neutralizing activity against LPS based on the cationic and amphiphilic character of the molecules.[26–28] The variation of the sequence of amino acids that conform the peptides and the optimization of their properties resulted in peptides with an increased affinity towards LPS in vitro and capable of conferring protection against sepsis in vivo.[29]

The aim of this work is to present the design, fabrication, characterization and validation of an integrated and portable platform for endotoxin detection. The system proposed is based on the electrochemical detection of LPS using a synthetic peptide as ligand. The use of a microbiosensor integrated in a single use assay cell and the development of a portable potentiostat/galvanostat renders a simple, competitive and easy method for endotoxin determination. The fact of combining thin-film technologies, biochemical immobilization methods and electrochemical techniques provides a new and sensitive alternative for the implementation of Lab-On-a-Chip and Point-Of-Care devices that industry and health systems are demanding.

Materials and fabrication methods

Biosensor fabrication

The biosensor developed in this work complies with the specifications of electrochemical devices, integrating the three microelectrodes in one single chip.[30] Four-inch oxidized silicon wafers were used as substrate and the sensors were fabricated employing standard microsystem processes. The working electrode (WE) and the counter electrode (CE) were made of gold by RF sputtering (Edwards ESM-100) and patterned as a disk of 2 mm of diameter the first and with the shape optimized in a former work the latter,[30] both with a thickness of 200 nm. A concentric semicircle of silver was deposited by DC sputtering (Pfeiffer Classic 500) between the CE and WE. This layer of 600 nm of thickness acts as a pseudo-reference electrode and enables the integration of the three electrodes in the structure of the microdevice.[31] With the aim of protecting the inactive parts of the biosensor, a 600 nm coating of SiO2 was deposited by plasma enhanced vapour deposition (Oxford Plasmalab 80 plus). To fit the required sizes of the exposed areas of the electrodes, sensors were subjected to a wet SiO2 etching.

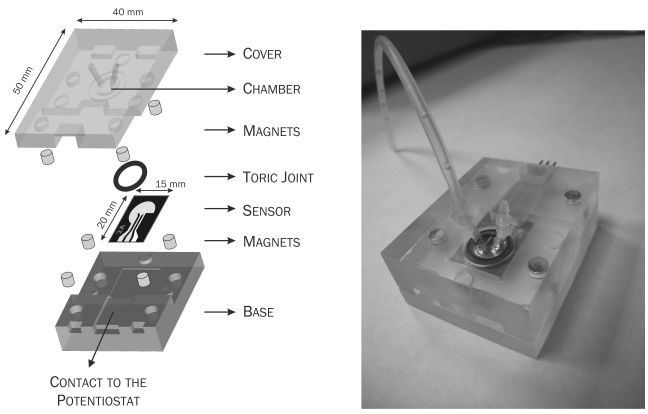


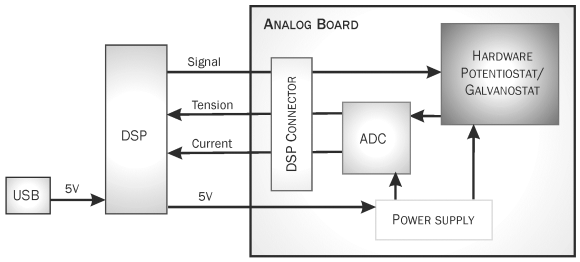
Fig. 1 a) Scheme of the fabricated biosensor and measurement cell, b) Image of the complete system

In order to assure the cleanliness and to minimize the possible influence of environmental conditions in the experiments, a measurement cell of 40 mm × 50 mm × 20 mm was designed and fabricated. This testing platform is made of methacrylate and includes a 40 µl chamber with a height of 1 mm, sealed with an O-ring, where the assays take place. The base and the cover of the cell are kept together with magnets. The use of this microfluidic chamber minimizes both the sample volume and the amount of reagents needed for detection, reducing thus the cost of the assay.

Potentiostat/Galvanostat fabrication

The prototype implemented combines both the hardware and the software needed to carry out the electrochemical measurements. The acquisition, generation and control actions have been conducted with a microprocessor and a series of converters both analogic and digitals. The potentiostat is the main part of the electronic system and its duty is to apply an electrical voltage and convert the electrochemical signal between the electrodes into an analogic input. This potentiostat contains an analogical digital converter ADS1256 from Texas Instruments (ADC), which turns the signal and sends it to the microcontroller composed of a FreeScale MC56F8037 commercial card (DSP, Digital Signal Processor). Finally, the data are sent to the control software by means of a USB connexion. Figure 2 shows a scheme of the measurement module. The software application, based on Labview (National Instruments), includes the tasks of interface, signal generator, control system, filtering of the acquired signal and visualization of the output. Supplementary figures S1 and S2 show some of the interfaces of the program. Technical specifications of the potentiostat/galvanostat are detailed in the supplementary tables T1 and T2.

a)



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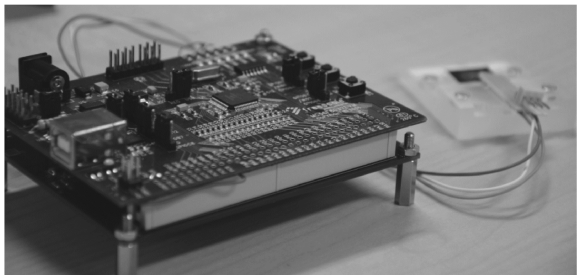

Fig. 2. a) Block diagram of the measurement module. b) Fabricated potentiostat/galvanostat

Reagents

Sulphuric acid, pyrogen free water, 2-mercaptoethanol (2-ME) and potassium ferrocyanide ($K_3Fe(CN)_6$) were purchased from Sigma-Aldrich. Acetone 99.5% pure and ethanol 99.5% pure were supplied by Panreac. Mercaptopropionic acid (MPA), 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-Hydroxysuccinimide (NHS) were purchased from Thermo Scientific. Trichlorethylene was obtained from Alden. Hellmanex II was supplied by Hellma. Ultra pure water of 18.2 MΩ resistivity was obtained from a Milli-Q Water System (Millipore Corp.). The synthetic peptide contains 7 hydrophobic amino acids, 4 polar amino acids and 7 positively charged amino acids in its sequence.[26] This composition provides the molecule both cationic and hydrophobic properties, essential for its binding to the endotoxin.

Lipopolysaccharide preparation

Lipopolysaccharide (LPS) of E. coli ATCC 35218 was obtained from the aqueous phase of a water-phenol extract according to a published procedure.[32] The use of these natural endotoxins is due to the aim of better mimicking the real targets. LPS extracts were dialyzed, lyophilized and purified following published protocols to remove traces of nucleic acids or proteins that could interfere with endotoxin detection.[33]

Experimental section

Sample preparation

In order to eliminate contaminants, the sensors were cleaned thoroughly following a standard procedure before any experiment. The process consists of three sonication steps of five minutes each with triclorethylene, acetone and ethanol. The gold electrodes were electrochemically activated via CV in sulphuric acid 0.05 M to clean and prepare the sensors for the ligand immobilization. The potential was scanned from 0 V to 1.2 V with a 0.1 V/s scan rate. After each polishing step, the sensors were rinsed with deionized water and dried with N_2.

Mixed self-assembled monolayers were built on the surface of the working electrodes to enable the correct immobilization of the bioreceptor. The sensors were incubated with a mixture of MPA 1 mM and 2-ME 1 mM for 2 hours and then washed with ethanol to remove unbound molecules. SAMs were activated incubating the sensors with EDC and NHS 46 mM for 1 hour and then rinsed with water to avoid unspecific bindings.

The synthetic peptide was immobilized onto the gold surface after the activation of the SAM. For this purpose, the sensors were incubated with 100 µl of peptide (100 µg/ml) for 2 hours, and washed once the step had finished.[18] Figure 3 shows the biofunctionalization protocol step by step.

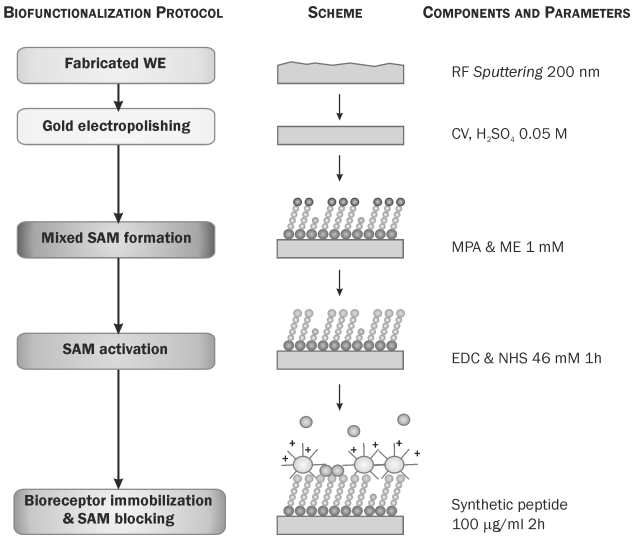

Fig. 3. Block diagram and scheme of the biofunctionalization protocol

LPS molecules form aggregates that differ in shape and activity, hence prior to the immobilization of the sample, the solution containing endotoxin was subjected to three alternating cycles of heating (10 minutes at 56 ºC) and cooling (3 minutes at -20 ºC). Afterwards, 100 µl of the solution were placed into the chamber for 30 minutes. Different concentrations of the analyte (0.01, 0.1 and 1 ng/ml) as well as pyrogen free water solutions as control were tested in order to analyse the response of the biofunctionalized biosensor.

Electrochemical measurements

The cyclic voltammetry (CV) experiments were monitored both with the Autolab Potentiostat PGSTAT 128N using the Nova 1.6 software version (Eco Chemie) and the portable potentiostat/galvanostat designed and fabricated. All voltammetry assays were performed under the same conditions: at room temperature (23 ºC) and in a 25 mM $K_3Fe(CN)_6$ solution. The measurements were carried out in a range of -0.5 V to 0.6 V, with a scan rate of 0.1 V/s. The output signal was recorded after at least 5 stable cycles, calculating the average of 4 consecutive cycles and ruling out the first one.

Results and Discussion

The results obtained along this work are summarized in three sections focused on the following aspects of the research: the electrochemical activation of the gold electrodes, the electrochemical validation of the device in terms of detection limit and the ability to test real sample and the study of the performance of the portable potentiostat-galvanostat in comparison with the commercial Autolab Potentiostat PGSTAT 128N.

Electrochemical activation of the gold electrodes

The gold surface of the fabricated sensors was activated by means of electrochemical polishing to get clean working electrodes. As it is reported by several authors experts in gold electrochemistry, when subjecting these electrodes to acid media they change both in terms of surface composition and topography because the superficial layer and sub-layers of gold oxide are activated.[34]

The electrochemical surface area (ESA) of the electrodes was determined from the voltammograms to study the roughness factor (Rf) of the gold surface. The chosen indicator of the microscopic surface area of gold was the measurement of the oxygen adsorption.[35] The determination was done integrating the gold oxide reduction peak from the voltammetry curves referred to the electrode area (Qexp). The standard reference charge of gold electrodes is 390 ± 10 μC cm^{-2} (Qstd).[36] ESA is the ratio between the experimental charge of the gold electrodes and the theoretical one; whereas the roughness factor is that value expressed per unit of geometric surface area (Equation 1).[37]

$$ESA = Qexp / Qstd \qquad \text{Eq. 1}$$

Eq.1. Electrochemical surface area of the WE

The calculated electrochemical surface area for the working electrodes was $3.01 \cdot 10^{-2} \pm 3.62 \cdot 10^{-3}$ cm^2 (mean value and standard deviation of 8 electrodes) and the roughness factor (Rf) took the value of 1.5 ± 0.18 (non-dimensional). This ESA value improves the ones obtained by other research groups with high reproducibility.[37,38] As the active area of the gold working electrode was increased, the electric current that passed through the sensing surface was enhanced and this lead to a better performance of the developed biosensor.[39]

Comparing this value with the one obtained in a previous work that led to a Rf value of 1.75 in sensors with a platinum counter electrode, the activation of the working electrode was slightly lower.[40] However, the activation of the area of the sensing gold surface was good enough, as the active layer increased by a factor of 50%. Nevertheless, the key step of the improvement of the biosensor performance was due to the fabrication process. As both the counter and the working electrode were made of the same precious metal (gold), the fabricated sensor suffered only two sputtering and lift-off processes instead of three, which clearly influenced the structural properties as well as the sensing behaviour of the electrodes.

Validation of the portable potentiostat/galvanostat

The validation of the fabricated potentiostat/galvanostat was carried out measuring the performance of a dummy cell (that has two resistances and a capacitor) and comparing the response of the potentiostat with the return of a commercial one. The output signal of both potentiostats is showed in supplementary figure S3. The results showed that the signal obtained in both units follows the same tendency, which makes its performance equivalent. However, in the current observed in the fabricated portable potentiostat, a sinusoidal shape can be inferred, caused by the fact that the operational amplifier is reaching saturation.

Besides this internal validation of the potentiostat, some clean electrodes were analysed to check the correct performance of the measurement module. Some examples of the measurements are shown in Figure 4.

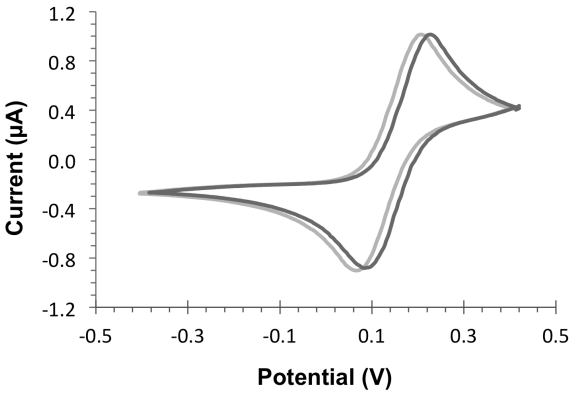

Fig. 4. Cyclic voltammetry experiments of clean electrodes measured by the fabricated potentiostat/galvanostat

The voltammograms show well-defined oxidation and reduction peaks corresponding to the ferro/ferricyanide redox couple. The anodic to cathodic peak currents ratio is near- unity, which indicates the reversibility of the reaction. The slight shift in the oxidation peak position is due to the fact that the integrated RE is a pseudo-reference one, as indicated before, instead of an absolute reference electrode. In any case, the experiments showed that the designed and fabricated portentiostat/galvanostat is able to perform cyclic voltammetry experiments with high accuracy and repeatability.

Calibration curve calculation

After checking that the design of the sensor and the pre-treatment of the working electrode are appropriate for the development of the sensing tool, the calibration curve of the biosensor was obtained. As described in section 3, the biofunctionalization process consists of several steps. The characterization of the detection stack was carried out adding known concentrations of LPS and measuring the biological interaction by means of CV.

In order to quantify this biological interaction, the amount of endotoxin bound to the peptide was analysed in terms of the change in the anodic peak current (Ipa) before and after the addition of the LPS samples. As the peptide is immobilized on the surface of the

sensor the signal of the produced current increases, due to the conductive properties of the molecule that enable a better electron transfer of the used redox couple (Fe^{3+}/Fe^{2+}). However, when the LPS is added and it gets bound to the bioreceptor there is a decrease in the current (a drop in the Ipa) due to the neutralization of the negative charges of the LPS by the cationic residues of the peptide and the presence of insulating chains in the lipid portion of the LPS. The insulating groups of the molecule partially block the electron transfer of the surface inducing the decrease in the current. This reduction in the signal reflects the interaction of the biological molecules and can be selected as the monitoring parameter of the sensing device.

To calculate the calibration curve of the detection system, samples with known endotoxin concentrations were added to the completely functionalized biosensor. Examples of the voltammograms obtained for each concentration are shown in supplementary figures S4-S7. Samples with no LPS content, just pyrogen-free water, were added as negative controls and to determine the detection limit of the system (dashed and continue parallel grey lines in Figure 5). Control assays that demonstrate the specificity of the biosensor exposing the bare gold electrodes (non-functionalized) to LPS solutions of different concentrations were carried out in a previous work.[40] The results of the calibration curve determination are shown in Figure 5.

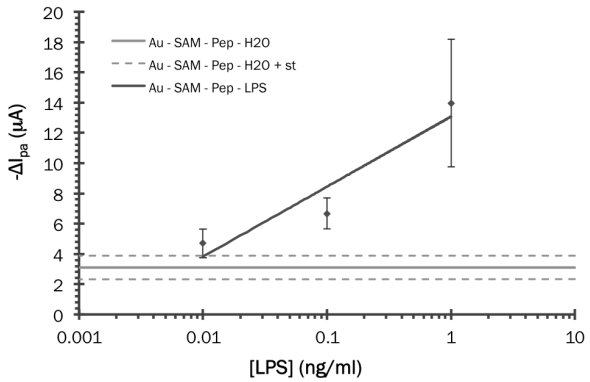

Fig. 5. Calibration curve of LPS detection via CV and control assays

Figure 5 shows the dependence of the output signal on the endotoxin concentration. The drop in the reduction peak has a clear correlation with the amount of LPS present in the sample. As the concentration of the samples gets lower, the variability among the different experiments (at least three repetitions of each concentration were made to achieve statistical values) decreases. The LOQ (Limit of Quantification), calculated as the concentration that gives a signal ten times the standard deviation of the background signal of the system, is 339 pg/ml. Therefore, considering that the LOL (Limit of Linearity) is 1 ng/ml, the dynamic range of the biosensor is 661 pg/ml. This range reaches values sufficient to analyse the 31% of the products that have to be tested to assure the lack of endotoxins.[6]

Furthermore, the LOD (Limit of Detection), calculated as the concentration that gives a signal three times the standard deviation of the background signal of the system, is 21.8 pg/ml. As it has been mentioned before, 100 pg correspond to 1 EU, which means that the LOD of the biosensor is 0.218 EU/ml. This value makes the developed biosensor a feasible alternative, in terms of sensitivity, to the actual methods for endotoxin detection that do exist in the market. For instance, the Hek-Blue test from Invivogen has a sensitivity of 3 EU/ml and the Pyrogent ™ developed by Lonza presents a sensitivity of 0.25 EU/ml a value close to the detection limit of the device implemented in this work.

Validation of the detection system with field samples

As a final validation of the detection system, experiments with field samples of recombinant A protein (rAP) containing an endotoxin contamination (provided by 3PBiopharmaceuticals, Noain –Spain-), were carried out. The concentration of LPS in the samples was 613.4 EU/ml, while the concentration of the rAP was 48.39 mg/ml. This LPS concentration was determined in the company laboratories by the chromogenic LAL test of Lonza. As the concentrations of the calibration curve are slightly lower than the sample concentration, several dilutions were carried out. As control or background assays, samples with the same synthetic rPA concentration in pyrogen-free water were prepared. Besides this, one more control assay was carried out to analyse the response of the biosensor when measuring a laboratory sample of LPS prepared with the same concentrations of both rPA and endotoxin as the real sample.

The aim of this study was at first to analyse the response of the detection system to the presence of a very concentrated amount of rPA, which in the sample acts as an interference. Once the signal obtained is measured, the response of the sensor when there is also some LPS is expected to be higher and in the range of the reference assays carried out with synthetic laboratory samples of the same concentration (dashed lines). Figure 6 shows the results obtained in the validation of the detection system with real samples.

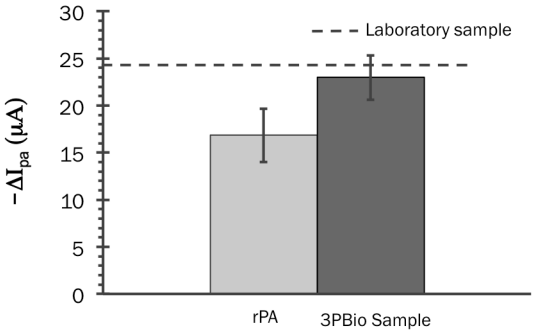

Fig. 6. Response of the biosensor to real samples containing LPS as contaminant. Background signal and standard deviation -st- (grey), laboratory sample signal (dashed line) and real samples signal and st (dark grey)

As it can be seen in the plot, the reference sample containing only rPA produces a signal, which evidences the non-specific interaction of the protein with some components of the device. However, the response of the biosensor to the laboratory prepared mixture of rPA and LPS is significantly higher, indicating the binding of LPS to the biosensor (dashed line). The real samples provided by 3PBio produce a signal higher than the one obtained with rPA, the

variability of the measurements allows the discrimination among the experiments and it matches the value of the response of the biosensor to the synthetic sample of rPA and LPS.

Besides this, further validations of the specificity of the sensing device were preformed analysing the output of the system with different samples containing widely described interfering compounds (DNA and glucose). Supplementary figure S8 shows the results of this preliminary study. The values obtained indicate that the response of the sensor does not shift in the presence of both DNA and glucose, confirming the specificity of the biosensor.

These results show the effectiveness of the implemented device to detect LPS concentrations in the ng/ml range in real samples of products that may content endotoxins and, therefore, must be subjected to a strict quality control.

Conclusions

Endotoxin detection in drugs, chemical compounds and pharmaceutical products is essential in the fabrication process and manipulation of a wide range of products. The emergence of alternative detection techniques is making an impact in the field of endotoxin detection systems and opening a sector that was limited to LAL based kits. In this paper, the design, fabrication, development and validation of a high sensitivity affordable technological solution for endotoxin detection based on electrochemical thin-film biosensing techniques is presented.

The integration of the three electrodes in a single chip allows the miniaturization of the transductor that makes possible its adaptation to the needs of the final user. The electrochemical device fabrication is based on standard techniques used in microtechnologies so it can be upgraded to an industrial level with high reproducibility and without incurring significant costs.

The use of the electrochemistry for both the pre-treatment of the electrodes and the detection system, vests the platform with the robustness and simplicity needed for the measurements. Electrochemical polishing improves the surface finish, increasing the active area of the electrodes and enhancing their sensing features. Cyclic voltammetry has been proven to be a suitable technique for endotoxin detection, reaching sensitivity values comparable with those of some commercial kits available nowadays. This sensitivity is mainly a result of the optimized functionalization protocol that, even though it is simple and direct, provides the sensitivity and selectivity needed to be a competitive alternative.

The fabrication of a portable potentiostat/galvanostat and the adaptability of both the software and the user interface make the system an integral and flexible solution for endotoxin detection. The implementation of the measurement module has enabled the adjustment of the whole detection system to the completion of field experiments, providing essential characteristics when trying to place a new endotoxin detection method in the market.

Finally, the biosensor has been tested with real samples, indicating that the implemented platform is a contrasted and reliable method for LPS quantification. The developed system can be the first prototype of a new generation of highly sensitive, portable and easy-to-use devices for endotoxin quantification that health care systems, pharmaceutical companies and industries are demanding.

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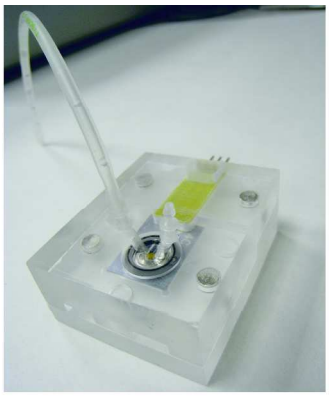
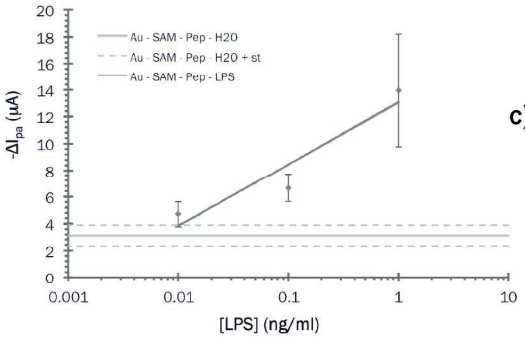
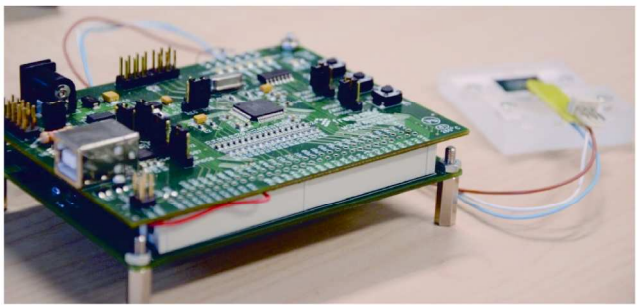
a)



c)



b)



a) Detection system. b) Fabricated potentiostat-galvanostat. c) Calibration curve of LPS detection via CV and control assays
299x213mm (300 x 300 DPI)