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A low-cost electrochemical biosensor platform for C-reactive protein detection

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ABSTRACT

Antibiotic resistance is a worldwide problem aggravated by the overuse of prophylactic antibiotic therapies and lack of real-time biosensing equipment to differentiate viral from bacterial infections at the Point of Care (POC), particularly in rural areas with limited access to clinical laboratory facilities. As recent studies reveal the potential of C-Reactive Protein (CRP) and other acute phase biomarkers to achieve early determination of etiology in acute febrile illness, novel biosensing equipment become a plausible approach in preventing the unnecessary prescription of antibiotics. A low-cost experimental platform was engineered to measure CRP concentrations in 50 µL samples of buffer and enriched plasma, using label-free antigenic probes, legacy electrochemical methods and open-source hardware. The prototype presents a portable, cost-effective device for use at the POC with a simplified user interface that can be used wirelessly by healthcare professionals using a mobile phone or laptop. Based on the measured CRP levels, the device suggests if the acute febrile episode is likely to be of bacterial etiology.

1. Introduction

C-reactive protein (CRP) was first documented in the serum of patients affected by pneumonia induced by *Streptococcus pneumoniae* in 1930, by Tillet and Francis at the Rockefeller Institute. Its name was initially derived from the protein's specific capacity to bind to polysaccharide C at the pneumococcus cell wall [1]. Different inflammatory pathologies exist in association with increased levels of CRP: such as infectious disease processes, rheumatoid arthritis, and cardiovascular conditions. Other conditions are also known to increase in serum CRP levels, such as cigarette smoking, age, gender, serum lipid levels, obesity, and blood pressure [2]. Plasma levels of CRP rising from basal concentration at 1 µg/mL up to a thousand-fold within 48 h have been related to a physiological prototype of an acute phase reactant, yet recent studies also report its novel diagnostic potential as an important modulator of the humoral response [3].

The medical relevance of CRP during infectious disease processes is well documented as a marker for diagnostics and follow-up, given its rapid appearance, short half-life (19 h) and the fact that it is poorly affected by other physio-pathological conditions, offering a highly negative predictive value [4,5]. In bacterial pneumonia, some authors outline the role of CRP in clinically differentiating a typical bacterial infection from the Respiratory Syncytial Virus (RSV) infection, exposing a clear relationship between these two disease scenarios and their respective correlation to levels of CRP, suggesting that CPR may be a good biomarker for epidemiologic studies. These results correspond to Pneumonia Etiology Research in Child Health (PERCH).

The main disadvantage of CRP as a predictive biomarker relies on its specificity and sensing capabilities compared to other acute phase reactants (such as procalcitonin), particularly in patients presenting sepsis [6]. Sensibility and specificity of CRP in the diagnostic of bacterial and viral infections is 73% and 81% compared to Procalcitonin, which was measured to 82% and 88% respectively. However, the negative predictive value of CRP increases when interpreted together with other variables, like the absence of fever or normal leucocyte levels in blood.

In clinical laboratories, CRP is determined by expensive methodologies such as immunonephelometric or immunoturbidimetric assays [7], that require pre-treatment of samples, moderate technical skills and sophisticated instrumentation. These factors led to the search for alternatives that would allow obtaining CRP concentrations quickly employing label-free, low cost and easy to use hardware. Some immunosensors based on optical [8,9]], piezoresistive device and

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piezoelectric [10,11], and fluorescent [12] systems have been developed for CRP determination. Electrochemical biosensors have received great attention for this purpose due to their relative lower cost, potential for portability, high sensitivity, reusability and stability.

Earlier concepts of successful immunosensors for measuring CRP have been reported [13–15], however, such platforms were found to focus primarily in the biochemical and molecular principles for detection and measurement rather than a fully-featured device with clinical usability. Likewise, these platforms were found to be made of expensive, non-portable and highly specialized hardware: such as industry-grade potentiostats, laboratory glassware, disk electrodes and entire desktop computers; as well as other laboratory components outside the scope of a low-cost, portable device. From an engineering standpoint these components not only remain a limiting factor for portability, but remain out of reach for medical facilities in rural communities with limited budget and infrastructure.

As for their functional characteristics, existing devices were not found to consider the problem of etiology in acute febrile illness by correlating the levels of antigenic response biomarkers nor designed to suggest a clinical diagnosic by automating the conversion of amperometric measurements to estimate the biomarker concentration; a key-feature for healthcare professionals at the POC.

The aim of this study was to explore well established electrochemical methods for the integration of a label-free, portable, real-time biosensor to determine the etiology of acute febrile illness at the POC by measuring the antigenic biomarker CRP.

2. Materials and methods

2.1. Materials and reagents

The reagents used in this study were isolated Human CRP (OPPA01434) and Polyclonal CRP Antibody (anti-CRP) (OAIA00117) purchased from Aviva Systems Biology, (San Diego, CA, USA). Surface functionalizing assays for the biosensing system were performed using 10 mM glycine HCl, pH 2.0 (B G20-50), pH 2.4 (B G25-50), pH 3.0 (B G30-50) and 10 mM glycine HCl, pH 9.4 (K D-500) from Xan Tec Bioanalytics GmBH (Düsseldorf, Germany). Amino K AN-50 Coupling Kit (containing 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride, 0.05 M N-hydroxysuccinimide (NHS) and 1 M ethanolamine HCl, pH 8.5) and 0.2 M 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) from GE Healthcare (Buckinghamshire, UK).

1× Phosphate Buffer Saline (PBS) was used as running buffer at pH 7.4 and consisted of 140 mM NaCl, 2 mM KH_2PO4, 10 mM Na_2HPO_4, and 2 mM Sodium acetate buffer (0.01 M) at pH 4.0 to 5.5. Carbonate-bicarbonate buffer (0.05 M) pH 10.0 and 1× PBS pH 7.4 were used for pre-concentration and immobilization assays; prepared in the laboratory by using a 0.22 μm filter and degasified before use. All solutions were prepared with Milli-Q distilled water obtained from a EMD Millipore Direct-Q 3 UV system (Merck KGaA, Darmstadt, Germany) and filtered daily using 0.22 μm Millipore Express Plus system.

The anti-CRP pAb was used as positive control and 1× PBS pH 7.4 buffer as negative control. A reference cell containing 50 μg/mL Fetal Bovine Serum (FBS) at pH 10.0 from Sigma-Aldrich (St. Louis, MO, USA) was used without immobilized material for suppressing background noise. A self-assembled monolayer (SAM) of 11-Mercapto-1-undecanol (MUD) and 16-Mercaptohexadecanoic acid (MHDA) obtained from Sigma-Aldrich (St. Louis, MO, USA) was used to modify the electrode surface for anti-CRP pAb immobilization. ELISA tests were developed using polystyrene plates MaxiSorp®, from Fisher Scientific (Loughborouhg, UK). An incubator/Shaker from Labsystem iEMS was used for temperature incubation. A Elx800 21 plate reader was used at appropriate wavelengths, BiotekInstruments (USA).

2.2. Software, hardware instrumentation and engineering design methodology

The selection of materials and critical hardware, such as the electrodes, potentiostat and onboard computer was carried out using an individual decision matrix for each critical component in accordance standard engineering design principles. The weight values of each decision matrix were adjusted to favor technical suitability, portability, low-cost and ease of manipulation, as well as processing capabilities, sensitivity, power consumption and feasibility to be interfaced with other components (See supplementary Material 1).

Two types of Carbon Screen Printed Electrodes (CSPEs) functionalized with Au Nanoparticles (NPs) CSPE-AuNPs (Model OHT-001) and carboxylic acid-modified Au nanoparticles (CSPE-COOH-AuNPs) (Model OHT-003) were purchased from Orion High-Technologies SL (Madrid, Spain). A scanning electron microscope (SEM) PHENOM ProX with an electron energy adjusted to 10 kV was used to obtain morphological images of the electrode samples.

The open-source potentiostat "*RodeoStat*" from IO-Rodeo (Pasadena, CA, USA) was selected as core electrochemical platform from a list of 3 candidate devices (See Supplementary material 1). A "type B" electrode adapter from the same manufacturer was used as electrode holder. The device incorporates a Teensy 3.2 MCU with a 120 MHz ARM® Cortex®-M4 CPU and a Kinetis K64F microcontroller, powerful enough to compute a wide range of standard amperometric protocols. Seamless integration and power supply (5 V, 0.01A) was delivered via simple USB connectivity. The prototype was energized with a 1A/5 V adaptor but batteries were considered for a future design to improve portability. The potentiostat's firmware was upgraded to the most recent version on the manufacturer's website (01-04-2018) before performing experiments.

The "*RaspberryPi ZeroW*" (Raspberry PI Foundation, United Kingdom) was used as embedded microcomputer for the platform. Several modifications were issued to the Linux operating system (raspbian) in order to meet computing and design specifications. The wireless driver of the board was set to operate as "Access Point" to provide Wi-Fi connectivity for external devices to operate the biosensor. The onboard HDMI port was kept as an option to connect a screen or TV. The Apache2 web server and NDISwrapper linux packages were used to provide a captive portal for end users. The captive portal was programmed in HTML and PHP 5.3 providing a "*platform agnostic*" user interface (UI) to launch experiments from any mobile phone or computer with WiFi cappabilities. A secondary UI for desktop computers was built for the purpose of development, calibration and standard laboratory work. Fig. 1 shows the conceptual biosensor design.

Origin Lab v8.1 from Originlab Corporation (Northampton, MA, USA) was used for analyzing the electrochemical biosensor data.

2.3. Strategy of interaction of anti-CRP and human C-reactive protein CRP

To evaluate the CRP concentration in samples enriched with human CRP, the electrical pattern of the binding interaction of anti-CRP with human CRP was measured at the working electrode (W), where the antibody (or probe) was immobilized.

Three immobilization techniques were carried out: i) immobilization of the anti-CRP via amino-coupling (NHS / EDC 1:1) on electrode type CSPE-COOH-AuNPs (OHT-003); ii) immobilization of anti-CRP via self-assembled monolayers (SAM) using mixtures of MHDA and MUD (1:3) on electrode type CSPE-AuNPs (OHT-001) via amino-coupling - and iii) immobilization of anti-CRP by direct adsorption, also on electrode type CSPE-AuNPs (OHT-001) (Fig. 2).

The reason for assessing different immobilization strategies was to compare which electrode and surface chemistry yielded the most suitable signal when measuring CRP.

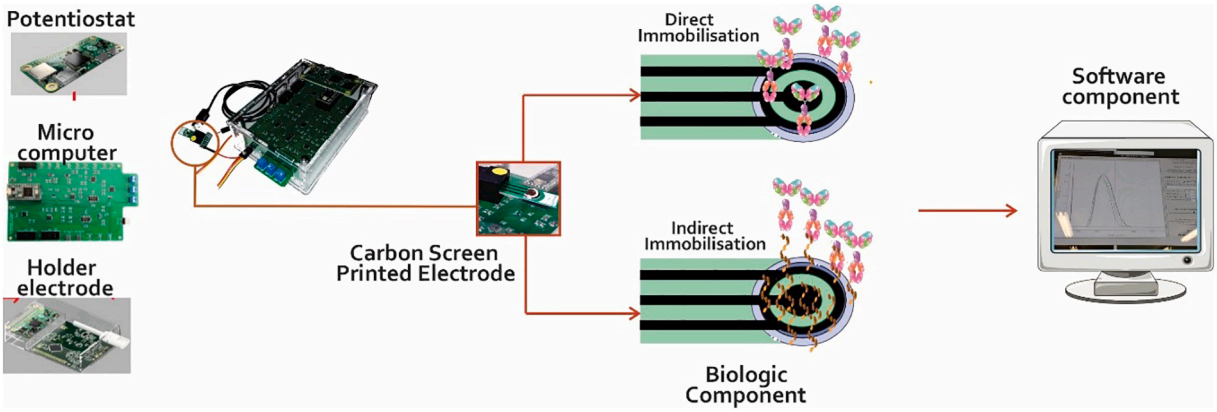

Fig. 1. Conceptual design overview of the proposed biosensor.

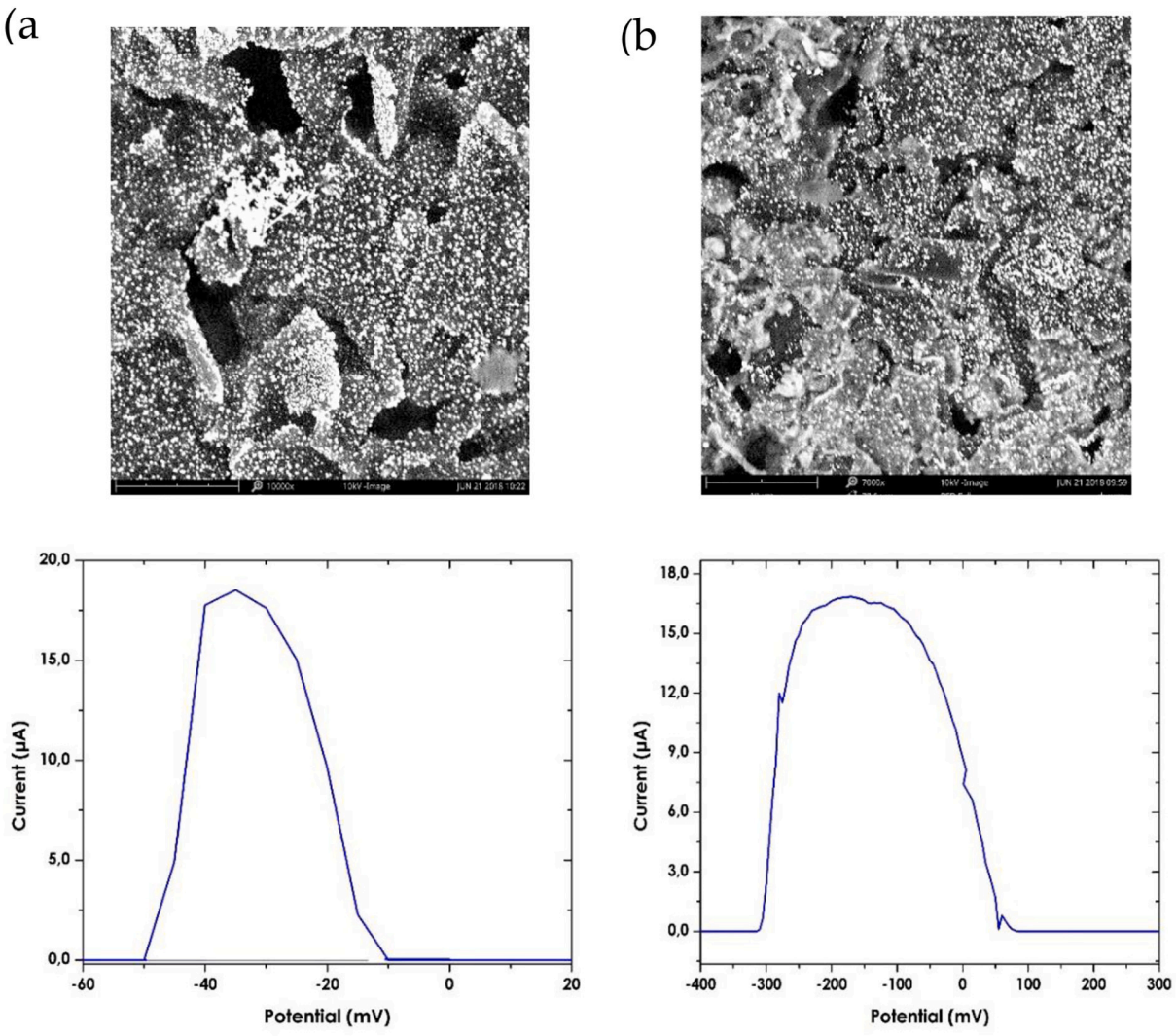

Fig. 2. Scanning Electron Microscope (SEM) images of (a) bare CSPE-COOH-AuNPs and (b) CSPE-AuNPs. The electrochemical response (SQWV) in 1× PBS pH 7.4 at a scan rate of 5 mV / s is shown for each of the electrodes.

2.4. Establishing conditions for immobilizing anti-CRP (pre-concentration assays)

2.4.1. Optimizing CRP assay on the carboxylic acid-modified AuNPs electrode

The optimization of electrode type CSPE-COOH-AuNPs using covalent interaction was carried out to find the best conditions for immobilizing anti-CRP. The parameters evaluated in this assay were 0.001 M sodium acetate buffer in 3 pH values (4.1, 4.5, 5.0), 0.05 M carbonate-bicarbonate buffer pH 10 and 1× PBS buffer pH 7.0. Each buffer pH (4.1, 4.5, 5.0, 7.0 and 10.0) was explored at two different anti-CRP concentrations (50 and 10 μg/mL). The incubation time for each sample was evaluated between 5 and 20 min at room-temperature (RT). A regeneration solution (1 M ethanolamine hydrochloride, pH 8.5) was used between each sample deposition as an agent for removing electrostatically adsorbed CRP from the electrode surface. The electrode was gently washed with 1× PBS pH 7.4 three times before air-drying.

2.4.2. Optimizing CRP assay on the AuNPs electrode

Two immobilization assays were performed on electrode type CSPE-AuNPs.

For the first assay, electrode type CSPE-AuNPs was functionalized with a mixture of 7.5 mM MUD and 2.5 Mm MHDA (3: 1) to allow the formation of a SAM. The reagents were diluted in absolute alcohol. 50 μL of this mixture was deposited on the electrode ‘s surface overnight at 4 °C and protected from light. Amperometric measurements of the CSPE-AuNPs-SAM were taken to establish a base signal before proceeding to the immobilization of anti-CRP.

The second assay consisted in the direct adsorption of anti-CRP onto the surface of the CSPE-AuNPs. The incubation time of the antibody to allow adsorption on the surface was evaluated from 5 to 20 min at RT. The adsorption of the antibody onto the CSPE-AuNPs was measured and monitored with periodic Square Wave Voltametry (SWV) cycles.

2.5. Immobilization of antibody anti-CRP

A critical step during the determination of a biomarker in an electrode is the immobilization of the probe. The anti-CRP antibody was immobilized in both electrode types CSPE-AuNPs and CSPE-COOH-AuNPs using an amino coupling protocol.

This strategy allows a covalent interaction of the antibody onto the sensor surface with high sensitivity and selectivity. The carboxy chains of the electrode were activated with an NHS / EDC mixture that allowed the formation of NHS-esters, forming NH2-containing ligands. In this way, the formation of covalent bonds allows anti-CRP to bind to the electrode surface.

The sensing surface of the electrode was activated by incubating a 1: 1 mixture of NHS / EDC for 30 min. 50 μL of antibody anti-CRP was diluted in buffer at a pH which had previously been selected in the preconcentration assay. Excess carboxyl groups remaining activated were blocked with ethanolamine hydrochloride (pH 8.5).

For the CSPE-AuNPs-SAM, the same amino immobilization procedure was used. Once the antibody was immobilized and measured (amperometrically) the electrodes were vigorously washed three times, using 1× PBS pH 7.4. Excess carboxyl groups remaining activated were blocked with ethanolamine hydrochloride (pH 8.5) for 30 min. Finally, the ready-to-use surfaces were stored at 4 °C.

2.6. Regeneration assay

Propper regeneration of the sensing surface is critical. After performing a measurement, the analyte (CRP) must be removed without affecting the ligand (anti-CRP) from the electrode surface. Surface regeneration was carried out according to previously reported protocols considering the chemical structure of the analyte. 50 μL of 10 mM glycine hydrochloride at a pH of 2.0 was used as regeneration agent. The effect of each regeneration solution was measured amperometrically.

2.7. Electrochemical-based real time quantification

Serial dilutions of human CRP protein in a concentration range of 100 to 1 μg/mL diluted in 1× PBS pH 7.4 were prepared for the construction of a standard curve. 50 μL of each dilution of CRP was incubated for 20 min on the electrodes. The range of linearity, binding capacity, limit of detection (LOD) and limit of quantitation (LOQ) were determined for each assay. LOD was determined by the following equation: (LOD = 3 SD/S) where SD = Standard deviation of the response based on either the standard deviation of the blank and S = Slope of the calibration curve. LOQ determined by the equation: (LOQ = 10 3 SD/ S). Human CRP was used as a control and was evaluated at 0.01, 0.1 and 1 mg/mL concentrations to determine specificity study.

Five plasma samples were spiked with CRP in concentrations of 25, 5 and 1 μg/mL. Regeneration was carried out after each measurement for 5 min to ensure the removal of the analyte, as well as biological and non-biological material. All biosensor assays were done in triplicate.

2.8. Verification and comparison of the electrochemical biosensor with ELISA

The label-free CRP assay on electrode type CRPS-AuNPs was compared with ELISA, based on an assay previously reported in the literature [16]. Briefly, the serum samples were diluted 1: 100 in 1× PBS pH 7.4. Five samples with known concentrations were prepared by spiking with CRP into the serum samples. Standard curves were determined using human CRP at concentrations ranging from 0 to 100 μg/mL, where a linear relationship could be observed with respect to their optical density (OD). OD was measured using a microplate reader BioRad with 0.001 OD photometric resolution set at 450 nm. ELISA experiments were repeated twice.

3. Results and discussion

3.1. Morphological characterization of the electrodes

As introduced in the Methodology (2.2), a SEM was used to characterize the electrochemical deposition of de AuNPs onto CSPE. The images of both electrode types CSPE-COOH-AuNPs and CSPE-AuNPs are shown in Fig. 2. AuNPs (i.e. the small bright dots) are distributed onto the surface of electrodes. The composite film shows porous surface, deposition of gold nanoparticles and net structure, which is favorable for electron transfer. The gold deposition is seen uniformly on virtually all surface electrodes, increasing the effective area and therefore, the proper interaction of the antibody on the electrode surface [17,18].

An average size of 80 nm of AuNP can be observed deposited on the surface of both CSPE. For each image of the electrodes, the Fig. 2 shows the Square Wave Voltamogram (SQWV) using 1× PBS buffer pH 7.4 at a scan rate of 5 mV/s. The electrode type CSPE-COOH-AuNPs exhibits higher current in comparison to electrode type CSPE-AuNPs. However, both displayed good conductive properties.

3.2. Pre-concentration assay

To verify the successful quantification of CRP, step-by-step tests were performed. In accordance with our direct and indirect immobilization strategies, a first preconcentration assay onto electrode type CSPE-COOH-AuNPs was carried out. Fig. 3a. shows the changes of electrostatic interaction with anti-CRP at different values of pH (4.1, 4.5, 5.5, 7.0, 10.0) in two concentrations (50 and 10 μg/mL). It is observed that the current transfer increased along with antibody concentration and pH value, suggesting that higher pH values promote electrostatic interactions. The weakest interaction was observed at pH 4.1 for the two

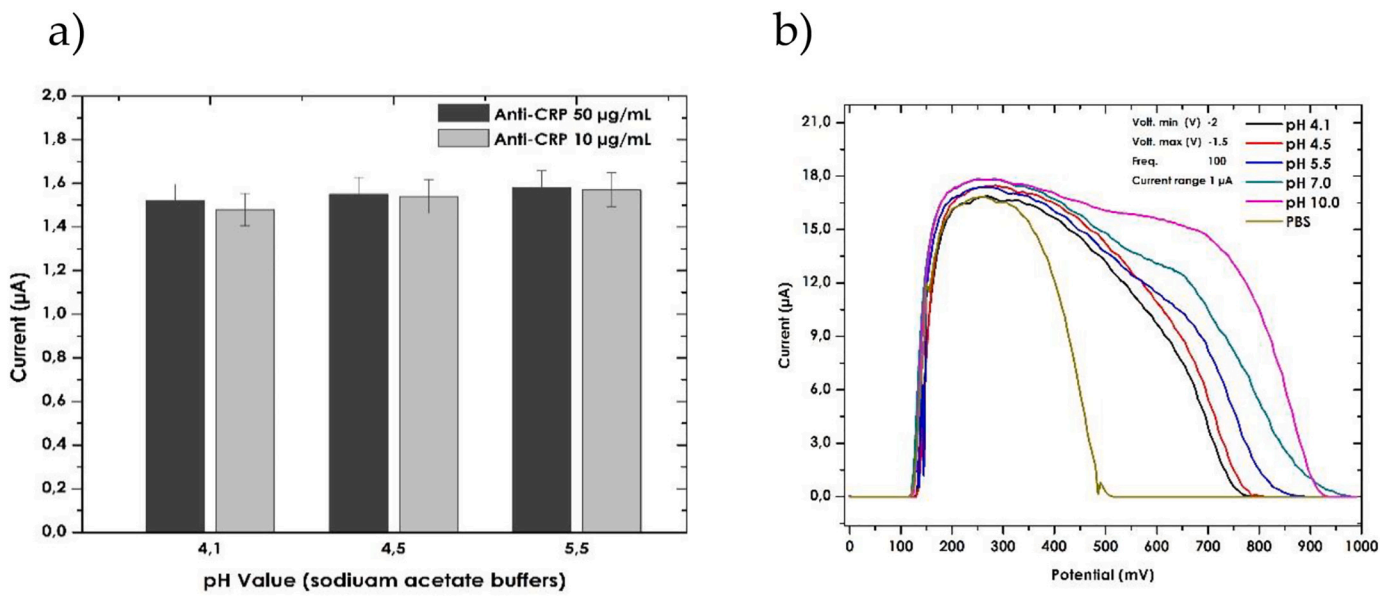

Fig. 3. Optimization of pH buffer and concentrations conditions of anti-CRP onto CSPE-COOH-AuNPs sensor. a) pH scouting using sodium acetate buffers with incubations time 5 min (pH 4–1-4.5). b) pH scouting using Sodium acetate buffer (0.01 M), at pH 4.0 to 5.5, carbonate bicarbonate buffer (0.05 M) pH 10.0 and 1× PBS pH 7.4 with incubations time 20 min and 50 µg/mL of antibody.

established concentrations.

Fig. 3b shows the anti-CRP electrostatic interaction influenced by pH using the highest antibody concentration (50 µg/mL) and producing the greater adsorption on carboxylated surface ~18 µA at pH 10. Incubation times were evaluated from 5 min to 20 min. pH scouting is often performed to optimize the conditions for the protein interaction with the sensing surface. This makes the immobilization of the protein to the surface to be more efficient [19].

Immobilization is often problematic due to the inappropriate use of acidic or alkaline buffer solutions with respect to the isoelectric point of the ligand and its amino acid residues [20]. In this line of thought, optimization of the preconcentration conditions should be available before final ligand immobilization, as otherwise, the sensing surface may result dysfunctional or impaired to produce consistent data, leading to ambiguous results and a wasted electrode. The work performed for the determination or quantification of biomarkers must use preconcentration assays [21,22].

3.3. Immobilization assay

The immobilization results of the ligand into electrode types CSPE-COOH-AuNPs and CSPE-AuNPs-SAM are shown in Fig. 4a and Fig. 4b respectively. The obtained results in the preconcentration assay worked to achieve a successful immobilization. It is necessary to consider the relevance of incubation times to the covalent bond formation of the antibody with the activation agent of the matrix via amino coupling. The current was seen stabilizing after increasing the incubation time of the activation agent to 20 min, as seen in Fig. 4a. Once the matrix was

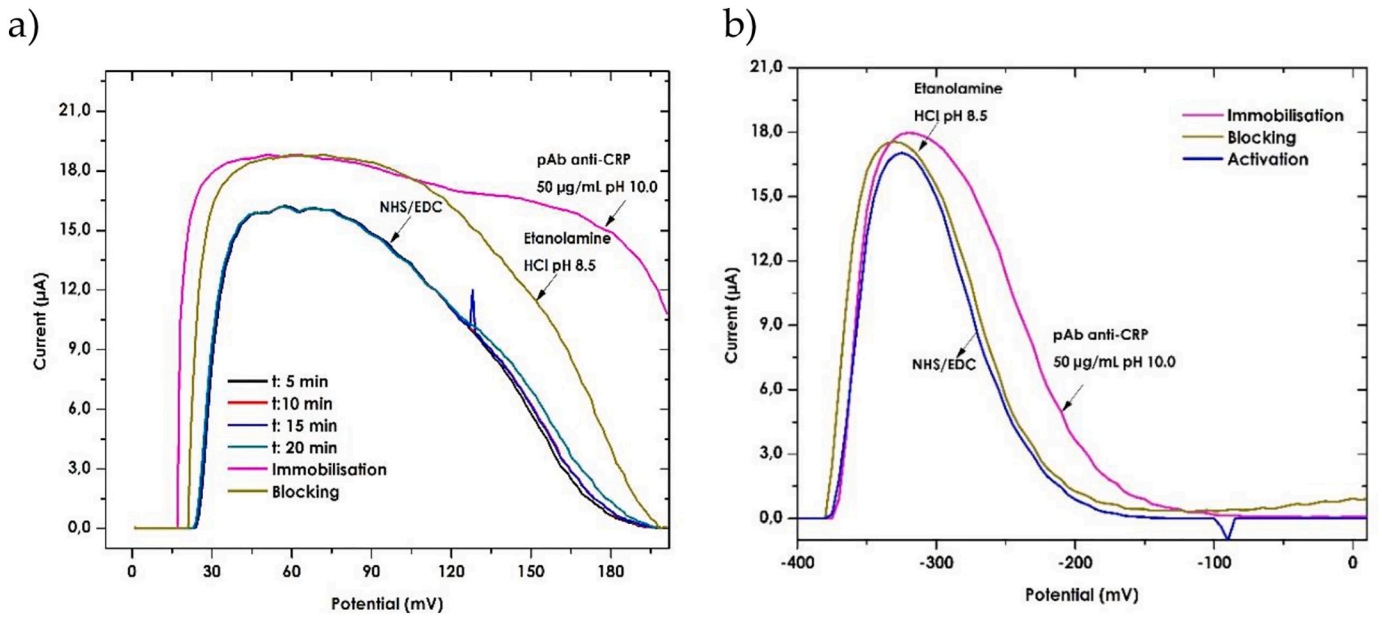

Fig. 4. Immobilization of anti-CRP pAb. a) immobilization anti-CRP into CSPE-COOH-AuNPs via amino coupling. b) Two additional strategies of immobilization into CSPE-AuNPs-SAM. Conditions: 50 µg/mL anti-CRP, carbonate bicarbonate buffer pH 10.0, time activation and incubation 20 min.

active, the response of the anti-CRP immobilization reached a maximum of 18 μA, observing an increase of 2 μA with respect to the matrix activation.

In order to observe and evaluate other immobilization strategies using the formation of covalent bonds on a surface, CSPE-AuNPs-SAM was used. The results were obtained using the same standard procedure as in CSPE-COOH-AuNPs and showed similar results at around ~18 μA for both surfaces. Ethanolamine HCl pH 9.5 was used to block the remaining active sites on the surface without immobilized anti-CRP.

SAMs provide a great platform for protein immobilization. Other studies that determine CRP use SAM formation as immobilization strategy: SH-ssDNA [15], polyethylene glycol (PEG)-thiol HS-C11-(EG) 3-OCH2-COOH [23], SBP-SPA fusion protein [24,25] and 11-MUA and DTDPA [26]. This technique acts by modifying the physical-chemical properties of the sensing surface [27] to provide binding ligands for antibodies. A mixture of SAMs favors that the molecules over the surface either create artifacts or become packed, having good chances of increasing sensitivity of the protein-protein interaction of the antibody and the analyte [28]. Substances with carbonated chains of assorted lengths, as well as the reaction of the terminal COOH groups in the MHDA (alkanethiol) and the terminal OH groups of the MUD with the ligand, minimize unspecific bonding and increasing protein stability.

Both CSPE-COOH-AuNPs and CSPE-AuNPs-SAM were seen to produce usable signal currents due to amino-coupling immobilization.

3.4. Regeneration assay

In order to re-use the electrodes after each measurement, different regeneration agents were tested. The conditions that these agents had to fulfill were: i) bioavailability of its ligand to ensure attachment to the analyte, and ii) interact in a way that would prevent damage to the sensing surface. After following the protocols reported in the literature [29,30], multiple glycine regeneration solutions were tested with a pH of 2.0, 2.4, 3.0 and 9.4. Changes in the current associated to each regeneration solution candidate showed that glycine at a pH of 2.0 successfully recovered the baseline signal after each iteration. At a pH 9.4, the observed current would significantly decrease, which can be explained by damage in the matrix, or the formation of artifacts on the electrode surface (Fig. S1).

Previous studies show that incomplete and partial desorption affect the regeneration of the surface, thus limiting electrode reusability. A fundamental part of this assays may yield reabsorption of detached molecules and other types of non-specific adsorptions [31]. Other studies have attempted to use direct electrochemical methods to perform desorption, reporting achieving similar results by applying a negative potential over the sensing surface.

This study used glycine induced regeneration, which has been widely reported to be successfully used in proteins of this size. In order to opt for this regeneration agent, the isoelectric point (pI) of the CRP protein was taken into consideration (pI: 5.45). The use of the amino acid glycine is a low-cost alternative within a pH range of 2.0 to 7.0, suitable for acidic buffers to avoid extreme values of pH. Having both positive and negative charge, glycine is considered an agent of choice to detach molecules from the sensing surface [32]. An opposite case occurs with acid-base regeneration, where pH variations are reported to affect the enthalpic state of the system as a consequence of changes in the relative electrical charge between the analyte and the bioreceptor.

It is well known that changes in pH may alter the base signal. Despite some authors having previously reported using glycine for effective regeneration with minimal detrimental effects on its original sensitivity [33], other studies mention that the use of glycine may irreversibly alter the biosensor signal permanently [32]. Nevertheless, such effects were not observed in our regeneration assays when using glycine at a pH of 2.0. Alterations of the base electrical signal was never observed changing above 5% after the 4th regeneration cycle. The Table 1 shows the optimal conditions in each of the biosensor development steps.

Table 1
Optimal conditions to quantification of CRP.

Preconcentration	
Concentration Ab anti-CRP	50 μg/mL
Buffer immobilization	0.05 M carbonate-bicarbonate buffer pH 10
T incubation	20 min
Volume (μL)	50
Immobilization	
Electrodes	- CSPE-AuNPs - CSPE-COOH-AuNPs
Direct	CSPE-COOH-AuNPs
Indirect	CSPE-AuNPs-SAM mixture of 7.5 mM MUD and 2.5 Mm MHDA (3: 1)
Regeneration	
Glycine hydrochloride	pH 2.0 10 mM

CSPE-AuNPs: Carbon Screen Printed Electrodes functionalized with Au Nanoparticles (NPs), CSPE-COOH-AuNPs: carboxylic acid-modified Au nanoparticles, SAM: self-assembled monolayers; MUD: 11-Mercapto-1-undecanol; MHDA: 16-Mercaptohexadecanoic acid.

3.5. Measuring algorithm and electrochemical platform

Linear Sweep Voltammetry (LSV), Potential Step Voltammetry (PSV), Cyclic Voltammetry (CV), Square Wave Voltammetry and Chronoamperometry (CA) are well established electrochemical techniques to measure and study electron transfer kinetics on electrode surfaces, comprising particularly useful and accurate analytical tools for screening electrolysis and redox reactions [34]. Adequate steps were taken and evaluated for the biosensor validation using standard strategies to perform electrochemical measurements.

These techniques have been employed successfully for biosensing applications [35–37] with many different functionalized surface chemistries like ssDNA probes, aptamer-based probes and immunoprobes to the detection of specific protein analytes in a sample. Instead of a single electrochemical probing method, as seen in most reported electrochemical biosensors, our analytical approach consisted in a stepwise integration of 3 electrochemical methods (SWV, PSV and CA) to find a combined measurement of the concentration.

Given that the maximum electron transfer of the system occurs at specific voltages particular to each medium, chemistry and nature of the probe-analyte interaction, peak currents become relevant to perform characteristic correlations if studied as a function of such specific voltage, as exposed in the Cottrell Eq. [28].

Series of short SQWV experiments were performed in order to find the ideal experimental voltage of the sample, yielding the maximum electron transfer potential. Having found the ideal voltage, a combination of PSV/CA experiments measured the current over time at that particular voltage, for a period of 300 s using 1 sample per second (sps) interval. It is important to mention that the CA algorithm of our biosensor was crafted manually from a constant voltammetry template instead of using the potentiostat's factory default, mainly due to limitations of the original version in allowing changes in its working parameters.

Short SQWV experiments used a range of potentials from −2.5 V to 2.5 V to find the best voltage. Constant voltammetry-based PSV probes would start at a voltage of 0 V for 1 s (quietValue 0.0, quietTime: 1000 ms), probing the electrode with the sample from 0 V to the ideal voltage found on the previous step, using 1 sps intervals. The ideal voltage for the CA was found to be 2.1 V.

Measurements for all concentrations were performed in triplicate and each concentration readout was given a different line color (Fig. 5). After the measurements, resulting current values (mA) were converted to their absolute values in order to compensate for the arithmetic effect of negative voltages. The final reference current values correspond to the average of the entire measurement over time, for each CRP

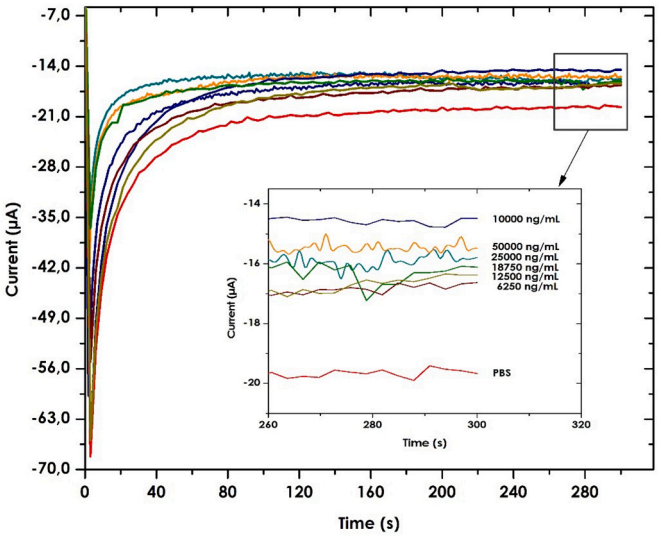

Fig. 5. Experimental current measurement runs (PSV, CA) at a voltage of 2.1 V of a serial dilution of CRP from 1 to 100 µg/mL, using a CSPE-COOH-AuNPs electrode. Inset bottom: Magnified region of the resulting currents, showing each sample with a different CRP concentration in a different color.

concentration. This operation was performed to approach a more realistic value by mitigating the effect of noise.

Peak currents over time for each concentration of CRP is seen in inset bottom of Fig. 5, exposing some visible overlapping on the upper and lower boundaries, differentiable current thresholds in the 1 to 5 µg concentration range and also in the 50 to 100 µg concentration range. A 2 mA (aprox) difference is observed between the control medium alone without CRP (Inset bottom, red line), and the rest of the samples containing CRP (Inset bottom, rest of the colors). Surface regeneration was performed in between each sample as stated in Section 3.4. The procedure was performed in triplicate, finding consistent and reproducible results.

Measuring peak currents with CA at 2.1 V over a period of at least 200 s proved adequate to obtain the reference current value for each concentration. With this information, a calibration curve of concentration vs electrical current was constructed and programed into the software as a reference to compute the concentration CRP in the sample, as seen in Sections 3.7 and 3.9.

The experimental voltage (2.1 V) exposing the maximum current measurements was unusually high with respect to similar experiments, however, negative effects were not observed on the sensing surface or reproducibility of the measurements.

Noise was observed in every measurement regardless of the concentration. The effect of noise is seen increasing as the concentration approaches 1 µg/mL. This behavior is expected as the CRP concentration approaches the LOD. The overall error induced by noise, presumably from electrical or physical sources (matrix effect) is reported in Table 2.

3.6. Calibration curve

A standard calibration curve was built to measure the analyte using serial dilutions of CRP diluted in 1× PBS pH 7.4, with concentrations ranging from 1 to 100 µg/mL. Standard curves were built for both electrode types CSPE-COOH-AuNPs and CSPE-AuNPs-SAM. All measurements were performed by triplicate. The bonding data of the different CRP measurements (time vs current) are shown.

Fig. 6 shows the calibration curves for both electrode surfaces, which were linearized with a linear fit. LOD concentration was estimated in 0,058 µg/mL for CSPE-COOH-AuNPs and 0,085 µG/mL for CSPE-AuNPs-SAM. LOQ (10*S/slope) was calculated at 0.203 µg/mL and 0.178 µg/mL respectively. The obtained data in µg for each of the concentrations

Table 2
Correlation between known concentration of CRP, immunosensor measurements and ELISA measurements of CRP-enriched Human Plasma.

Sample	CRP measured (µg/mL)					
	Biosensor				Immunoasay	
	CSPE-COOH-AuNPs	SD	CSPE-AuNPs-SAM	SD	ELISA	SD
1	12.34	0.131	11.56	0.415	12.24	0.453
2	3.42	0.075	3.49	0.642	3.85	0.184
3	2.35	0.156	2.89	0.373	2.56	0.134
4	11.36	0.066	13.52	2.245	12.05	0.283
5	39.46	0.015	38.02	0.698	38.95	0.042
Negative Control	0.52	0.061	0.95	0.021	0.62	0.007
Positive Control	80.2	2.128	78.95	1.275	81.20	0.983

SD: Standard deviation.

analyzed by triplicate showed correlation coefficient with a high precision ($R^2 > 0.90$) and variation coefficients with good reproducibility (0.001% a 0.0011%).

3.7. Comparing electrochemical-based real time quantification of CRP in serum samples against ELISA

The applicability of this immunoassay was confronted using human serum samples (diluted with PBS buffer to a ratio of 1:5) enriched with CRP. Table 2 shows the concentrations of CRP that were known at the time of dilution, along with the measured concentrations found by the immunosensor and confirmed by ELISA [26]. Each value in the table represents an average of 3 measurements. Measurements of enriched plasma were performed in both CSPE electrode types (COOH and SAM).

Fig. 7 shows a 0.99 R2 correlation regarding response values obtained by electrochemical biosensor and traditional ELISA methodology. Such results suggest that our Biosensor had similar sensitivity to a standardised ELISA in quantifying CRP in plasma samples.

3.8. User interface (UI) and conversion of calibration curve into a diagnostic data

The CRP concentration reference values used to differentiate etiologies of febrile illness was obtained from the literature [38] and hardcoded into the device memory. These values were used by the device to compute the likelihood of a CRP level being correlated to a particular etiology (bacterial or viral).

The linear equation of the calibration curve in Fig. 6 was hardcoded into the program memory. In order to yield the concentration of CRP from the sample, amperometric redings are replaced dynamically into the equation after the measurement is performed, displaying the calculated concentration in ug/mL for any given electric current value. The program compares this value with the clinical database table [38], formulating a clinical recommendation to the healthcare professional, as seen in Fig. 8.

The illustrated sequence 10A-D summarizes the biosensor's UI. From left to right, the mobile phone connects to the biosensor's WiFi hotspot (10A). The initial screen (captive portal) appears automatically and presents an action button to start the measurement routine of the sample (10B). The platform assumes that the plasma sample is already placed in the electrode. Once the button has been pressed, the measurement routines are deployed and the biosensor asks the user to wait for the measurement to take place (10C). Finally, the biosensor displays a familiar result indicating the possible etiology of the febrile illness correlated to the concentration of CRP (10D). For the case scenario, a concentration of 49 µg/mL was found, indicating a high probability of bacterial infection, rather than a viral infection. A button allows to

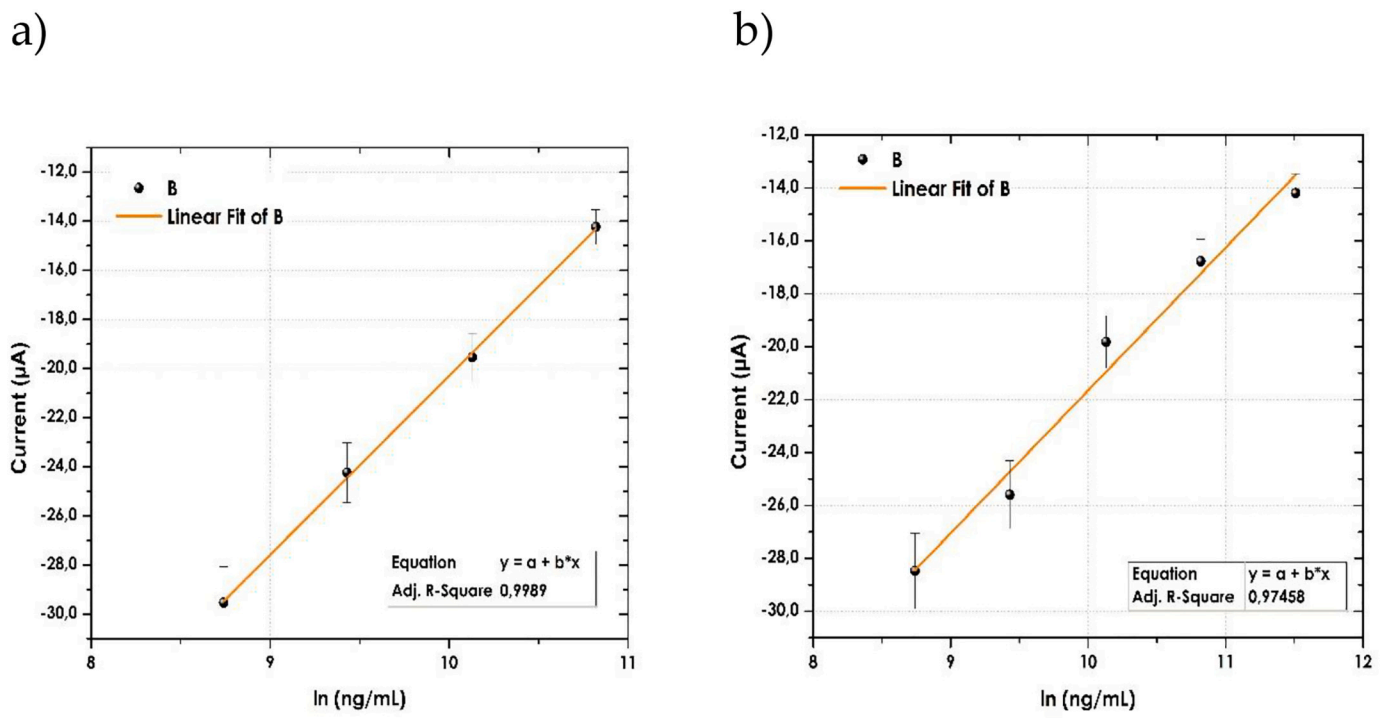

Fig. 6. Calibration Curves over a) CSPE-COOH-AuNPs. Concentration ranging from 1 to 100 μg/mL, $R^2 = 0.974$ and b) CSPE-AuNPs-SAM, concentration ranging from 6,25–50 μg/mL, $R^2 = 0.9989$. The obtained values were averaged from 3 measurement attempts. Graphs are plotted in a logarithmic scale.

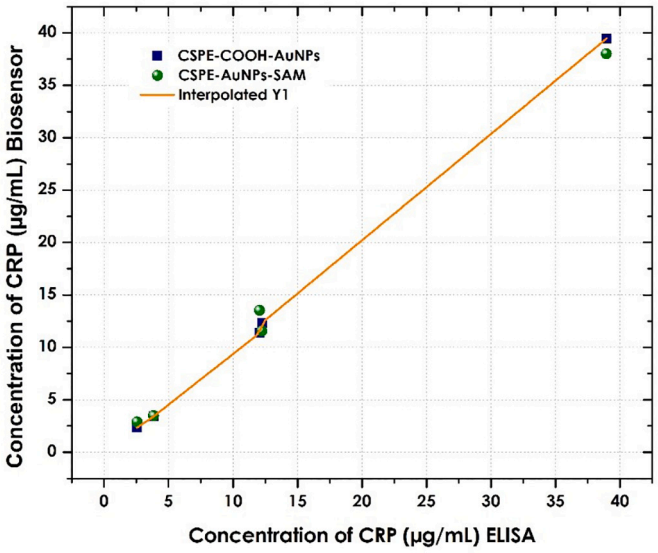

Fig. 7. Correlating electrochemical biosensor and ELISA concentrations ($R^2 = 0.99$).

restart and repeat the measurement if necessary.

To exit the program, the user simply disconnects from the biosensor's WiFi hotspot.

3.9. Differences with other devices in literature

The biosensing technique of our device was built with on top of earlier experimental science available in the literature [15,17,26,39], however, the aim of this device was not only to measure CRP levels in the POC without the need of a clinical laboratory, but to inform the healthcare professional on the possible etiology of acute febrile illness by correlating concentration of the analyte towards clinically established thresholds [40]. This feature was not identified or attempted in similar devices.

The device was conceived by applying engineering principles to fulfill the context of healthcare professionals, offering a simple and comprehensive UI that allows taking measurements with the push of a simple button; creating an abstraction layer between the technical complexity of amperometric methods and the final correlated results, which are calculated and displayed in familiar SI units. As it turns out, other devices in the literature use the potentiostat's default graphical interface, which is highly technical and unfamiliar for most healthcare professionals.

As seen in similar devices [41], the potentiostat is a critical component of an electrochemical biosensing platform and often one of the most budget-limiting components. As opposed to similar devices in the field [42,43] using industry grade potentiostats, implementing an open-source potentiostat lowered the overall costs (including reactants, electrodes and peripherals) well below $1000 USD per unit. Despite choosing a commercial grade potentiostat (RodeoStat) over other plausible options, future developments of these platforms could positively improve the cost effectiveness and offer new functionalities, like evaluating additional analytes (ie. procalcitonin) and increased current sensitivity.

3.10. Advantages, disadvantages and limitations and future work

The design offers several improvements over similar platforms [42,43] in terms of portability, accuracy, reusability and correlating output measurements into clinical diagnostic results. An easy-to-use interface with wireless access capabilities provides end users with a fast and yet simple tool to measure CRP concentrations in enriched plasma, as well as an automated tool to determine acute febrile etiology based on predictive values of CRP, a feature that can serve in preventing unnecessary antibiotic prescriptions at the POC.

The open-source nature of the software and hardware allows other researchers to easily tailor the device for other biomarkers or improve its

Fig. 8. Sequential view of the biosensor end user UI (in spanish) displaying the process of measurement and clinical assessment, as seen in a mobile phone.

design, in contrast with closed source technology. Likewise, the platform is cost effective and affordable enough for most budgets. As better materials come into availability, such as manufacturer improvements or upgrades, their benefits would be inherited by extension to the platform without having to change its software architecture or biological probe.

Given the biological CRP concentration range required to issue relevant clinical diagnostics (1 to 100 µg/mL), the LOD is considered adequate for the intended application. If required, sensitivity could be modulated by altering the electrode chemistry or improving surface properties like orientation of the antibodies during immobilization [11].

Other limiting factors, such as the reusability of the electrode, was measured and found to decay as expected in accordance with similar models. Further testing and robustness assessment would necessary to accurately measure the repeatability under different conditions, as well as fine tuning the process of immobilization in key aspects like ideal antibody concentration in relation to the usability of the electrode and its lifecycle. Other biological probes, such as CRP aptamers, could be considered instead of antibodies as a strategy to avoid degradation and increase reusability [41].

Material resistance, durability, innocuity, industrial design variables such as ergonomic factors were not tested or evaluated in this study. Nevertheless, having that the device has yet to be tested in a clinical environment suggests that further engineering and design would likely be necessary to achieve a fully functional medical grade device.

4. Conclusions

An electrochemical immunoassay has been successfully designed and tested for measuring CRP in clinically relevant concentrations, using two types of CSPE electrodes and their respective immobilization methods (COOH and SAM). The electrochemical biosensor shows an adequate detection range, which was found to be sufficient in terms of sensitivity and reproducibility.

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Declaration of Competing Interest

The authors declare no conflict of interest.

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