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ElectroELISAProbe: Low-cost and miniaturized cell for on-site electrochemical microtiter plate readout

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ABSTRACT

The development of portable devices for bioassay readouts has revolutionized point-of-care diagnostics by enabling rapid and decentralized testing of biomarkers, pathogens, and contaminants. Despite the sensitivity of traditional methods like ELISAs, their reliance on bulky and costly optical plate readers limits their applicability in resource-constrained settings. To address these challenges, we have developed the ElectroELISAProbe, a novel electrochemical readout system designed for microtiter plate-based assays. This portable, cost-effective, and user-friendly platform combines mass-manufactured, ultra-low-cost metallic pins and simple electronics as electrodes to achieve robust and adaptable performance across various assays. The ElectroELISAProbe comprises a three-electrode cell housed in a 3D-printed holder, designed for compatibility with standard microtiter plates. The device was characterized in terms of electrochemical features, demonstrating excellent analytical performance, with high reproducibility (RSDs < 7%). The validation was conducted for detecting Epstein-Barr Virus (EBV) anti-VCA IgG in spiked serum and the matrix metalloproteinase-9 (MMP9) in saliva samples, achieving high recovery rates. Its decentralization potential was endorsed using a portable potentiostat paired with a smartphone interface, enabling field-based applications without performance losses. Furthermore, the versatility of the developed probe supports multiplexing and adaptation to other bioassays requiring electrochemical readouts. These features highlight the ElectroELISAProbe as a promising tool for enhancing accessibility and efficiency in diagnostics, bridging gaps in healthcare, biomedical research and environmental analysis.

1. Introduction

Portable readout devices for bioassays have become essential for providing rapid and on-site detection of biomarkers, pathogens, and contaminants in settings where laboratory access is limited. These systems offer the ability to perform analysis directly at the point of interest, delivering results without the need for complex equipment or specialized personnel, thereby enabling quick decision-making and early intervention. This is especially important in remote or resource-constrained areas, where obtaining rapid results can help improve outcomes while reducing costs and challenges associated with centralized analysis.

In this aspect, the importance of immunoassays is more than widely known, becoming one of the main tools in biomedical and (bio)chemical research for quantifying a vast range of molecules and biomarkers. Commonly, immunoassays, such as enzyme-linked immunosorbent assays (ELISAs), rely on microtiter plates as the physical support of the assay, and in optical readouts, mostly using plate readers. While these methods are highly sensitive, the instrumentation is bulky and expensive, based on components such as lasers, filters or photodetectors that increase the manufacturing and maintenance costs. This limits the use of ELISA for point-of-care diagnostics or on-field applications where portability, simplicity and low cost are crucial. Furthermore, current analytical trends are moving the analysis volumes towards smaller ones (in the μL scale), with the aim of reducing the required sample volume, but also the volume of reagents and the waste generated. However, one of the key factors on which optical determinations rely is the path length. Thus, very small sample volumes might hinder the use of optical

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ELISA when cheap and simple analysis are required.

Currently, benchtop plate readers are not the only option available for optical readout since many portable methods have been developed to make this readout process more affordable and decentralized. Some examples are based on novel platforms aimed to readout on conventional microtiter plates using the camera of a phone [1], while some others focus on creating novel microtiter plate-like devices where the reaction is performed and then easily quantified using either a smartphone [2] or a custom-made reader [3]. Furthermore, commercial plate readers under 1 kg of weight are also available [4]. These approaches overcome the lack of portability of most commercially available plate readers. However, other common limitations in optical measurements, as they can still be affected by ambient light and sample turbidity, might hinder optical point-of-need determinations, especially when performed by non-specialized operators.

In this aspect, electrochemical readout is a suitable alternative to optical methods, particularly for applications that demand low-cost, portable readout systems and low sample volumes. Usually, in immunoassays with electrochemical readout, the binding of antigen and antibody can be followed through changes in the electrochemical properties of the system (e.g. conductivity or charge transfer resistance), or by determining either an electroactive label on the antigen/antibody, or a molecule produced as a result of the immunoreaction (such as in enzyme-coupled immunoassays). These strategies are sensitive, allowing detection of analytes at low concentrations, and have been widely used in the field of biosensors. Nevertheless, they involve taking the solution from the well after performing the immunoassay to an external electrochemical cell [5,6], hampering the determination by adding another step to the immunoassay. For integrating the measurement step and achieving a portable device, an immunoassay can be performed directly on a flat electrochemical cell, such as a screen-printed electrode, using it as the substrate [7–9]. However, this approach is specific to a particular application, and it cannot be applied to most commercial ELISA kits, as these are usually sold with the capture antibody already immobilized on the wells of the microtiter plates. These same drawbacks apply to commercial microtiter plates with 96 individual electrochemical cells integrated at the bottom of the wells [10]. Moreover, these 96-electrochemical cell plates considerably increase the cost of the assay, and they are not reusable, as the assay usually involves a binding between the plate and the reagents of the immunoassay. Therefore, more versatile and adaptable devices are needed in order to provide a universal platform that can be easily adapted to the changing demands of today's world.

In this context, an 8-channel, two-electrode insertable system has been developed for the determination of immunoassays in microtiter plates [11]. Although reusable, this system presents limitations in terms of applicability, as the two Pt electrode system only allows determinations with low current intensities. Furthermore, the price could be reduced, and the fabrication process simplified by using mass-produced, low-cost materials.

In this work, we have developed a novel probe-like electrochemical cell (ElectroELISAProbe) designed as an alternative approach for the readout of microtiter plate-based assays. The cell consists of two gold-plated metallic wires (from commercial electronic connections) acting as pseudo-reference and counter electrodes, and a stainless-steel sewing pin modified with carbon ink serving as the working electrode. The three electrodes are inserted into a 3D-printed holder that fits perfectly into the wells of microtiter plates. After characterization, the developed electrochemical cell has been applied to two different immunoassays: for the determination of IgG antibodies against the Viral Capsid Antigen (VCA) of the Epstein-Barr virus, and of Matrix Metalloproteinase 9 (MMP9). The Epstein-Barr Virus is a lymphotropic γ-herpesvirus of which more than 90% of the population has been infected through their lifetime [12]. One of the main antigens of this virus is the Viral Capsid Antigen (VCA), mainly expressed during the lytic phase of viral replication [13]. This virus has been proven to be the causal factor of numerous diseases, spanning from minor illnesses such as strep throat or tonsillitis [12], to immunological anomalies and cancer, such as multiple sclerosis [14] or B-cell lymphomas [15] and neurovascular diseases such as stroke [16]. Meanwhile, MMP9 has been widely studied for the diagnosis and prognosis of several diseases, such as cancer [17], infectious diseases [18], rheumatoid arthritis [19], neurovascular [20] and cardiac diseases [21,22], among others. Also, the presence of this biomarker has been studied in several fluids across the human body, such as serum [18,22], saliva [23], cerebrospinal fluid [24], tears [25] and synovial fluid [19].

The newly developed electrochemical probe showed high analytical performance, and its usefulness was demonstrated with its application to the two aforementioned immunoassays. Moreover, the ElectroELISAProbe has been designed to overcome the limitations of current systems by: i) having a portable and lightweight design, making it ideal for point-of-care analysis and on-field applications; ii) being cost-effective, as it is based on widely-available low-cost materials and simpler electronics, compared to optical readers; iii) being versatile in terms of design which makes it easy to construct multiplexed platforms; iv) being easy-to-use, as the device requires minimal training to operate, making it suitable for non-specialized personnel; and v) being easy to adapt to a wide variety of bioassays, allowing its use with almost any commercial ELISA kit, even those in which the capture antibody is immobilized on the wells.

2. Materials and methods

2.1. Chemicals

Potassium ferrocyanide, multi-walled carbon nanotubes (MWCNTs) functionalized with carboxylic acid (Product No. 755125), and graphene oxide (GO) dispersion in water (Product No. 777676) were purchased from Sigma Aldrich. Carbon paste (Product No. C10903P14) was purchased from Gwent Group. N,N-dimethylformamide (DMF) and sulfuric acid were purchased from Merck. Disodium hydrogen phosphate, sodium dihydrogen phosphate and isopropyl alcohol were purchased from VWR. TMB (3,3′,5,5′-tetramethylbenzidine) solution was purchased from Abcam. All chemicals were of analytical reagent grade. For all experiments, ultrapure water, purified by a Direct-Q UV 3 purification system (Merck Millipore) was used.

2.2. Electrochemical cell materials and measurements

For the fabrication of the electrochemical cell, gold-plated electronic connections (PBC35SFDN, Sullins Connector Solutions) were used as pseudo-reference (RE) and counter (CE) electrodes; stainless-steel sewing pins (Folch, ref. AIN265925) and gold-plated steel sewing pins (Folch, ref. AAO346005) were evaluated as working electrodes (WEs). The shaft diameter of both pins is 0.6 mm. The heads of the sewing pins were cut before using as electrodes, and their side with the sharp tip is the zone used as electrode. Before use, the stainless-steel pins were cleaned by sonication in isopropyl alcohol (20 min).

All electrochemical measurements were performed at room temperature. The initial setup (Figure S1) consisted of a μAUTOLAB TYPE III (Metrohm) potentiostat to which the cell was connected through hook connectors (purchased from DigiKey). The electrode holder piece and its shaft were fabricated using a Flashforge Creator 3 3D printer. The designs were made on AutoCAD Tinkercad and sliced with FlashPrint5. Clear thermoplastic polyurethane (TPU, Geetech) was used for the electrode holder piece and polylactic acid (PLA, Flashforge) for the shaft. For the decentralized setup, Dupont female cables (from Amazon) were used as interface between the ElectroELISAProbe and a Micrux ECSens portable potentiostat (from Micrux Technologies), that was connected to a Xiaomi Redmi Note 7 smartphone running the ECSens Smart app.

The electrochemical measurements with the ElectroELISAProbe were performed in the wells of a 96-well microtiter plate, in which

200 μL of measuring solution were placed. For the characterization of the electrochemical cell and the evaluation of its analytical performance, cyclic voltammograms (CVs) in potassium ferrocyanide prepared in 10 mM phosphate buffer pH 7.0 (PB), or in TMB solution in 0.1 M sulfuric acid were used.

2.3. Modification of the WE

To modify the pin acting as WE with carbon ink, a 40% carbon ink was prepared by dispersing the carbon paste in DMF using a sonication bath (Elmasonic P, Elma) for 1 h at 37 kHz. Then, around 1 cm of the shaft of the pin was modified by covering it with the carbon ink, either by immersion or by manual painting using a brush. For the immersion coating protocol, the WE was immersed into the carbon ink for 10 s in each modification step. For the brush painting method, the WE was pinned onto a stable foam surface, and one layer of carbon ink was painted on top of the electrode with a painting brush. Then, the electrode was cured in a Sanyo Mir-162 incubator, at an appropriate temperature and time.

For the modification of the WE with MWCNTs, the carbon modified

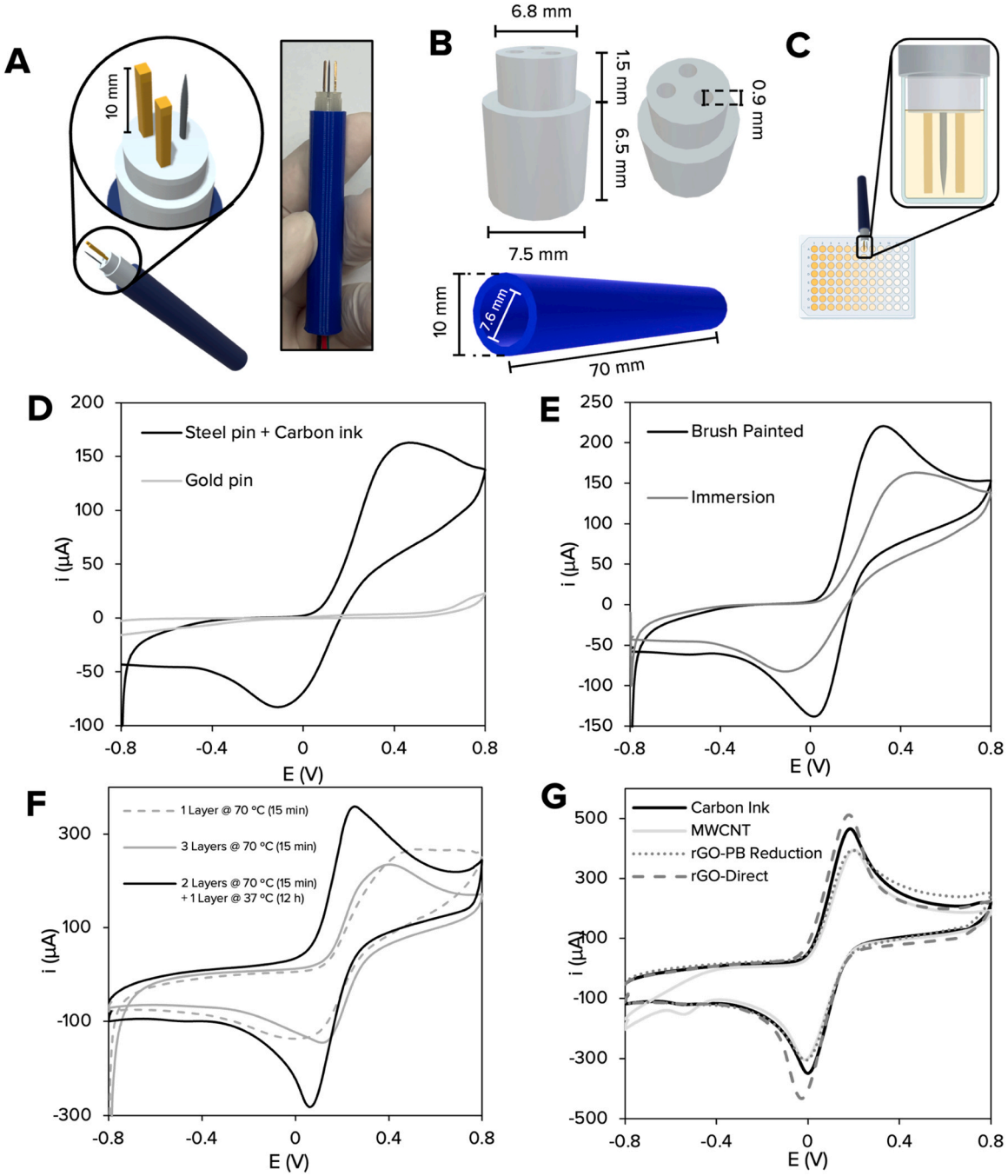


Fig. 1. Design and characterization of the ElectroELISAProbe. **A**: Scheme and photograph of the final configuration of the device. Details show the disposition of the WE, RE and CE. **B**: Design of the 3D-printed pieces of the electrochemical cell (TPU and PLA pieces are in gray and blue colors, respectively). **C**: Detail on the configuration of the cell within a microtiter plate well. **D, E, F** and **G**: CVs performed, in a 20 mM potassium ferrocyanide solution in PB pH 7.0 buffer, from −0.80 to + 0.80 V at 0.1 V/s, to assess electrochemical cells with different WE. **D**: comparison between carbon-ink stainless-steel pin and bare steel gold-plated pin. **E**: comparison between two stainless-steel pins modified with carbon ink following different procedures. **F**: Evaluation of the effect of the number of layers and curing temperatures and times to modify stainless-steel pins with carbon ink. **G**: Assessment of the modification of carbon-ink stainless-steel pins with nanomaterials.

WE was immersed into a 1 mg/mL MWCNTs dispersion (in DMF) for 10 s, and then cured at 70°C for 15 min. In the case of rGO-modified WE, two approaches were used. The first one consisted of immersing the carbon-modified WE into a 1 mg/mL dispersion of GO (in water) for 10 s, and then, cyclic voltammetry (from -1.4 to 0.0 V, at 50 mV/s, 10 scans [26]) in PB pH 7.0 was performed (rGO-PB reduction). The other approach (rGO-direct) consisted of immersing an electrochemical cell with a carbon-modified WE into a 1 mg/mL GO dispersion and then electrodepositing rGO onto the WE by performing the same electrochemical procedure as before (CV 10 scans from -1.4 to 0.0 V, at 50 mV/s). Then, in both cases, the WE was cured at 70 °C for 15 min.

2.4. Immunoassay procedure

Two fast ELISA kits were used in this study. For the detection of VCA-IgG, a kit from MyBiosource (MBS3804559) was used. In the case of MMP9, a kit from Abcam (ab246539) was employed. Both provided all standard, antibody, buffer, TMB and stop solutions. Immunoassays were carried out according to the manufacturer's instructions (detailed protocols can be found in SI). To perform the measurement with the ElectroELISAProbe, after adding the stop solution, the device was placed in the plate well, and a chronoamperometry at −0.1 V for 30 s was performed. The average of the currents obtained in the time range 25–26 s of the chronoamperogram was used as the analytical signal for quantification purposes. Limit of detection (LOD) and limit of quantification (LOQ) of the methods are calculated as 3 and 10 times of the deviation of the intercept of the regression, respectively, divided by the slope of the regression.

Samples for VCA-IgG consisted of human artificial serum (h6914, Sigma Aldrich) spiked with VCA-IgG provided with the ELISA kit. In the case of MMP9, saliva from 4 healthy volunteers was collected by spitting into a 5 mL tube. These samples were collected in accordance with the recommendations of the Ethical Committee on Research of the Principality of Asturias (Study 2024.016). The saliva samples were validated with the same ELISA kit via optical readout with an Eon™ plate reader, using the Gen5 software (BioTek).

3. Results and discussion

3.1. Design and characterization of the electrochemical probe

The electrochemical cell proposed in this work (Fig. 1A) is composed of three electrodes inserted into a flexible TPU electrode holder (Fig. 1B). This piece is inserted into the PLA shaft that can be conveniently used to grip and insert the device into a microtiter plate, where the electrochemical determination can be easily performed (Fig. 1B,C). The overall device is pencil-like shaped and can be easily held and operated with one hand. Additional pictures of the parts of the device are shown in Figure S2.

In this device, gold-plated electronic connections (hereinafter referred to as gold connections) were used as the RE and CE. They have been previously reported as a very convenient option for miniaturized devices [27–29]. The first step in this work was thereby to select the material for the WE. For this, the three electrodes of the cell were inserted into the 3D-printed holder and used for performing electrochemical measurements following an initial setup displayed in Figure S1. The electrodes were placed in the holder, made of flexible TPU, adjusting the inserted length until they protruded 10 mm from the TPU holder. On the other side of the TPU piece, the electrodes were connected to the potentiostat via hook connections.

The choice of material for the WE was initially assessed by using two different sewing pins: a stainless-steel pin modified with carbon ink and a gold-plated steel pin. In both cases, the shaft length immersed in the measuring solution was 5 mm (immersed area ≈ 9.7 mm^2). Before placed in the TPU holder, the carbon modification was performed by the immersion of the pin into a 40% carbon ink three times (10 s each time) with a curing step (at 70°C for 15 min) after each immersion. When the performance of these different WEs was compared by recording CVs of ferrocyanide, a well-known redox probe, (Fig. 1D), it can be clearly seen the gold-plated steel pin did not show any of the characteristic peaks of ferrocyanide. However, the carbon ink-modified stainless-steel pin presents a clear oxidation (i_{pa} 91 μA, E_a 0.46 V) and reduction (i_{pc} −90 μA, E_c 0.08 V) process. This material has been successfully used as a WE [30–33], and thus, it was chosen as the WE for this work.

Furthermore, the preparation of the carbon-ink modified stainless-steel pin was optimized. Two different carbon ink modification procedures, immersion coating and brush painting, were evaluated (Fig. 1E) with the latter showing better defined oxidation and reduction processes, higher peak current intensities (i_{pa} 74 μA *vs.* 154 μA, i_{pc} −67 μA *vs.* −134 μA) and lower difference between anodic and cathodic peak potentials (ΔE 0.44 V *vs.* 0.18 V). Additionally, after selecting brush painting procedure, the number of layers and the curing temperature and times were optimized (Fig. 1F). Three conditions were tested: one layer cured at 70°C for 15 min, 3 layers curing each one at 70°C for 15 min, and 3 layers with the first two cured at 70°C for 15 min, and the last one cured at 37°C for 12 h. As can be seen in Fig. 1F, for the three protocols, both oxidation and reduction process appear. However, the last protocol was chosen to continue the work since it showed the best-defined oxidation and reduction peaks with the higher peak current intensities (Table S1). Although curing at higher temperatures provides faster solvent removal, the final 12 h step likely promotes complete solvent evaporation and gradual carbon matrix consolidation, resulting in improved electrochemical performance [32].

Finally, and in order to improve the electrochemical performance of the device, the possibility of modifying the carbon-ink WE with nanomaterials was also studied. Fig. 1G shows the CVs of ferrocyanide solution obtained with a bare carbon-ink WE, a carbon-ink WE modified with MWCNTs, and carbon-ink WEs modified with rGO following two modification procedures: one involving GO modification and its subsequent reduction in PB (rGO-PB reduction), and another consisting of the direct reduction in a GO dispersion (rGO-direct). (All the modification protocols are detailed in Section 2.3). When peak potentials, and current intensities were compared (Table S2), it was observed that the higher peak current intensities were achieved using a rGO-WE modified by direct reduction. However, the WE without nanomaterials (i.e. unmodified carbon-ink WE) showed similar results in terms of peak current intensities, and moreover, the ΔE was slightly lower. Furthermore, when RSD values of peak current intensities are compared, it can be observed that the unmodified carbon-ink WE provided lower values than rGO-direct WE modified (for anodic peak, 0.2% *vs.* 4.2%), and much lower than the other WEs nanomodified. The complete precision results regarding the nanomaterial modification study are included in Figure S3 and Table S2. Thus, since none of the modifications with nanomaterials provided significant enhancements over the unmodified WE, no modifications were applied in subsequent studies, which also helped to keep the fabrication process as simple and cost-effective as possible.

This systematic comparison of fabrication parameters (material selection, carbon-ink deposition strategy, curing conditions, and optional nanomaterial incorporation) highlights the step-by-step optimization process followed to obtain the most robust and reproducible configuration of the working electrode.

3.2. Analytical features of the ElectroELISAProbe

The final design of the ElectroELISAProbe, based on the optimizations of the previous section, consisted of a 3-electrode cell, where the RE and CE are gold-plated electronic connections and the WE is a carbon-ink-modified stainless-steel pin (modified with three layers, two cured at 70°C for 15 min, and the last one at 37°C for 12 h). The fabrication costs of the device are below 1€, being the value of the electrochemical cell itself c.a. 0.15 €. The analytical features of this device were then analyzed to prove its usefulness for the desired

applications. Detailed comparison with other approaches for transduction (optical and electrochemical) of immunoassays is shown in Table S3.

To further emphasize the reliability of the ElectroELISAProbe, the reproducilibity of the device was evaluated assessing the inter-device precision of the cell. For that purpose, 15 independently fabricated devices were tested by recording CVs of a ferrocyanide solution (Fig. 2A), yielding RSD values of 6.8% and 6.0% for the anodic and cathodic peak current intensities, respectively. These results confirm the reliability of the device.

Then, a calibration curve for potassium ferrocyanide was performed using the anodic peak current intensity as analytical signal (Fig. 2B), obtaining the equation i_{pa} (μA) = 11.1 [$[Fe(CN)_6]^{4-}$] (mM) + 5.2 with a good coefficient of determination (R^2 = 0.992).

After that, the electrochemical behavior of TMB using the ElectroELISAProbe was evaluated, as this compound is the enzymatic substrate used in the immunoassays. In the immunoassays, the enzymatic reaction where TMB becomes oxidized by HRP is usually stopped by addition of concentrated sulfuric acid. Thus, the behavior of TMB was evaluated in acid media. As can be seen in Fig. 2C, when a CV of TMB in 0.1 M H_2SO_4 was recorded, the typical anodic and cathodic process appears at 0.55 V and 0.50 V, respectively, with peak current intensities of 25.7 μA and −25.3 μA, respectively [34]. Then, a calibration curve was obtained recording CVs of different dilutions of TMB in 0.1 M H_2SO_4 (Fig. 2D). The cathodic peak current was taken as analytical signal since in the immunoassays the electrochemical quantification will be performed using as analytical signal the current intensity due to the reduction of the TMB enzymatically oxidized by the HRP. (In Figure S4, the calibration curve taken the anodic peak current as analytical signal is also represented). As the provider of TMB solution does not specify the starting concentration (nor the inclusion of hydrogen peroxide), the concentration of TMB is given in % of TMB in the H_2SO_4 solvent. As can be observed in Fig. 2D, the device shows an excellent response to TMB concentration according to the equation: i_{pc} (μA) = -0.31 [TMB] (%) - 7.38, R^2 = 0.993. This shows that the ElectroELISAProbe can accurately determine TMB, supporting its further application in HRP-TMB-based immunoassays.

3.3. Application to commercial ELISA readouts

In order to prove the applicability of the ElectroELISAProbe to commercial ELISA kits, two analytes and therefore two distinct ELISA kits were tested. The initial application of this ElectroELISAProbe was focused on the determination of Anti-VCA IgG. Given the aforementioned complications and diseases that can result from EBV infection, a rapid and accurate analysis is key to determine whether an individual is infected or has passed the infection, not only to treat the patient but also to control the spread of the virus, which is key in most virus infections. Thus, the determination of anti-VCA IgG was used as an initial approach for the use of the ElectroELISAProbe.

The commercial ELISA here evaluated is a fast assay that uses a microtiter plate in which the wells contain immobilized the VCA. Into the wells, the standard solutions (or the sample) presenting anti-VCA IgG (the analyte) are added, and then, the anti-VCA IgG is sandwiched with an antibody labeled with HRP. When the substrate solution, containing TMB, is then added, the enzyme HRP catalyzes the oxidation the

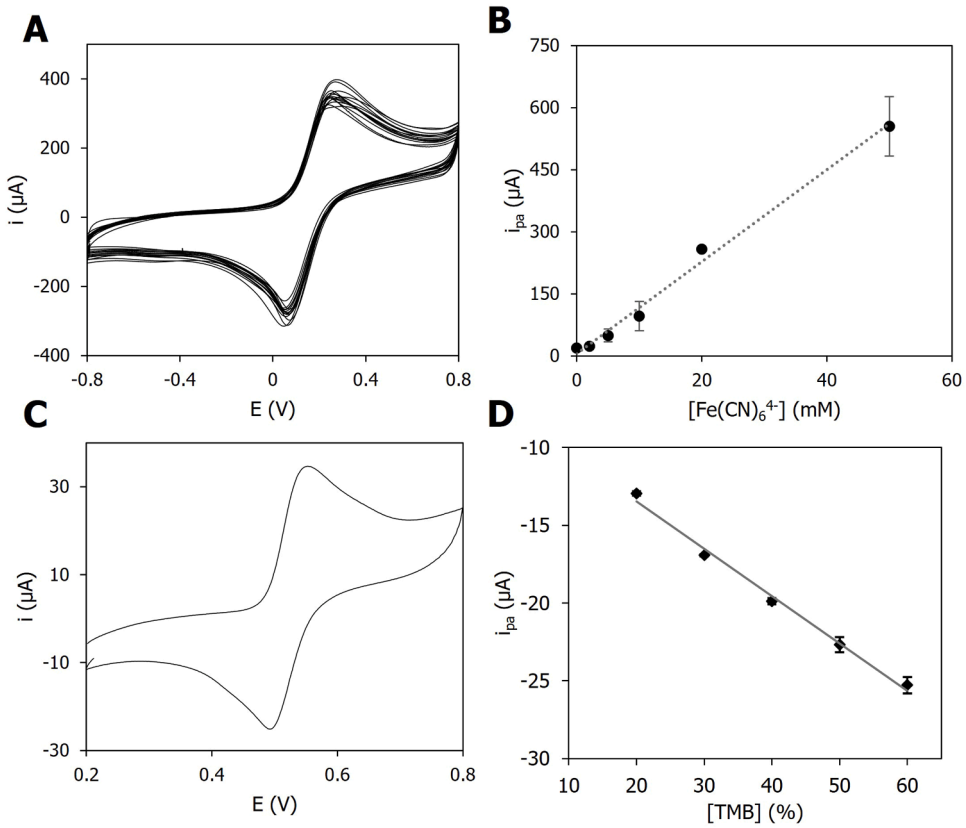


Fig. 2. Electrochemical characterization of the ElectroELISAProbe. A: CVs of a 20 mM potassium ferrocyanide solution (in PB pH 7.0 buffer) recorded with 15 different devices, from −0.80 V to + 0.80 V at 100 mV/s. B: Calibration curve obtained by representing the anodic peak current intensity obtained recording CVs of potassium ferrocyanide solutions (in PB pH 7.0 buffer) of different concentrations (0, 2, 5, 10, 20 and 50 mM). C: CV of a TMB solution in acidic media (1:1 TMB: H_2SO_4 0.1 M) scanning from + 0.2 V to + 0.8 V, at 100 mV/s. D: Calibration curve obtained by representing the cathodic peak current intensity obtained recording CVs of TMB solutions in 0.1 M H_2SO_4 at different dilutions (20, 30, 40, 50 and 60%). Error bars represent the standard deviation (SD) of measurements obtained with 3 different devices.

TMB which can be quantified by reducing it electrochemically by chronoamperometry using the ElectroELISAProbe (Fig. 3A). Therefore, it is a simple ELISA protocol that can be easily performed without any complex lab equipment. The resulting calibration curve obtained using the ElectroELISAProbe can be seen in Fig. 3B, with a linear relationship, in the concentration range 12.5 – 400.0 ng/mL, following the equation i (μA) = -2.1 log [anti-VCA IgG] (ng/mL) + 1.7, with an R^2 of 0.992, an LOD of 2 ng/mL and a LOQ of 8 ng/mL.

To further prove this methodology, human artificial serum was spiked with increasing concentrations of anti-VCA IgG and then analyzed with the immunoassay combined with the ElectroELISAProbe. Results of this validation can be observed in Fig. 3C, where the concentration obtained with this methodology is compared with the expected (spiked) concentration. The detailed results are included in Table S4. It can be observed that results correlate linearly, with an average recovery of 106 ± 2% (estimated by the slope of the linear regression, R^2 = 0.9996), demonstrating the applicability of the ElectroELISAProbe for accurate quantification in complex matrices such as serum. The strong agreement between spiked and measured values further supports the robustness of the platform and its suitability for immunoassay readout.

The usefulness of the ElectroELISAProbe was also assessed with another commercial immunoassay, in this case for the determination of MMP9, as this protein is a widely studied biomarker related to several illnesses, from cancer to infectious diseases or neurovascular diseases. This, along with its presence in human fluids with a non-invasive sampling, such as saliva, makes MMP9 an interesting biomarker for decentralized determination. Therefore, a fast (90 min) one-step MMP9 ELISA was chosen to determine this biomarker in saliva, which in combination with the ElectroELISAProbe represents a very adequate platform for decentralized analysis. This study aimed to demonstrate the versatility of ElectroELISAProbe to be applied to different commercial immunoassays and to different kinds of biological samples.

This ELISA kit provides a 96-well plate pre-coated with a capture antibody, and in its wells, the standard solutions (or the sample) containing MMP9 (the analyte) and a detection antibody labeled with HRP are added in a single step. Then, the substrate solution, containing TMB, is added and, after adding the stop solution, the electrochemical determination using the ElectroELISAProbe is performed as in the assay for anti-VCA IgG (Fig. 3D). The achieved calibration plot obtained can be seen in Fig. 3C, with a linear relationship, in the concentration range 106 – 6750 pg/mL, following the equation i (nA) = -132 log [MMP9] (pg/mL) + 135, with an R^2 of 0.996, an LOD of 3 pg/mL and an LOQ of 23 pg/mL.

This MMP9 assay was validated using real saliva samples collected from four volunteers, and the electrochemical results were compared with the optical readout of the same ELISA kit. Compared results can be seen in Fig. 3F, with the detailed results collected in Table S5. As can be seen, both determination methods correlate in a linear trend, with a slope revealing an average recovery of 84 ± 2% (estimated by the slope of the linear regression, R^2 = 0.9990). This not only demonstrates the applicability of the ElectroELISAProbe to this assay, but also the agreement between this novel detection system and the widely used optical plate reader.

These results prove the applicability of the device developed here not only to two types of immunoassays with different immunoreaction configurations, but also to two different types of sample matrices (serum and saliva), and two validation approaches: one based on recovery study of spiked samples, and the other based on comparison with the widely established optical detection using the conventional benchtop

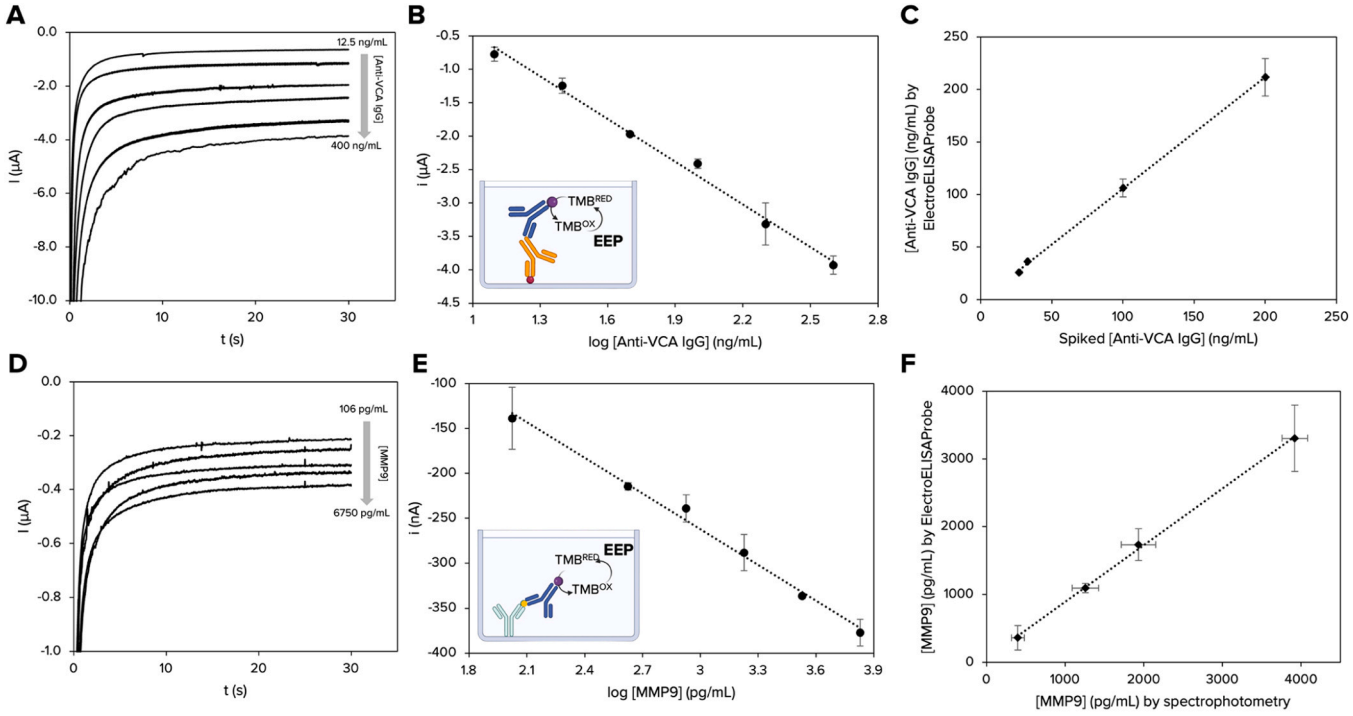


Fig. 3. Application of the ElectroELISAProbe to commercial ELISA immunoassays. A: Chronoamperomgrams recorded at −0.10 V for 30 s using the ElectroELISAProbe for the anti-VCA immunoassay at different analyte concentration (12.5, 25.0, 50.0, 100.0, 200.0, 400.0 ng/mL). B: Calibration plot for anti-VCA Ig achieved by chronoamperometry using the ElectroELISAProbe. Inset: Scheme of the anti-VCA Ig immunoassay format and the electrochemical reaction for determination. C: Representation of the anti-VCA Ig concentrations determined in spiked human artificial serum samples (in the range 27 – 200 ng/mL) using the immunoassay combined with ElectroELISAProbe, plotted against spiked concentration. D: Chronoamperomgrams recorded at −0.10 V for 30 s using the ElectroELISAProbe for the MMP9 immunoassay at different analyte concentration (106, 422, 844, 1688, 3375, 6750 pg/mL). E: Calibration plot for MMP9 achieved by chronoamperometry using the ElectroELISAProbe. Inset: Scheme of the MMP9 immunoassay format and the electrochemical reaction for determination. F: Representation of the MMP9 concentrations found in saliva samples determined by the immunoassay using the ElectroELISAProbe plotted against those determined by the immunoassay using a bench microplate spectrophotometer. Error bars represent the SD of 3 different measurements. EEP: ElectroELISAProbe.

instrumentation. This is a very interesting starting point for ElectroELISAProbe application to the readout of other plate-based bioassays whenever an electrochemical determination is possible, such as enzymatic assays, not only for biomedical [35] applications, but also for environmental analysis [36] and food safety [37], or even cell viability assays [38] or DNA amplifications [39], among others.

3.4. Possibilities for decentralization and multiplexing

In order to assess its possibility for a decentralized use, the initial setup of the ElectroELISAProbe was shifted into a more portable one (Fig. 4A), where the computer has been replaced by a smartphone and the benchtop potentiostat for a USB-C based miniaturized potentiostat, that was successfully coupled to the cell using the same gold-plated connectors used for the RE and CE. The performance of this setup was evaluated by recording a CV of a TMB substrate solution in acidic conditions (1:1 with 0.1 M H_2SO_4), as shown in Fig. 4B. It can be observed that both expected electrochemical processes, oxidation and reduction, appear, and the i_{pa}/i_{pc} ratio and the ΔE_p is very similar as the obtained with a benchtop potentiostat (1.2 in both cases, and 62 mV *vs.* 50 mV, respectively)

Furthermore, its simple design combined with the low-cost mass-produced materials used for its construction, provide ElectroELISAProbe with high versatility, not only regarding its application, as has been proved, but also allowing the simple construction of multiplexed platforms. To demonstrate this, an 8-cell ElectroELISAProbe was designed and fabricated, as shown in Fig. 4C, allowing for the multiplex determination of 8 wells using an appropriate multichannel potentiostat. This also paves the way for achieving up to a 96-cell ElectroELISAProbe device, by simply fabricating an appropriate adapter. In this case, the adapter is comprised of a single TPU piece, where 8 individual TPU holders are aligned and spaced to fit a complete column of a microtiter plate. The design of the 8 well probe adapter is shown in detail in Figure S5.

4. Conclusions

The ElectroELISAProbe, an innovative portable electrochemical tool, has been here presented and validated to be an appropriate candidate for the decentralized electrochemical determination of microtiter plate-based assays. This device addresses critical limitations associated with traditional optical-based immunoassays, such as high costs, bulkiness, and vulnerability to light interferences, while retaining analytical performance. The ElectroELISAProbe demonstrates significant advantages, including portability, cost-effectiveness, and ease of use, making it a convenient alternative suitable for point-of-care diagnostics and field-based applications. These features are especially relevant in contexts where rapid decision-making is essential and access to centralized laboratory infrastructure is limited or impractical.

After an exhaustive optimization of the fabrication protocol, ElectroELISAProbe exhibits high precision and sensitivity across various analytical tests. Given that the probe operates directly inside standard 96-well microtiter plates, it is broadly compatible with the majority of commercial ELISA kits that use this format and an electroactive substrate. To prove its versatility, it was successfully used to quantify two biologically relevant targets using standard HRP/TMB-based ELISA protocols: a viral antigen associated with Epstein-Barr virus (mononucleosis), and the protein biomarker MMP9. The results correlated adequately with conventional optical detection approaches, showing strong linearity and satisfactory recovery rates in both real saliva samples and spiked artificial serum. In the case of infectious diseases such as mononucleosis, the portability and simplicity of the device enable the decentralization of immunoassays, facilitating rapid screening in schools, sports clubs, and other community settings. This could significantly reduce the time between sample collection and result delivery, helping to contain outbreaks more efficiently. For non-infectious targets like MMP9, the device could support localized monitoring in chronic conditions, post-operative recovery, or rehabilitation programs, where timely and repeated measurements are essential for clinical decision-

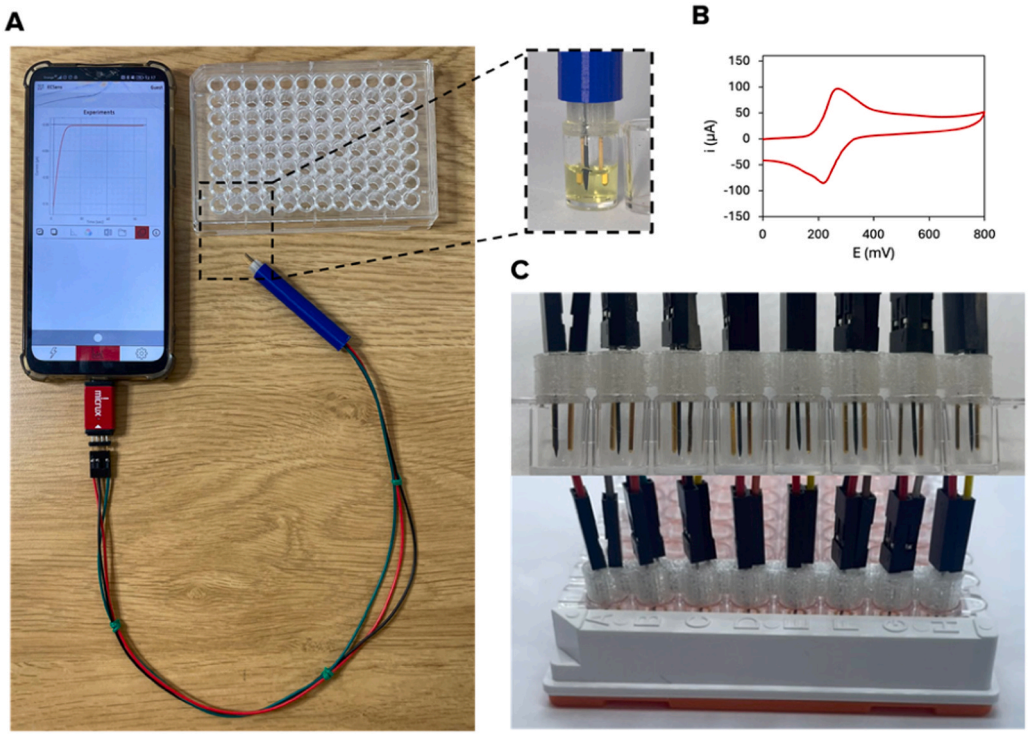


Fig. 4. Toward a decentralized use of the ElectroELISAProbe. A: Photograph of a portable setup for the ElectroELISAProbe. Detail: Device inserted into a microtiter-plate well with 100 μL of a ferrocyanide solution. B: CV of a TMB solution in acidic media (1:1 TMB:H_2SO_4 0.1 M) recording using a USB-C portable potentiostat C: Multiplexed design for the simultaneous determination in 8 microtiter-plate well. Top: detail of the cells inside the wells. Bottom: detail of the layout within a plate.

making.

The versatility and modular design of the ElectroELISAProbe allow for straightforward adaptation to any microtiter-plate-well-based electrochemical assays, as well as the possibility of being adapted to multiplexed formats. All of this highlights the capability of ElectroELISAProbe to accommodate to diverse sample matrices and applications, broadening its potential utility in clinical diagnostics, biomedical research, and public health surveillance.

Therefore, the ElectroELISAProbe represents a significant step toward democratizing bioassay readouts by bridging the gap between laboratory-grade performance and field-level accessibility. This innovative platform holds promises for improving healthcare delivery in a rapidly evolving diagnostic landscape, but also for environmental monitoring and food safety control. Furthermore, the potential for reuse, either by reusing the entire probe or by replacing only the working electrode , could offer a cost-effective and sustainable approach for transitioning between assays. While full reuse in clinical settings may be limited due to contamination risks and regulatory constraints, disposing of the whole cell could be a highly viable option due to its low cost (approximate 0.15 €).

CRediT authorship contribution statement

P. Rioboó-Legaspi: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Visualization, Writing – original draft. **M. Cerrato-Alvarez:** Formal analysis, Investigation, Methodology, Writing – review & editing. **A. Fernández-Quesada:** Formal Analysis, Investigation, Methodology, Visualization. **M.T. Fernández-Abedul:** Conceptualization, Funding acquisition, Methodology, Resources, Supervision, Writing – review & editing. **E. Costa-Rama:** Conceptualization, Data curation, Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.snb.2026.139872.

Data availability

The data that support the findings of this work and the .STL files for 3D printing are available at the Repository of the University of Oviedo (RUO) at the URL: https://hdl.handle.net/10651/83187, with the following DOI: 10.17811/ruo_datasets.83187.

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