Original Research

Immobolisation of electrochemically active bacteria on screen-printed electrodes for rapid in situ toxicity biosensing

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A B S T R A C T

Microbial biosensors can be an excellent alternative to classical methods for toxicity monitoring, which are time-consuming and not sensitive enough. However, bacteria typically connect to electrodes through biofilm formation, leading to problems due to lack of uniformity or long device production times. A suitable immobilisation technique can overcome these challenges. Still, they may respond more slowly than biofilm-based electrodes because bacteria gradually adapt to electron transfer during biofilm formation. In this study, we propose a controlled and reproducible way to fabricate bacteria-modified electrodes. The method consists of an immobilisation step using a cellulose matrix, followed by an electrode polarization in the presence of ferricyanide and glucose. Our process is short, reproducible and led us to obtain ready-to-use electrodes featuring a high-current response. An excellent shelf-life of the immobilised electrochemically active bacteria was demonstrated for up to one year. After an initial 50% activity loss in the first month, no further declines have been observed over the following 11 months. We implemented our bacteria-modified electrodes to fabricate a lateral flow platform for toxicity monitoring using formaldehyde (3%). Its addition led to a 59% current decrease approximately 20 min after the toxic input. The methods presented here offer the ability to develop a high sensitivity, easy to produce, and long shelf life bacteria-based toxicity detectors.

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1. Introduction

Environmental pollution has become one of the world’s major concerns over the past few decades. Many toxic compounds, originating mostly from industrial, domestic and agricultural activities, are being released to our environment. Therefore, early detection and monitoring of the inflow of toxic substances has become a pressing necessity [1].

None of the currently available methods is ideal. Analytical methods that rely on separation techniques such as gas or high-performance liquid chromatography coupled to a mass spectrometry detection system are widely used for toxicity analysis of natural waters [2,3]. However, they are expensive, require complex sample pre-treatment and separation, and require highly-trained technicians [4]. Alternative biomonitoring methods [4–7], do not seem to offer much improvement over these aforementioned techniques, as they generally involve long detection times and low reproducibility, stability and sensitivity, and they may be challenging to perform or unsuitable for online monitoring applications.

Bioelectrochemical systems (BESs) are based on enzymatic or microbial bioelectrocatalysts [8,9]. More specifically, microbial BESs rely on the capability of electrochemically active bacteria (EAB) to freely exchange electrons with an electrode. Thus, the current density generated by the microbial electrode is directly proportional to the metabolic rate of the EAB. The presence of toxic compounds induced a dysfunction leading to a change in current density (i.e., typically a loss). This mode of sensing is unspecific and, although the biosensing abilities of BES have not been sufficiently investigated, they are relatively easy to implement, making them potentially very useful. They can also complement classical...
analytical techniques in water quality assurance [10], particularly as rapid methods in low-resource settings.

In most reported cases, the microbial electrodes proposed for sensing are colonised by anodic, heterotrophic EABs oxidising organics to produce current [11]. However, Prevot et al. [12] have also suggested autotrophic cathodes for toxic detection in oxygen-rich environments devoid or deficient in organic compounds.

Efficient electron transfer between bacteria and electrodes is particularly important to increase current density as well as improve the measurement range in response to a toxic input. One way to do this is by means of redox mediators [13–16]. Redox mediators promote electron transfer between microorganisms and electrodes [17–20]. Ferricyanide is the most commonly used redox mediator because of its low toxicity, high solubility in water, and because it enables the use of highly concentrated bacteria in toxicity assays [17,19]. Additionally, we decided to use bacterial strains such as Escherichia coli (E.coli) because you can use a vast array of biosensing organisms without needing an inherent capability for extracellular electron transfer (EET) as you can work with a mediator.

However, results in the BES literature are highly variable, with no significant response to toxic inputs whatsoever in some cases [21], due to biofilm formation conditions [22]. Growth conditions affect biofilm composition, density, porosity and extracellular polymeric substances (EPS) content. These factors influence the sensitivity of the biosensor to a given toxic compound [23] and affect the success of BES-based biosensors [21]. Besides this, spontaneous biofilm growth leads to longer device production times [24–26], and has higher risks of reference electrode biofouling and destabilising the system by changing the working electrode applied potential [27].

Forced immobilisation techniques may overcome some of these problems. Different types of bacterial immobilisation have been reported such as adsorption [28,29], entrapment within a porous matrix [29,30], self-aggregation (natural [29,31] or with cross-linking agents [29]), and cell containment behind barriers [29,32–34]. Among them, cell entrapment may be the most suitable option since it is a cost-effective and straightforward method. This technique is based on the immobilisation of cells within a porous matrix to prevent their diffusion into the surrounding medium, while still allowing the mass transfer of nutrients and metabolites [34].

Immobilisation techniques eliminate the need for biofilm formation via controlled deposition of bacteria on the working electrode. Nevertheless, during biofilm formation, bacteria gradually adapt for direct electron transfer [35,36], leading to faster electrode kinetics [35]. This does not happen during controlled immobilisation of the cells, which requires new strategies to reduce the time necessary for bacteria adaptation to the electrode and to obtain large and stable readout currents [34,37].

The reference electrode in a three-electrode configuration microbial biosensor enables the control over the potential applied to a microbial modified electrode, which becomes an amperometric microbial biosensor [27,38]. Three electrode systems can avoid issues inherent to 2-electrode BES systems, which can suffer from electrode potential drifts and provide a more stable current response. Therefore, facilitating the detection of toxic substances affecting microbial activity at the electrode [27]. A handful of studies exist that deal with the development of miniaturised microbial biosensors in three electrode configurations [25,39–41]. In all cases, these biosensors needed a long start-up time ranging from 7 days to 1 month due to the necessity of a formation and stabilisation of a natural biofilm on the working electrode.

The aim of this study was the development of a method to fabricate bacteria-modified electrodes, as an alternative to the long times required for spontaneous biofilm growth, but without compromising the response time of the sensor. An immobilisation process is developed based on the controlled deposition of E. coli using cellulose as an immobilisation matrix. To reduce the startup time of the biosensor, this process is accompanied by the application of a potential of +0.3 V (vs Ag). We demonstrate the potential use of these sensors in toxic biosensing and their long-time stability after up to one year of storage of the bacteria-modified electrodes. A single-use miniaturised microbial biosensor based on a screen printed three-electrode cell and a lateral flow membrane is designed and tested as the biosensing platform.

2. Material and methods

2.1. Reagents and bacterial culture

Potassium hexacyanoferrate (III), glucose, formaldehyde (ACS Reagent 37%wt. in H2O), Xanthan gum, 2-Hydroxyethyl cellulose and Poly(ethyleneimine) solution (PEI) were obtained from Sigma-Aldrich. Ashland (Ashland, USA) kindly provided the Gafquat™ 755N. All the solutions were prepared with M9 minimal medium [42] as the solvent.

E. coli ATCC 10536, obtained from the American Type Culture Collection were grown overnight in an 8 mL Luria-Bertani (LB) broth (Sigma-Aldrich) at 37 °C. The number of colony-forming units per milliliter (cfu mL⁻¹) was plate-counted after incubating the plates at 37 °C overnight. The number of cells of the culture had an average concentration of 10⁶ cfu mL⁻¹.

2.2. Electrode fabrication

The chip layout was designed using Vectorworks 2016 (Techlimits, ES). This design consisted of a central 2.5 mm diameter working electrode graphite disc surrounded by a graphite auxiliary electrode and a silver pseudo-reference electrode, as shown in Fig. S1 (Supplementary Material). Electrodes were screen printed directly on a 0.5 mm thick polyethylene terephthalate (PET) substrate (Autostat, MacDermid, UK), using a custom-made manual press, using 20 × 20 cm screens meshed 90 threads cm⁻¹. The snap-off distance was 0.5 mm for conducting inks and 1 mm for the dielectric coating. Silver paste Electrotag 725A (Henkel, ES) was used to print the pseudo-reference electrodes, tracks and contact pads. The working and auxiliary electrodes were printed using Carbon paste C2030519P4 (Gwent Electronics materials Ltd, UK). A layer of UV curable dielectric Electrotag PF-455B (Henkel, ES) was used to insulate the conducting tracks between the contact pads and the electrodes and to define the electrode area.

2.3. Immobilisation matrices analysis

2.3.1. Electrochemical characterisation

Xanthan gum, 2-Hydroxyethyl cellulose, Poly(ethyleneimine) solution (PEI) and Gafquat™ 755N pastes were characterised electrochemically using a DropSens µStat 8000 multipotentiotstat and DropView 8400 software (DropSens, ES). For this, cyclic voltammetry of the modified electrodes with different paste concentrations (2, 5, 10%wt.) were run in a 100 µL drop of potassium hexacyanoferrate (III) (5 mM) between −0.5 V and +0.6 V (vs Ag) at a scan rate of 20 mV s⁻¹.

2.3.2. Bacterial viability test

ELISA 96 Microwelldish (Nunc-ImmunoMicroWell96-well plate, SigmaAldrich) were inoculated with E. coli at a final concentration of 10⁵ cfu mL⁻¹ suspended in 100 µL LB growth medium with glucose (20 mM) as the carbon source and the different pastes at
different concentrations (2, 5, and 10 %wt.). Growth was monitored by measuring the absorbance at 550 nm using an ELISA reader (Multiskan EX, Thermo Scientific). The absorbance values were taken over 16 h, giving enough time to observe a stabilisation of the absorbance values and, therefore, of the bacterial growth. Results were compared with blank samples. The blanks consisted of 100 μL LB growth medium and glucose with the different pastes at a maximum concentration as a no-growth control and a sample with E. coli in LB growth medium with glucose without any paste as the maximum growth control. All conditions were studied in triplicate.

2.3.3. Distribution analysis

A mixture with E. coli bacterium and the different immobilisation pastes at a final concentration of 2% was prepared. A drop of 5 μL of each bacteria-paste mix containing about 10^7 E. coli cells was deposited on the working electrodes and dried at room temperature for 1 h. After this, the Hoechst 33342 fluorescent stain (Fisher Scientific) was used on the samples following protocols recommended by the manufacturer. Confocal microscopy (Leica TCS SP2 AOBS, DE) confirmed microbial distribution within the different matrices.

2.4. Bacteria-modified electrodes preparation and test

Two different methods for bacteria-modified electrodes preparation were studied and tested with clean water samples.

2.4.1. Strategy 1: Extracellular electron transfer adaptation of bacteria before immobilisation

An E. coli overnight culture was supplemented with ferricyanide (5 mM). After 12 h, the growth of E. coli was quantified, yielding a concentration value of 10^8 cells mL^-1. Then, 1 mL of the E. coli culture was centrifuged (4500 G, 15 min) using an OrtoAlresia BioCen20 Eppendorf centrifuge and suspended in 50 μL of cellulose paste (2 %wt.). A drop of 5 μL of this suspension was deposited on the working electrode by drop-casting, assuming a number of bacterial cells immobilised on the working electrode of about 10^7. The electrode with bacteria immobilised was dried at room temperature for 1 h.

Bacteria-modified electrodes were tested by rehydrating the electrodes with a drop of 100 μL of a solution containing 20 mM glucose as carbon source and 5 mM ferricyanide as redox mediator.

2.4.2. Strategy 2: Extracellular electron transfer adaptation of bacteria during the immobilisation process

In this strategy, E. coli was grown overnight in LB in the absence of ferricyanide. After bacteria immobilisation, following the same process explained previously in strategy 1, a drop of 100 μL of M9 medium supplemented with glucose (20 mM) and ferricyanide (5 mM) was added on the electrode. A fixed potential of +0.3 V (vs Ag) was applied until the drop was dried. Bacteria-modified electrodes were tested by rehydrating the electrodes with 100 μL of distilled water.

In both strategies, the electrodes were tested and compared by measuring the amperometric response of E. coli at +0.3 V (vs Ag). This process was repeated until the drop was dried. The oxidative current obtained was close to zero. Note that ferricyanide is the most stable species of the Ferro/ferri couple, and that its spontaneous conversion to ferrocyanide can be neglected [43].

2.5. Bacteria-modified electrodes long term stability tests

Forty electrodes were modified using the drying process under polarization explained in Section 2.4.2. After this, the electrodes were stored in the fridge at 4 °C. At different intervals over a year, two electrodes were rehydrating with water. Current response at a fixed potential of +0.3 V (vs Ag) of these stored modified electrodes was studied. Percentage of decreased activity of modified electrodes was measured as the drop in current output recorded during immobilisation and after storage at 4 °C during the rehydration of the electrodes with water.

2.6. Toxicity shock monitoring

Bacteria-modified electrodes were rehydrated with 100 μL of distilled water, and a fixed potential of +0.3 V (vs Ag) was applied. After a stabilisation time, a drop of 10 μL of formaldehyde (30%) was added to reach a final formaldehyde concentration of 3%.

Current change (ΔI), and inhibition ratio (IR) provide an estimation of the sensor response to toxicity [10]. The ΔI corresponds to the value of the current drop after exposure to toxic agents following equation (1):

\[
\Delta I = I_{\text{nor}} - I_{\text{tox}} = (I_{E \text{ coli}} - I_{\text{blank}}) - (I_{E \text{ coli}} \text{ after toxic input} - I_{\text{blank after toxic input}})
\]

(1)

Where \(I_{\text{nor}}\) is the current generated before the exposure to toxic agents, normalised by the control signal and \(I_{\text{tox}}\) is the current output following the introduction of a toxic [10] normalised by the control signal.

On the other hand, the IR is defined as the percentage of current drop normalised to the stabilized current before exposure to toxic agents [10], and calculated as equation (2):

\[
IR(\%) = 100 \times \left( \frac{(I_{\text{nor}} - I_{\text{tox}})}{I_{\text{nor}}} \right)
\]

(2)

2.7. Lateral flow biosensing platform design and toxicity test monitoring

The lateral flow platform, including the electrodes, was designed and printed following the same procedure described previously in the Materials and Methods Section “Electrode Fabrication”. The lateral flow chip features two working electrodes with an area of 0.01 cm² each, two auxiliary electrodes and a common reference electrode shared between the working electrodes. The electrodes were printed on PET substrates. Paper channels consisted of laser-cut Fusion 5 (GE Healthcare, ES) pieces. The device was sealed using pressure-sensitive adhesives (Adhesives Research, IE) with different thicknesses and surface properties (i.e., hydrophilic cover). Further, a custom holder containing spring-loaded connectors facilitated the experiments.

The electrodes were modified as follows: E. coli cells were resuspended in 50 μL of 2-Hydroxyethyl cellulose (2%), and a drop of 5 μL of this paste-bacteria mix was deposited on one of the working electrodes and dried at room temperature for 1 h. The other working electrode was used as a blank depositing a drying a 5 μL of 2-Hydroxyethylcellulose (2%) without bacteria. Then, Fusion 5 paper channels were placed over the chip. A 100 μL M9 solution supplemented with glucose (20 mM) and ferricyanide (5 mM) was placed in the platform inlet to flow until the electrodes by capillarity. At the same time, a computer-controlled CH Instruments 1030A multipotentiostat applied a fixed potential of +0.3 V (vs Ag).
For bacteria toxicity shock monitoring, a drop of 100 µL of formaldehyde at a final concentration of 3% in distilled water was introduced in the inlet. Bacteria-modified electrodes response to the toxic was measured by applying a fixed potential of +0.3 V (vs Ag).

3. Results and discussion

3.1. Immobilisation matrix analysis

Different pastes applied in products like detergents, adhesives, food additives, water treatment agents or cosmetics at different concentrations were studied. Gafquat [44], a positively charged polymer, Xantham Gum [45], a polysaccharide produced by the bacterium Xanthomonas campestris, Polyethyleneimine (PEI) [46], a cationic polymer, and 2-Hydroxyethyl cellulose [47], a poly- saccharide derived from cellulose, were used to immobilise bac-
teria on the working electrode. This matrix must confine the microbial cells while allowing free passage for the reaction prod-
ucts (carbon source and redox mediator) with no damage to the microorganisms.

The pastes gafquat, xantham gum, cellulose and PEI used to immobilise the bacteria on the electrode were analysed in terms of electrochemical response, bacterial viability and bacteria distribution. Cyclic voltammetry in ferricyanide (5 mM) of electrodes modified with different concentrations (2, 5 and 10 %wt.) of the pastes were compared to a non-modified electrode. Fig. 1 shows an example of these voltamnograms for each type of paste. Additionally, Supplementary Material (Figs. S2–S4) provide more detailed information about the voltamnograms performed for each modified-electrode and the variability between identical electrodes.

When bare screen-printed electrodes were used, the oxidation-reduction peaks of ferricyanide appeared at potentials of +0.18 ± 0.01 V (vs Ag) and +0.90 ± 0.01 V (vs Ag) with a current peak of 587.27 ± 2.14A and -737.6 ± 42.1 µA cm⁻² for oxidation and reduction peaks, respectively, with a variability between elect-
rodes under 12% (Fig. S4).

Changes in the oxidation-reduction of ferricyanide brought about by the modification with the different pastes were studied. When electrodes were covered with different polymers, oxidation-reduction peaks were observed but, with a mild influence on the peak potential and current depending on the paste covering the working electrode. In general, the modification of the electrodes produced a voltammogram shift towards negative voltages. Additional-
ly, the variability found between electrodes modified with the same paste was high, above all in the case of Xanthan and PEI-modified electrodes. These electrodes showed variability in the peak potential above 50%, even higher in the case of Xanthan and PEI-modified electrodes (Fig. S3). Thus, only cellulose-modified electrodes maintained similar peak potentials than non-modified electrodes with variability between electrodes under 16%.

The Xanthan-modified electrodes (Fig. 1a) showed low current densities of the ferricyanide oxidation-reduction peaks. When a 5% matrix concentration corresponded to a reduction of up to 80%, compared to the unmodified electrodes. This suggests that the mediator cannot diffuse readily through the matrix. Immobilisation by entrapment can show diffusion resistance caused by the entrapped material, which might result in lower sensitivity [24,48].

Additionally, concentrations tested turned out too high for Gafquat paste, above all the 10% concentration. The high viscosity of Gafquat paste at this concentration prevented its use as an immo-
ibilisation matrix.

PEI-modified electrodes showed an increase of the current oxidation densities with the increased concentration with 672.87 ± 86 µA cm⁻² at 2%wt. to 797.5 ± 43 µA cm⁻² at 10%wt. (Fig. 1b, Fig. S3). In these electrodes, an additional oxidation peak at 0.4–0.5 V (vs Ag) appeared.

The electrochemical response to ferricyanide of the Cellulose (Fig. 1c) and Gafquat-modified (Fig. 1d) electrodes did not change in terms of current density, and the reproducibility between elec-
rodes was good (Fig. S4). Only, a slight decrease of the current reduction density was observed in cellulose-modified electrodes when the concentration of the paste increased (Fig. S3).

The pastes used must entrap bacteria without affecting cell viability. To demonstrate this, E. coli growth tests were carried out in presence of different concentrations of each matrix (2, 5 and 10 %wt.). The decrease in bacterial growth compared to the E. coli growth without paste, is represented in Fig. 2a.

The results showed differences in E. coli growth both between the different pastes and between concentrations. In general, E. coli growth was slower when the pastes were added to the growth medium. The bacteria growth with PEI was close to zero with a growth decrease of about 90% for all the concentrations analysed. This agrees with other works in which the antimicrobial chemical activity of PEI has been studied and demonstrated at high con-
centrations [49,50].

E. coli grew in the presence of Gafquat, although it was reduced by 31.9 ± 6.5–46.6 ± 1.6%. Gafquat is a film-forming cationic polymer designed primarily for use in personal care. It has been used in enzyme biosensors [44], but there are no records of it as a biocide. In the case of cellulose, the smallest decrease in growth was observed proportional to the concentration used. Thus, the growth was reduced by 37.6 ± 9.6%, 22 ± 3.6% and 14.9 ± 8 with a concentration of 10, 5 and 2%, respectively.

Due to the density of the matrix, no reliable results were obtained by absorbance in the case of the E. coli samples with Xanthan Gum paste. Thus, a growth decrease with high variability between replicates, between 5 and 50%, was found. As an alternative to the growth curves, plate counts of E. coli growth cultures with Gafquat were performed. The results indicated a small effect of this polymer in E. coli viability, showing a reduction of growth of about 20% (Data not showed).

The distribution of bacterial cells inside the matrix was analysed by confocal microscopy. Interestingly, Fig. 2b shows a different distribution of E. coli within each paste. Gafquat paste (Fig. 2b, image C) led to thicker matrices with the majority of the cells near the electrode surface. The PEI matrix (Fig. 2b, image P) led to a similar distribution, with most of the E. coli cells in the bottom of the matrix. Cellulose and Xanthan pastes (Fig. 2b, image C and image X) generated a homogenous distribution of the bacterial cells across the matrix.

Based on the electrochemical response of paste-modified elec-
trodes, E. coli growth results and the homogenous bacteria distri-
bution in the matrix, cellulose was chosen for cell entrapment. Moreover, cellulose has good water solubility, high chemical sta-
tility and biocompatibility [51]. Since nearly no differences were observed between 2% and 5% concentrations, 2% cellulose was selected in the following immobilisation experiments.

3.2. Strategies for bacteria-modified electrode fabrication

After choosing the entrapment matrix, bacteria-modified elec-
trodes were prepared following different strategies. The goal was to fabricate electrodes able to produce a fast measurable signal. Fig. 3a shows a comparison of both strategies studied. In the first approach, bacteria were grown in the presence of ferricyanide before immobilisation. Ferricyanide and glucose were added before using the electrode. The second strategy consisted of polarising the electrode in the presence of ferricyanide and glucose after immo-
ibilisation. In this approach, bacteria-modified electrodes were ready to use immediately after rehydration.
3.2.1. Pre-immobilisation growth in ferricyanide strategy

Fig. 3b compares the current obtained at a fixed potential of +0.3 V (vs Ag) for two *E. coli* cultures growth overnight with and without ferricyanide (first graph). Before chronoamperometric analysis, *E. coli* cells were counted, and no differences were observed between both culture mediums growth with and without ferricyanide.
The current densities obtained between both samples differed by 93.19 ± 16.2 mA cm⁻² after 30 min of measurement (Fig. 3b, first graph). The bacteria culture grown overnight in the presence of ferricyanide yielded higher currents (153.83 ± 0.9 mA cm⁻²) compared to the bacteria sample growth without ferricyanide (34.43 ± 1.2 mA cm⁻²) already in the first 5 min of measurements. Next, the bacteria grown with ferricyanide were centrifuged so they could be isolated from the culture media before attaching them to the working electrode in a cellulose matrix. The bacteria-modified electrode response (grey line) showed a current decrease to no adapted cells values of 29.4 ± 0.6 µA cm⁻², and it was not recovered after 30 min (37.59 ± 0.28 µA cm⁻²). This current was due to the ferrocyanide accumulated in the medium. This ferrocyanide was produced by the bacterial activity overnight since bacteria separation of the medium and subsequent immobilisation caused the current to fall.

3.2.2. Electrode polarization strategy

Fig. 3a shows the second immobilisation strategy. In this case, E. coli was immobilised in the presence of both glucose and ferricyanide, and a +0.3 V (vs Ag) potential was applied to the electrode until the drop was completely dry. Fig. 3b (second and third graphs) shows the current obtained during the process.

The choice of an adequate sensor polarization potential is critical. The percentage of the stored charge recovered during the chronoamperometric analysis depends to a large extent on whether the potential used is high enough to oxidise all the redox compounds present in the system. In our case, an oxidation potential of +0.3 V (vs Ag) was selected considering the cyclic voltammetry data of our E. coli-modified electrodes (Supplementary Material Fig. S5) and selecting a potential high enough to oxidise the ferricyanide present in the medium at a sufficiently high rate.

Bacteria in the presence of glucose reduce ferricyanide, which is again oxidised at the electrode during the polarization producing a current increment. Thus, the current increased gradually from about 12.35 ± 4.2 µA cm⁻² to a maximum of 111.6 ± 8.5 µA cm⁻² after 170 min, when the current started to decrease as a result of the drop drying. In addition, the current density obtained by E. coli-modified electrodes in the absence of glucose was also analysed. Low current densities of 8.7 ± 4.04 µA cm⁻², close to those obtained by blank electrodes (1.14 ± 1.6 µA cm⁻²), confirmed that the current obtained by the bacteria-modified electrodes was due to the reduction of ferricyanide by the bacteria.

After this, bacteria-modified electrodes were tested. In this strategy, electrodes were modified with E. coli together with
glucose and ferricyanide. Only distilled water was necessary for their use. Then, bacteria-modified electrodes and blank electrodes (electrodes modified only with cellulose and electrodes modified with E. coli but without glucose) were rehydrated and a current density at +0.3 V (vs Ag) was applied. Fig. 3b (third graph) shows that bacteria-modified electrodes produced higher current densities over a shorter time period. Thus, values of $63.74 \pm 8 \mu A \ cm^{-2}$ after 38 min of measurements were reached while the current levels obtained in previous experiments did not exceed $31.6 \pm 6 \mu A \ cm^{-2}$ during the same timeframe. In addition, blank electrodes maintained lower current densities of $4.41 \pm 3.5 \mu A \ cm^{-2}$ and $15.3 \pm 3.92 \mu A \ cm^{-2}$ for electrodes with no E. coli and no glucose, respectively.

These results indicated that the strategy of bacteria-modified electrode fabrication allowed us to achieve higher currents over shorter times.

### 3.3. Improving immobilisation conditions

Experiments were carried out to improve the immobilisation conditions in terms of current output by testing different temperatures, applied potentials and bacterial concentrations (Fig. 4). Fig. 4a shows a series of chronoamperometric measurements performed at room temperature and at 37 °C, which is the optimal growth temperature of E. coli. Samples held at 37 °C showed a faster current drop since the higher temperature led to faster drying of the solution on the electrode. Likewise, the maximum current was also obtained faster by the samples at 37 °C, more specifically after 55 min, while in the case of samples held at room temperature the increase was slower, and it took 140 min to reach the maximum current density. Thus, E. coli-modified electrodes showed an increase in performance at 37 °C. These electrodes obtained higher current values ($131.07 \pm 8.03 \mu A \ cm^{-2}$) compared to the blank ($11.71 \pm 2.7 \mu A \ cm^{-2}$) in only 55 min, maintaining the reproducibility of the measurements. Likewise, E. coli-modified electrodes at room temperature showed only a difference with the blank samples of $78.2 \pm 14.1 \mu A \ cm^{-2}$ after 140 min when they reached the maximum current density, and the drop started to dry. Therefore, high metabolic bacterial activity performed at the optimal growth temperature (37 °C) can give good current signals and reduce the test time.

Regardless of temperature, blank samples without E. coli yielded low current values of $14.26 \pm 4.8$ and $11.7 \pm 2.7 \mu A \ cm^{-2}$ for samples at room temperature and 37 °C, respectively after 55 min of measurement, indicating no temperature effect on the measurements.

Different potentials between −0.2 and +0.3 V (vs Ag) were applied during the preparation of the bacteria-modified electrode at 37 °C to study their later effect on current production (Fig. 4b). Potentials higher than +0.3 V (vs Ag) were not analysed since oxidation-reduction peaks of ferricyanide (Supplementary Material Fig. S5) were observable between −0.1 and +0.2 V (vs Ag). Thus, increases in current applying potentials above +0.3 V (vs Ag) could be attributed to other causes derived from the immobilisation of bacteria on the electrode at 37 °C and not to the oxidation on the working electrode of the ferrocyanide produced by bacteria.

No differences were observed between the blank and E. coli-modified electrodes when the applied potential approaches to +0.3 V (vs Ag). In contrast, significant differences in current, about $55-59 \mu A \ cm^{-2}$, were observed relative to the blank, applying −0.1 V and +0.3 V (vs Ag). Potentials matching the oxidation and reduction peaks observed in the voltammograms of samples with ferricyanide and bacteria (Supplementary Material Fig. S5). However, the variability of the blank samples was very high with current density values of $-60.1 \pm 22.8 \mu A \ cm^{-2}$ when a potential of −0.1 V (vs Ag) was applied.

On the other hand, blank samples at +0.3 V (vs Ag) showed less variability ($41.64 \pm 5.6 \mu A \ cm^{-2}$). Consequently, we chose +0.3 V (vs Ag) as the optimum polarization potential.

Fig. 4. Study of bacteria-modified electrode fabrication conditions. a. Temperature: Chronoamperograms at +0.3 V (vs Ag) recorded at room temperature and at 37 °C. b. Applied potential: Current density values obtained after 40 min applying different potentials by chronoamperometry technique. c. Bacteria concentration: Chronoamperograms at +0.3 V (vs Ag) recorded using different bacteria concentrations to cover the working electrode. No E. coli, n = 2, E. coli n = 6.
The bacterial concentration immobilised on the working electrode is another critical factor to consider. The number of bacteria immobilised represents a compromise between two rate-limiting factors, namely carbon source and mediator diffusion into the bacteria-paste matrix, and the measured signal-dependent of the number of bacterial cells per unit volume (cell density). In our case, electrodes were modified with about $10^7$ E. coli cells. Assuming that the average size of an E. coli cell is 2 $\mu$m long and 1 $\mu$m in diameter, and that the area of our working electrode is about 0.05 cm$^2$, $10^7$ E. coli cells could stack into at least two layers of bacteria on the electrode surface. To verify that the bacterial concentration used was not limiting the current signal, the response obtained after reducing bacterial concentration both by half and by an order of magnitude was analysed and shown in Fig. 4c.

In these experiments, we observed that a decrease in the number of cells immobilised on the working electrode led to a decrease in current from a maximum of 131.07 ± 8.03 $\mu$A cm$^{-2}$, obtained by the electrode modified with about $10^7$ cells of E. coli, to 38.85 ± 10 $\mu$A cm$^{-2}$ when the number of bacterial cells was reduced by half. Additionally, when the working electrode was modified with $10^6$ cells of E. coli, which covered approximately half of the electrode, the current density values were very low (11.53 ± 4.6 $\mu$A cm$^{-2}$), becoming the difference with the blank electrode very small or negligible.

### 3.4. Toxicity tests

For shock monitoring, the amperometric response of E. coli at +0.3 V (vs Ag) immobilised on the working electrode was analysed in the presence of formaldehyde (3%). When bacteria are in contact with a toxic compound, the death or decreased activity of the microorganisms results in a drop of the oxidative current measured. Formaldehyde was chosen for toxicity analyses since it is a commonly used disinfectant and biocide, which allows us to observe the inhibition of bacterial metabolic activity [52]. Additionally, electrodes were analysed under glucose saturating conditions to ensure that a change in substrate concentration did not cause a variation in the current signal.

Thus, E. coli-modified electrodes and blank electrodes were rehydrated with water, and a constant potential of +0.3 V (vs Ag) was applied to obtain a current of 120.46 ± 4.3 and 26 ± 5.9 $\mu$A cm$^{-2}$ for E. coli-modified electrodes and blank electrodes, respectively (Fig. 5a). Then, formaldehyde at a final volume concentration of 3% was added. Fig. 5a shows that the current output of bacteria-modified electrodes was affected by the addition of this toxic compound. The current density from the E. coli-modified electrodes dropped from 120.48 ± 4.3 to 52 ± 7.45 $\mu$A cm$^{-2}$ after 20 min of the formaldehyde addition and yielded an inhibition ratio of 69.5 ± 0.4%.

The current decrease could also be attributed to a dilution of the ferrocyanide, previously produced by the bacterial reduction of the ferricyanide and not due to a toxic effect on the E. coli. Nevertheless, the quantity of formaldehyde added was only 10 $\mu$L, which could produce only about 10% of the current reduction. Likewise, the current supplied by the blank electrode was also decreased, but less than 10% (from 26 ± 5.84 to 23.7 ± 8.07 $\mu$A cm$^{-2}$).

### 3.5. Long-term shelf-life of bacteria-modified electrodes

For practical application, the immobilisation procedure must allow for microbial activity maintenance and stability [53] during long-term storage and operation. For this, in order to identify the storage stability of bacteria-modified electrodes, electrodes were modified using the drying process under polarization. After which, the blank and bacteria-modified electrodes were prepared, obtaining a current output of 10.9 ± 2.4 and 124.65 ± 39.2 $\mu$A cm$^{-2}$, respectively. Electrodes were then stored in the fridge at 4 °C until use. Current response at a fixed potential of +0.3 V (vs Ag) of these stored modified electrodes was studied at different intervals over a year by rehydrating with water.

Fig. 5b shows the apparent biological activity decay of the immobilised cell on storage. However, although the results after 2 months indicated a decrease of about 50% of the initial current, no further losses were observed after that for 1-year (decrease of 44.8 ± 3.6%). In addition, despite this signal reduction, the electrodes still showed differences with a blank without bacteria.

Fig. 5c shows a toxicity test using formaldehyde (3%) and two bacteria-modified electrodes and a blank electrode stored during a year. Electrodes were rehydrated with water and the current at +0.3 V (vs Ag) was recorded for 30 min. The output current values during the rehydration of the bacteria-modified electrodes were lower (average of 58 ± 1 $\mu$A cm$^{-2}$) than those obtained during the immobilisation process. Nevertheless, formaldehyde addition caused the current to drop about 27 ± 3.03% in both bacteria-modified electrodes after 10 min. In the same way, the blank electrode did not suffer any changes in the current values. The time for the formaldehyde addition depends firstly on the stability of the measurements and secondly on the sample volume due to the risk of evaporation.

Finally, an inhibition ratio of 60.59 ± 4.36% was calculated after 20 min of formaldehyde addition, which was slightly lower than the value obtained by the recently prepared electrodes (69.5 ± 0.4%). This observation indicates a slight reduction in electrode response to toxicity after storage.

These experimental results showed that our bacteria-modified electrodes were stable and bacteria immobilised on the electrode were viable after long-term storage.

### 3.6. Lateral flow biosensing platform for toxicity monitoring

Fig. 6a–b shows a lateral flow chip able to perform differential measurements. This chip featured two independent electrochemical cells with two working electrodes and two auxiliary electrodes sharing the same pseudo-reference electrode and a simple “Y” channel made from a lateral flow membrane.

Experiments were carried out immobilising bacteria on this chip design. To perform a differential signal, bacteria were immobilised only on the first working electrode while the twin-working electrode was modified only with cellulose as the blank. Next, the paper channel was placed over the chip and a sample containing ferri-cyanide (5 mM) and glucose (20 mM) was added through the inlet. The sample was distributed equally through both channels and reached both working electrodes spontaneously by capillary forces.

A constant potential of +0.3 V (vs Ag) was applied for 60 min. In Fig. 6c, a current density of 120 $\mu$A cm$^{-2}$ can be observed for the working electrode with the bacteria. There were no differences in current density in the absence of bacteria.

Current density values from the fluidic system were in line with those from the static three-electrode arrangement discussed previously. The three-electrode system showed an average maximum current densities of 124.7 ± 39.2 $\mu$A cm$^{-2}$, while the lateral flow device obtained a maximum current density of 120 $\mu$A cm$^{-2}$ before the liquid inside the platform was dried.

The response to the presence of formaldehyde (3%) was also investigated. For this scenario, the formaldehyde was added in the inlet of the bacteria-modified electrode. This exposure to the toxic compound resulted in a significant drop in the current output equivalent to a bacterial inhibition ratio of 99% after about 20 min, which was in agreement with the results shown above in Fig. 5.

To test whether formaldehyde caused this current drop in the ferricyanide signal, ferricyanide and glucose were added through...
the inlet, and the current was monitored. Fig. 6c shows that the current did not increase, demonstrating the toxicity effect of formaldehyde on the immobilised microorganisms.

4. Conclusions

A method to modify the electrodes with bacteria for toxicity biosensing has been developed and demonstrated. The proposed...
method consists of the immobilisation of bacteria on an electrode surface, preferably by the entrapment in a cellulose matrix, while the electrode is polarised. The proposed method is also fast, easy and leads to reproducible results relative to other approaches. In addition, high current levels are achieved in shorter times, pointing at its suitability for systems where bacteria need to adapt to electron transfer quickly. The shelf-life of these electrodes was also analysed, to the best of our knowledge, this is the first report of long-term stability of one year by storage at 4 °C of a BES-based sensor.

The application of these bacteria-modified electrodes for toxicity monitoring was tested on a lateral flow biosensing platform. It was possible to rapidly monitor the inflow of toxic compounds by analysing current patterns following the addition of formaldehyde to the sensor. Besides this, the toxicity sensor presented here is cost-effective and easy to fabricate.

Although further research is needed, the lateral flow microbial sensor developed here represents a platform with more promising potential for sensing applications such as toxicity warning system for rapid spill detection through controls that could be done routinely due to the lower cost of the sensor and its ease of use. Many biofilm-based sensors have been developed in the past [12,52]: Rasmussen and Minteer; [54], which could easily be adapted and perhaps made more attractive using a platform as the one presented here. In addition, these bacteria-modified electrodes can be extended to the field of microbial fuel cells with applications such as electrical power generation or pollutant removal, microbial electrocytosis to produce valuable chemicals, or solely for carrying out fundamental electrochemical studies of microbial activity.

Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

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