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Electro-addressable conductive alginate hydrogel for bacterial trapping and general toxicity determination

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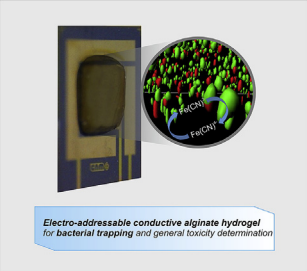
HIGHLIGHTS

- Electro-addressable conductive alginate hydrogels.
- Live bacteria electrotrapping for biosensing.
- Enhanced general toxicity assessment through ferricyanide respirometry.

GRAPHICAL ABSTRACT



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ABSTRACT

In biosensors development, alginate hydrogels are a first choice for enabling stable biomolecules entrapment in biocompatible membranes obtained under soft physiological conditions. Although widely exploited, most alginate membranes are isolating and poorly repetitive, which limit their application in biosensing. Significant steps forward on improving repeatability and conductivity have been performed, but to date there is no single protocol for controlled deposition of live cells in replicable conductive alginate layers. Here, cell electrotrapping in conductive alginate hydrogels is examined in order to overcome these limitations. Conductive alginate-coated electrodes are obtained after potentiostatic electrodeposition of graphite-doped alginate samples (up to 4% graphite). The presence of graphite reduces electrode passivation and improves the electrochemical response of the sensor, although still significantly lower than that recorded with the naked electrode. Bacterial electrotrapping in the conductive matrix is highly efficient (4.4×10^7 cells per gel) and repetitive (CV < 0.5%), and does not compromise bacterial integrity or activity (cell viability = 56%). Biosensing based on ferricyanide respirometry yielded a four times increase in biosensor response with respect to non-conductive alginate membrane, providing toxicity values completely comparable to those reported. Cell electrotrapping in conductive hydrogels represents a step forward towards in high-sensitive cell-based biosensors development with important influence in environmental analysis, food and beverage industry as well as clinical diagnosis.

1. Introduction

Biosensors development requires stable immobilization of biomolecules, including enzymes, antibodies, aptamers and cells,

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among others, on the transducer surface. A good immobilization method may allow to control and maintain the amount of biomolecules without compromising the integrity and activity of the biomolecule or the biosensor performance. Cell immobilization is particular because, apart from previous stability and reproducibility requirements, the immobilization methods should ensure cell viability and metabolic activity [1] The methods for cell immobilization include adsorption [2–4], covalent binding [5], encapsulation [6,7] and entrapment. Although this variety, cell entrapment in polymeric matrices is advantageous for preserving cell integrity and viability, at the time that ensures stability in a physiological environment [8,9].

A number of polymeric matrices have been used for entrapping cells, such as gellan/xanthan matrix [10], SiO_2 sol-gel [11], PVA/alginate [12], alginate [13] and polypyrrole [14,15], among others. From all of them, alginate is one of the preferred for enabling gel formation at very soft experimental conditions, i.e. room temperature, aqueous medium and pH 7, through a cation-mediated cross-linking process [13,16]. Additional advantages are in the low toxicity, low antigenicity and permeability of these membranes, allowing the diffusion of small molecules [16,17]. Main limitations of alginate hydrogels for biosensors are the lack of standardization and repeatability of the gelling protocols, and the electrical insulation of alginate membranes, particularly relevant in the development of electrochemical biosensors. It is worth mentioning that electrochemical transduction, for simplicity, cost-efficiency and miniaturization/integration capacities is still the first choice in biosensors development.

In recent years, significant efforts to improve hydrogels repeatability and conductivity have been performed in separate. Regarding repeatability and homogeneity of the hydrogels, electrodeposition techniques are preferred for providing strict control of the deposition region and the architecture of the hydrogel, i.e. size, shape, thickness and porosity [18–21], properties that can be modulated by adjusting the potentiostatic conditions [21]. Mammalian and bacterial cells have been successfully immobilized by alginate electrodeposition [22–25]. On the other hand, a growing trend to improve hydrogel conductivity is the inclusion of conductive nanomaterials within the polymer matrix [26]. Conductive hydrogels based on graphite [27], graphene [28,29] and carbon nanotubes [30], have attracted a great interest [31]. These conductive matrices combine advantages of hydrogels and conductive composites in a hybrid material, the properties of which can be modulated by shifting the ratio between the alginate and the doping conductive nano-material [26]. These conductive hydrogels have been used to immobilize bacteria and to demonstrate their electroactivity for energy production [32]. However, to the best of our knowledge, both improvements have been never combined in a single electrodepositable and conductive alginate hydrogel for cell trapping.

In this work, bacterial electrotrapping in conductive alginate hydrogel-coated electrodes is examined and applied to biosensing. Graphite microparticles are used as conductive doping material. The analytical performance of the conductive hydrogel-coated electrodes is characterized and hydrogel thickness is measured by profilometry. The biocompatibility and reliability of the electrotrapping protocol is assessed by confocal microscopy and plate counting. Biosensing is performed based on ferricyanide respirometry [33–36] and applied to toxicity assessment.

2. Materials and methods

2.1. Chemicals

Alginic acid, calcium carbonate, calcium chloride, potassium ferricyanide, potassium ferrocyanide, glucose, graphite, 3,5-dichlorophenol, potassium di-hydrogen phosphate and di-potassium hydrogen phosphate 3-hydrate were purchased from Panreac (Spain) and were of analytical grade and all the solutions were prepared with distilled water.

2.2. Alginate electrodeposition and electrochemical analysis

Gold screen printed electrodes (SPE; Dropsens 220BT) integrating a round gold working-electrode (4 mm of diameter), a gold counter-electrode and a Ag pseudo-reference electrode were used in this work. Alginate electrodeposition and electrochemical measurements were performed with a Dropsens μSTAT8000 potentiostat controlled by Dropview 8400 software.

Briefly, precursor solution containing 1% (w/v) sodium alginate, 0.125% (w/v) $CaCO_3$, graphite (between 0 and 4% w/v) and a bacterial concentration up to 1×10^9 colony forming units (cfu) per mL were prepared in distilled water. Graphite was pre-treated and introduced in the solution following the protocol described elsewhere [37].

A volume of 100 μL of the precursor solution was dropped on the SPE surface and electrodeposited at 1.5 V (vs. Ag pseudo-reference) for 90 s. Thick and stable conductive alginate hydrogels were obtained.

2.3. Microorganisms

Escherichia coli ATCC 10536 was grown aerobically in LB broth for 18 h at 37 °C in a shaker bath (110 rpm). Grown cultures were centrifuged at 10100 g for 10 min and re-suspended in distilled water. For cell quantification, optical density of re-suspended bacteria was measured at 550 nm in a SmartspecTM Plus spectrophotometer (BioRad). Agar plate counting was carried out for viable counting.

2.4. Confocal microscopy

Three-dimensional reconstructions of bacteria-containing conductive alginate hydrogels images were used to evaluate the biocompatibility of the electrodeposition protocol (through the number of live and dead bacteria) and the distribution of bacteria in the gel.

Electrotrapped bacteria were stained with the Live/Dead Invitrogen Kit BacLight (Invitrogen) following the protocol detailed by the supplier. The conductive alginate hydrogel was incubated in 100 μL of Live/Dead staining solution for 15 min, washed with water (three times) and imaged by confocal microscopy (Leica TCS SP5) at excitation wavelength of 470 nm. Z-stacks were acquired every 1 μm for a total thickness of 300 μm. Reconstruction of individual stacks was performed with the IMARIS software. In the three-dimensional reconstruction, live bacteria, stained with SYTO9, appeared in green (emission wavelength = 630 nm), while dead bacteria, stained with propidium iodide, emitted in the red region of the visible spectra (emission wavelength = 530 nm).

2.5. Profilometer

Hydrogel thickness was measured with an optical profilometer PLμ2300 from Sensofar controlled with PLμ Confocal Imaging Profiler.

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3. Results and discussion

3.1. Conductive alginate hydrogel

Conductive alginate hydrogels were produced by electrodeposition of precursor solutions doped with 1, 2 and 4% (w/v) graphite and compared with non-conductive hydrogels (Fig. 1a). Above 4% hydrogels presented poor colloidal stability and were discarded. The presence of graphite doping particles in the precursor solution increased the thickness (Fig. 1b), the electrodeposition current and the electrodeposition charge, the latter calculated as the variation of electrodeposition current over time (Fig. 1c). Concretely, the charge associated to the electrodeposition process and the hydrogel thickness increased linearly with the graphite concentration in the precursor solution (Fig. 1d). This result suggested that the presence of conductive graphite particles improve charge transfer during electrodeposition producing thicker, stable and repetitive ($n = 12$, $CV < 0.3\%$) hydrogels.

The electrochemical behaviour of the hydrogels was examined by cyclic voltammetry using equimolar mixtures of ferricyanide and ferrocyanide (10 mM). Cyclic voltammetry measurements of the hydrogels were carried out 5 min after sample inoculation to ensure diffusional equilibrium and compared with naked electrodes. Results are illustrated in Fig. 2. The presence of non-conductive alginate membranes reduced a 73% the anodic current of the electrode. A progressive increase was observed when increasing the graphite concentration in the hydrogel. Best performance was obtained by alginate hydrogels containing 4% graphite, which presented a reduction of only 36% with respect to the naked electrode. Graphite-doping, therefore, enhanced the electrochemical response of alginate-coated electrodes thus overcoming some of the traditional limitations of electrically passivating polymers. Hydrogels containing more than 4% graphite were expected to present better electrochemical behaviours, but those were not possible to obtain with the current electrodeposition protocol.

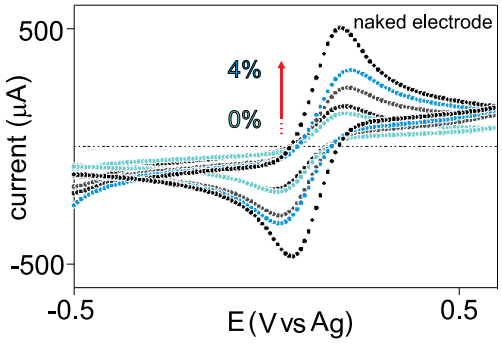

Fig. 2. Cyclic voltammograms corresponding to the measurement of equimolar mixtures ferricyanide/ferrocyanide (10 mM) with alginate hydrogels containing 0%, 1%, 2% and 4% graphite concentration. The cyclic voltammetry corresponding to the naked electrode (black dots) is included by comparison. Measurements are performed at a scan rate of 50 mV·s-1.

3.2. Bacterial electrotrapping in conductive alginate hydrogel

Bacterial suspensions of *Escherichia coli* (*E. coli*) from 10^6 to 10^9 cfu mL^{-1} were mixed with the precursor solution and electrodeposited in conductive (4% graphite) and non-conductive alginate membranes under optimal conditions. Hydrogel formation was

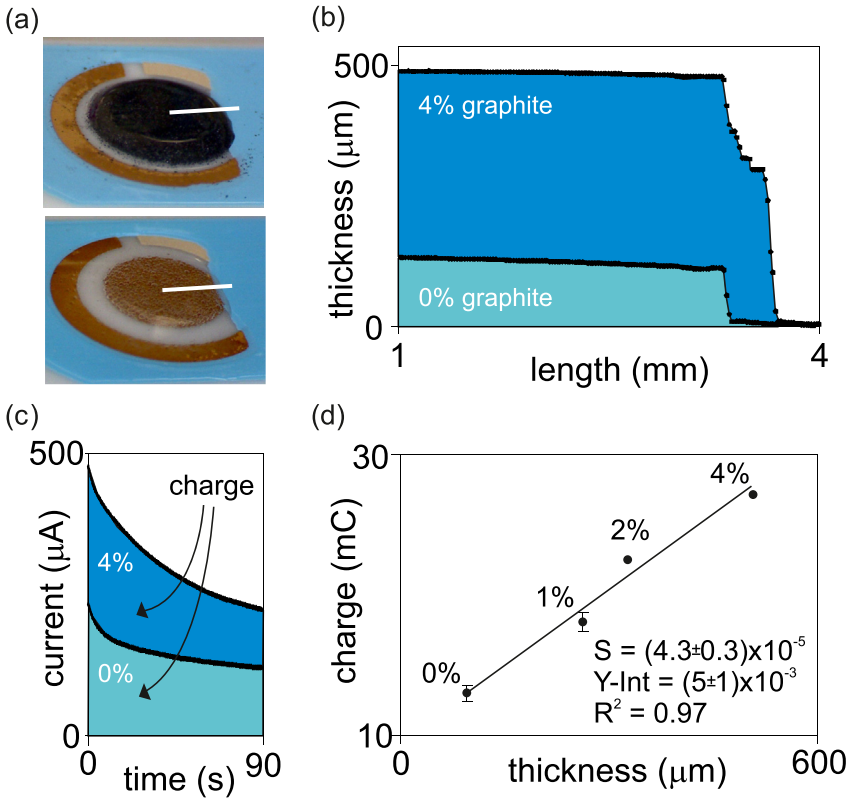

Fig. 1. a) Image of conductive (dark) and non-conductive hydrogel (transparent) electrodeposited on working electrode. Inset, a white line indicating the region analysed by profilometry. b) Profiles of the conductive (dark blue) and non-conductive (clear blue) hydrogels obtained by profilometry. c) Electrodeposition current curves for the two hydrogels, with the associated electrodeposition charge. d) Representation of the variation of the charge (mC) and thickness of hydrogels with 0%, 1%, 2% and 4% graphite concentrations. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

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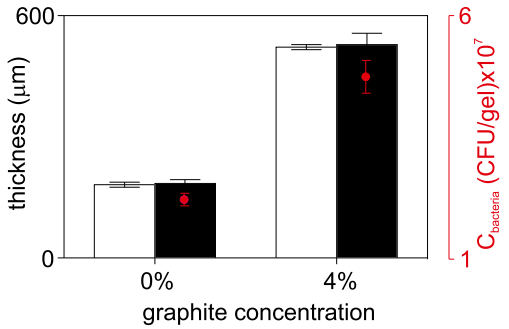

Fig. 3. Variation of the thickness of non-conductive (0%) and conductive (4%) alginate hydrogels in absence (white) or presence (black) of *E. coli* bacteria. Red dots in the plot correspond to bacterial concentration entrapped in the hydrogels determined by agar plating. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

achieved in bacterial suspensions containing up to (1.52 ± 0.02) x10^8 cfu mL^{-1}. In higher concentrations, bacteria isolation impeded electrodeposition and were discarded for future experiments. Unless otherwise stated, the maximum bacterial concentration enabling electrodeposition was used in subsequent experiment for providing maximum sensitivity in the development of general toxicity biosensors based on microbial metabolism.

To evaluate the effect of cells on electrodeposition of conductive and non-conductive hydrogels, cell viability and the thickness of bacterial hydrogels was determined and compared with that corresponding to hydrogels without bacteria. Results are presented in Fig. 3. As shown, the presence of bacteria had an almost negligible contribution to hydrogel thickness in any case. At the same time, plate counting of hydrogels dissolved with 0.1 M phosphate buffer demonstrate high cell viability in the hydrogels, i.e. 1.4×10^7 cfu gel^{-1} for non-conductive and $4{,}4 \times 10^7$ cfu gel^{-1} for conductive hydrogels ($n = 10$, CV < 0.1%), which demonstrated that electrodeposition did not compromise cell viability. The difference in the number of living bacteria in each case was consistent with the higher thickness of conductive alginate hydrogels, which retained more bacteria. In fact, it was a linear correlation between graphite concentration, hydrogel thickness and bacterial concentration (supplementary information) suggesting that electrotrapped cell quantity was proportional to hydrogel thickness (and thus to graphite content). Additionally, electrodeposited cell-containing hydrogels were stained with Live/Dead fluorophores and imaged by confocal laser microscopy. Due to the high opacity of graphite-doped hydrogels, only transparent non-conductive hydrogels were used in this case. The 3D reconstruction of the confocal images and three selected planes are illustrated in Fig. 4. The reconstruction showed a homogeneous vertical distribution of living cells (green) and dead cells (red) along the gel, which coincided with the analysis of individual stacks at different depths (0, 125 and 250 μm). The elongated morphology of the cells in the upper part of the image appeared due to depth of field limitations of the microscope. Quantitative image analysis revealed that $55 \pm 9\%$ of total electrotrapped bacteria were alive, which was in agreement with the percentage observed in *E. coli* overnight cultures ($66 \pm 15\%$).

Considering these results, the electrodeposition conditions may be considered harmless for cell entrapment and validate electrotrapping in the generation of cell-based biosensors with viable bacteria.

3.3. Cell-based conductive alginate biosensor

Electrodeposited conductive alginate hydrogels containing *E. coli* were employed in the production of biosensors for general toxicity assessment. Bacterial metabolism of entrapped bacteria is used to determine the toxicity of samples through ferricyanide respirometry [33–36]. Briefly, ferricyanide acts as a final electron acceptor for the bacterial metabolism. That is, living and metabolically active bacteria are able to reduce ferricyanide to ferrocyanide in their electron transport chains, whereas dead or metabolically inactive cells are unable to reduce this redox compound. Therefore, ferrocyanide production over time is directly proportional to bacterial metabolic activity and thus, to the number of live bacteria.

For toxicity assessment, a 100 μL 1 mM ferricyanide supplemented with 0.2% glucose was inoculated to the hydrogel-containing electrode. Chronoamperometry recordings were then acquired at 0.4 V (vs Ag pseudoreference), corresponding to the ferrocyanide oxidation potential, for a maximum of 90 min, to evaluate the accumulation of metabolically-produced ferrocyanide. Current values of conductive (4% graphite) and non-conductive alginate hydrogels were acquired at 0, 30, 60 and 90 min of assay (Fig. 5a). Both conductive and non-conductive hydrogels showed a progressive increase in current magnitude over time resulting from the metabolic production of ferrocyanide due to bacterial activity. The main difference between them was in the current magnitude.

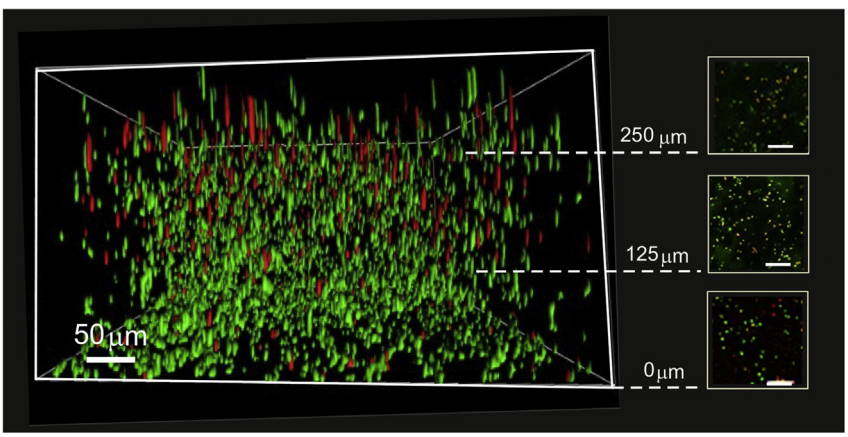

Fig. 4. Confocal 3-D image reconstruction image corresponding to a non-conductive alginate hydrogel containing bacteria. Due to the staining, live cells appear in green and dead cells in red. Three individual stacks corresponding to a height of 0, 125 and 250 μm versus the electrode surface are also included to demonstrate the homogeneous distribution of cells in the gel. Scale bar in the stacks corresponds to 25 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

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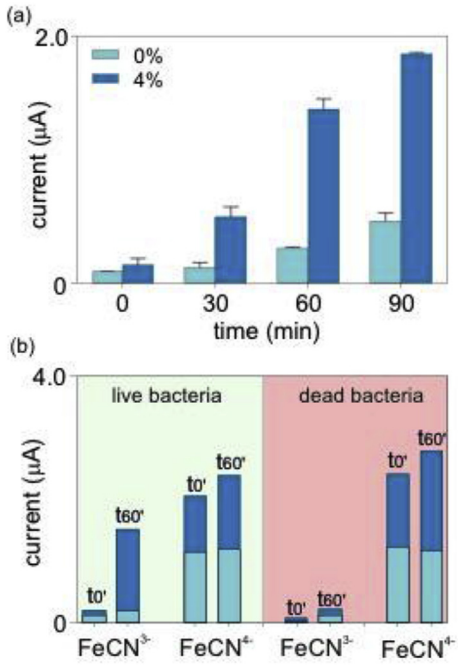

Fig. 5. a) Variation of the current values at 0.4 V with the accumulation time for conductive (dark blue) and non-conductive (clear blue) alginate hydrogels containing 1.5×10^8 cfu/mL bacteria. The accumulation time is defined as the time that entrapped bacteria are in contact with 1 mM ferricyanide (supplemented with 0.2% glucose) to metabolically produce ferrocyanide, which is finally measured at 0.4 V b) Current values of live and dead bacteria at the beginning of the experiment and after 60 min of incubation with ferricyanide or ferrocyanide solutions supplemented with 0.2% glucose. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Conductive hydrogels showed larger current values due to (i) the presence of graphite particles, which improve charge transfer between redox molecules and the electrode, and (ii) the number of bacteria, higher in the thicker conductive membrane as discussed before. It is worth mentioning that the hydrogels lost their consistency after 90 min of accumulation and the optimal time assays was set at 60 min.

To ensure the metabolic origin of ferrocyanide, the response of conductive and non-conductive hydrogels containing live and dead bacteria (by incubation with 4% glutaraldehyde) was compared. In both cases, measurements were performed in solutions containing either 1 mM ferricyanide or 1 mM ferrocyanide, the latter used as control. Results after 0 and 60 min of incubation are presented in Fig. 5b. Important differences between hydrogels containing live and dead bacteria were observed when comparing the response to ferricyanide. That is, hydrogels with live bacteria always presented higher current values associated to the production of ferrocyanide, which may confirm that this production was metabolic. However, dead bacteria hydrogels still presented a current that increased over time, probably due to the presence of some live bacteria in the gel. Control measurements with ferrocyanide provided similar results for live and dead bacteria hydrogels, which remained almost stable over time.

These results, therefore, confirmed the metabolic origin of the ferrocyanide in the sample as a consequence of live bacteria activity, as well as the potentiality of these simple cell-based biosensors for toxicity assessment based on ferricyanide respirometry.

3.4. General toxicity assessment

The cell-based conductive alginate hydrogel biosensor was finally applied to general toxicity assessment using the well-known toxic agent 3,5-dichlorophenol (DCP). Toxicity assessment involved in situ generation of the conductive alginate hydrogel membrane with bacteria and 15 min of incubation with the toxic solution. DCP concentrations from 0.1 to 100 ppm were evaluated. Then, initial solution was replaced by the sensing solution containing 1 mM ferricyanide and 0.2% glucose. After 60 min of incubation, the accumulated ferrocyanide was measured by chronoamperometry at 0.4 V. DCP toxicity results are presented in Fig. 6 as inhibition (in percentage) by comparison with the response of the biosensor to samples without toxic. As shown, there was a clear concentration-dependence in the response of the biosensor. At low toxic concentration the inhibition of the metabolic production of ferrocyanide was low, but it increased with the toxic concentration up to 10 ppm DCP where it reached a 100% of inhibition. The half-maximal effective concentration (EC_{50}) magnitude for DCP with the current biosensor was of 3.5 ppm, which was in agreement with the already reported values [38,39].

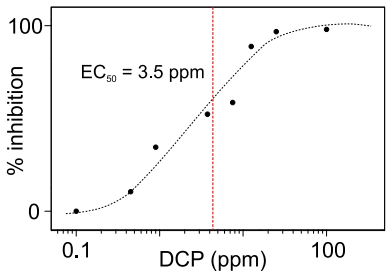

Fig. 6. Dose-response curves obtained by plotting the inhibition percentage against DCP concentration. Inset, the EC_{50} magnitude, corresponding to the half-maximal effective concentration of a toxic, is also included.

Therefore, the conductive alginate hydrogel presented in this work was suitable for stably and reproducible immobilization of viable bacteria which may be used for fast and simple determination of general toxicity through their metabolic activity and using ferricyanide as final electron acceptor.

4. Conclusions

Cell electrotrapping in conductive alginate hydrogels, containing up to 4% graphite microparticles, is here examined and applied to the development of general toxicity biosensors. Electrotrapping of bacteria (up to 1.5×10^8 cfu mL^{-1}) in the conductive hydrogels provides repeatability and simplicity of the entrapment process, and ensures cell stability in a biocompatible matrix that not compromises its integrity and activity. Hydrogels conductivity, moreover, improves charge transfer during electrodeposition, producing thicker hydrogels with more bacteria than non-conducting counterparts, and biosensing. Regarding the latter, the determination of metabolic ferricyanide reduction to ferrocyanide, used in the development of the general toxicity biosensor, is enhanced when using conductive hydrogels due to the increased bacterial number and the improved charge transfer between electrode and redox molecule. When applied to general toxicity, the biosensor presents a dose-response curve with a EC_{50} magnitude of 3.5 ppm for DCP, completely comparable to reported values. The presented entrapment approach represents a step forward towards reliable, controlled and repetitive cell immobilization and the production of simple and fast-response biosensors with enhanced sensitivity.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.aca.2018.06.062.

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