Immobilisation of Neuro-2a cells on electrodes and electrochemical detection of MTT formazan crystals to assess their viability

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Abstract

Marine toxins are potent toxic compounds that may reach humans and poison them. Therefore, their detection in seafood is crucial to prevent intoxication cases. Colorimetric cell-based assays (CBAs) have been developed to analyse marine neurotoxins, such as ciguatoxins (CTXs) and tetrodotoxins (TTXs), and are based on the toxicological effect of these toxins on the cells. Cell viability can be quantified by measuring the mitochondrial activity with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). With the purpose of moving forward in the development of cell-based biosensors (CBBs) for neurotoxins, Neuro-2a cells were immobilised on electrodes of different materials (carbon, carbon/polyaniline, carbon/poly-L-lysine, carbon/poly(3,4-ethylenedioxythiophene) and gold) and their presence and viability were assessed by the detection of MTT formazan crystals with cyclic voltammetry. Best results in terms of oxidation potential and current intensity were achieved with carbon and carbon/polyaniline electrodes. Light microscopy also proved the presence of immobilised and living cells on electrodes. Cell density, incubation time and MTT concentration were optimised. Appropriate electrochemical responses were obtained incubating 100,000 cells/electrode for 2 h and using 0.86 mg/mL MTT. The system was able to detect toxicity when exposed to CTX1B and TTX standard solutions as well as *Seriola dumerili* and *Lagocephalus sceleratus* fish extracts containing these toxins.

Keywords

Neuro-2a cell, electrode, tetrazolium salt, ciguatoxin, tetrodotoxin, cell-based biosensor.

1. Introduction

Marine toxins are natural compounds produced mainly by microalgae and in some cases by bacteria or other organisms. They enter the food webs, potentially reaching humans and causing poisoning, which can be mild but also severe and even lead to death. Although some marine toxins have been extensively described, studied and legislated, some others, emerging, still require further attention. The term "emerging" means recently discovered marine toxins, but also known marine toxins not fully characterised yet or marine toxins that appear in geographical areas or matrixes where they were previously absent [1]. In Europe, ciguatoxins (CTXs) and tetrodotoxins (TTXs) are considered emerging marine toxins. Ciguatera poisoning (CP) cases, caused by the consumption of fish contaminated with CTXs and usually associated to tropical and subtropical areas, have been recorded in the Canary Islands (Spain) [2-3] and the Selvagens Islands (Portugal) [4] in the last years. CTXs have also been detected in fish from the Canary Islands [5-6] and the Selvagens Islands [7-9]. Since 2004, *Gambierdiscus* and *Fukuyoa*, the microalgae responsible to produce CTXs, have been found in the Canary Islands [10-14] and Madeira [15-17], but also in the Mediterranean Balearic Islands [18-21], Greece [22-23] and Cyprus [23], which proves their geographical expansion. TTXs are usually found in some species of puffer fish and have been associated to poisoning cases in the Mediterranean [24]. Additionally, TTXs have been recently found in shellfish from several European countries, including the United Kingdom [25], Greece [26], the Netherlands [27], Portugal [28], Spain [29-30], Italy [31-32] and France [33-34], although usually at very low concentrations.

Since the emergence of these toxins in Europe is a serious concern for the public health, the evaluation and characterisation of their risk are a priority for the European Food Safety Authority (EFSA), which also encourages the search for occurrence data and the development of reliable and efficient analytical methods [35-36]. The toxicological mouse bioassay (MBA) was one of the first methods used to detect marine toxins and has been for years an important tool to manage seafood safety and prevent risk situations. However, due to its insufficient detection capability and ethical concerns, it is only used to analyse samples with unexplained toxicity. *In vitro* cell-based assays (CBAs) are good alternatives to animal assays, since they are based on the toxicological effect of toxins on cells and provide an estimation of the composite toxicity of a sample. Both CTXs and TTXs block the cell membrane voltage-gated sodium channels (VGSCs), but they have opposite mechanisms of action. CTXs block them in an open position, whereas TTXs block them in a closed position. Both neurotoxins can be detected with the CBA by using agonists or antagonists to promote or counteract their effects. Therefore, in the presence of ouabain and veratridine, CTXs cause cell mortality, whereas TTXs counteract their effect, increasing cell viability. In a conventional colorimetric CBA, cell viability is usually quantified by measuring the mitochondrial activity with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [37].

Cell-based biosensors (CBBs) are based on CBAs, and therefore provide toxicological information from a sample, and have added advantages such as higher sensitivity, shorter analysis times, miniaturisation, portability and automatisation. In the development of a biosensor, the immobilisation of the biorecognition element on the transducer, i.e., cells on electrodes in an electrochemical CBB, is key. Cells must be adhered to non-toxic biocompatible electrodes that allow them to remain viable, growth and respond to target analytes. Additionally, the presence of a cell layer must not inhibit the electron transfer and maintain the electrode conductivity.

Only a few CBBs have been developed for the detection of TTX [38-40] and only one has been applied to CTX [41]. These systems are based on impedance or extracellular potential measurements on multi-electrode arrays (MEAs). However, instrumentation is expensive and not always commercially available. The development of CBBs for the detection of these marine toxins using cheaper, simpler and still highly performant systems is desired. Although working with living material involves a certain degree of variability making standardisation and

harmonisation difficult, the advantages in terms of sensitivity and composite toxicological responses are undeniable.

The objectives of this work were the immobilisation of living Neuro-2a cells on electrodes of different materials, the electrochemical detection of their viability using MTT and the optimisation of the assay conditions, as a previous step to the development of electrochemical CBBs. The effect of CTX and TTX standard solutions on the immobilised cells was evaluated. Finally, the system was applied to the analysis of fish extracts containing CTX (*Seriola dumerili*) and TTX (*Lagocephalus sceleratus*), as a proof of concept. The strategy has been shown to be successful and thus paves the way towards the development of CBBs for the detection of marine neurotoxins.

2. Materials and methods

2.1. Reagents and materials

Neuroblastoma murine (Neuro-2a) cells were purchased from ATCC LGC standards (Manassas, VA, USA). Foetal bovine serum (FBS), ouabain, veratridine, phosphate buffered saline (PBS), penicillin-streptomycin, RPMI-1640 medium, sodium pyruvate, thiazolyl blue tetrazolium bromide (MTT), potassium hexacyanoferrate (III) and potassium hexacyanoferrate (II) were purchased from Merck KGaA (Gernsheim, Germany).

CTX1B standard was obtained from Prof. Richard J. Lewis (The Queensland University, St Lucia, Australia) and the standard solution was prepared at 2 µg/mL in methanol. TTX standard was purchased from Abcam plc (Cambridge, UK) and the standard solution was prepared at 1 mg/mL in 1% acetic acid. Okadaic acid potassium salt (OA) from *Prorocentrum concavum* was obtained from Merck KGaA and the standard solution was prepared at 100 µg/mL in methanol. Extractions of the flesh of an amberjack *S. dumerili* from the Canary Islands (Spain, Northeastern Atlantic Ocean) and the gonads of a silver-cheeked toadfish *L. sceleratus* from Denia (Spain, Western Mediterranean Sea) were previously described [42, 43].

Screen-printed carbon electrodes (110), polyaniline-modified screen-printed carbon electrodes (110PANI), poly-L-lysine-modified screen-printed carbon electrodes (110PLYS), optically transparent poly(3,4-ethylenedioxythiophene) (PEDOT) screen-printed electrodes (P110), screen-printed gold electrodes (220AT), and a cable connector (CAC) were purchased from Metrohm DropSens (Oviedo, Spain). The electrodes consist of 4 mm working electrodes, a carbon/gold counter electrode and a silver reference electrode. Electrochemical measurements were performed using an Autolab PGSTAT128N potentiostat and data were collected and evaluated with NOVA v2.1 software (Metrohm Autolab, Utrecht, The Netherlands).

2.2. Cell immobilisation on electrodes and MTT assay

Neuro-2a cells were maintained in RPMI-1640 medium (which also contained 10% FBS, 1% penicillin-streptomycin and 1% sodium pyruvate) in an incubator (BINDER Gmbh, Tuttlingen, Germany) at 37 °C in 5% CO_2 humid atmosphere. For the experiments, Neuro-2a cells were trypsinised and suspended in the same medium, but with 5% FBS instead of 10% FBS. Neuro-2a cells were seeded at 100,000 cells/electrode (also at 200,000, 50,000 and 25,000 cells/electrode in the electrochemical optimisation study) on untreated electrodes of different materials (i.e., C, C/PANI, C/PLYS, PEDOT and AU) for 2 h (also 4, 6 and 24 h in the electrochemical optimisation study) at 37 °C in 5% CO_2 humid atmosphere. After washing the electrodes twice with PBS to remove unbound cells, 60 µL of MTT at 0.86 mg/mL in RPMI medium (also ½ serially diluted and at 5 and 2.5 mg/mL in the electrochemical optimisation study) were placed on the screen-printed electrodes and incubated for 25 min at 37 °C in 5% CO_2 humid atmosphere. Electrodes were washed twice with PBS to remove excess of MTT. Some electrodes with immobilised cells were exposed to OA at 1 µg/mL (overnight at 37 °C in 5% CO_2 humid atmosphere) before MTT incubation.

2.3. Light optical microscopy

Cell immobilisation on electrodes was first characterised using light optical microscopy (Leica DMLB, Leica Microsystems, L'Hospitalet de Llobregat, Spain) with lateral light (cold light source PL 2000 from Optical Technology, Ryfag, Grenchen, Switzerland). Pictures were taken with DPController software 2.1.1.183 (Olympus, L'Hospitalet de Llobregat, Spain).

2.4. MTT electrochemical detection

The MTT electrochemical detection was used in the characterisation of the cell immobilisation, in the optimisation of the assay conditions and in the detection of toxins. Two electrochemical techniques were used: cyclic voltammetry (CV) and chronoamperometry (CA). CVs were recorded between -0.8 V and +1.0 V (vs. Ag or Au) at 50 mV/s. CA measurements were performed by applying a constant potential of +0.3 V (vs. Ag) for 5 s. Measurements were performed in quadruplicate.

2.5. Ferricyanide/ferrocyanide electrochemical detection

Cell immobilisation on C electrodes was also characterised electrochemically using the ferricyanide/ferrocyanide redox couple. To this purpose, after cell immobilisation on screen-printed carbon electrodes and washing with PBS to remove unbound cells, 50 µL of 1 mM $[Fe(CN)_6]^{3-}/^{4-}$ (1:1 potassium hexacyanoferrate (III):potassium hexacyanoferrate (II)) in PBS were placed on the screen-printed carbon electrodes and CVs were recorded between -0.4 V and +0.7 V (vs. Ag) at 50 mV/s. Bare electrodes and electrodes with cells exposed to OA at 1 µg/mL (incubated overnight at 37 °C in 5% CO_2 humid atmosphere) were also evaluated.

2.6. Toxin detection – proof of concept

In the experiments with toxin standards and natural samples, after cell immobilisation on screen-printed carbon electrodes, some cells were pre-treated with ouabain and veratridine (at 0.1 and 0.05 mM, respectively, for the experiments with CTX, and at 0.35 and 0.15 mM, respectively, for the experiments with TTX). Then, CTX1B standard solution, TTX standard solution, *S. dumerili* flesh extract and *L. sceleratus* gonad extract were evaporated, reconstituted in cell culture medium, and 10 µL were added to the electrodes with and without ouabain/veratridine pre-treatment, and incubated overnight at 37 °C in 5% CO_2 humid atmosphere. Cells were exposed to CTX1B standard solution at 2500 pg/mL, TTX standard solution at 2500 ng/mL, *S. dumerili* flesh extract at 2500 mg tissue equiv./mL of and *L. sceleratus* gonad extract at 200 mg tissue equiv./mL. Cell viability was then evaluated with MTT and CA. Electrodes not pre-treated with ouabain and veratridine, neither exposed to standard toxin solution or fish extract, were incubated with MTT and used as a positive control. Measurements were performed in quadruplicate.

3. Results and Discussion

3.1. Cell immobilisation on different electrode materials

In a conventional colorimetric CBA, the detection of insoluble MTT formazan, formed by the reduction of MTT by the mitochondrial dehydrogenases of living cells, requires lysing the cells and solubilising the MTT formazan crystals for the absorbance measurement. In this work, where microtiter plates have been replaced with electrodes with the final purpose to develop a CBB, the electrochemical detection of the MTT formazan crystals (not solubilised), precipitated and confined on the electrode has been pursued, trying to avoid the cell lysis step.

Electrode properties, including material, geometry, fabrication technique and surface roughness among others, may significantly affect the cell adhesion, proliferation and viability. Screen-printing provides a rough surface which may favour cell adhesion. Therefore, in this work,

screen-printed electrodes of different materials were used, and cells were immobilised by adsorption, which allows direct contact with the transducing surface.

3.1.1. Characterisation with optical light microscopy

The immobilisation of cells on the electrodes was first evaluated with optical light microscopy. The transparency of the PEDOT electrodes allowed the direct visualisation of the cell immobilisation (Fig. 1), which was also observed after incubation of the cells with MTT (Fig. 2A2). On the contrary, C and Au electrodes required the incubation of immobilised cells with MTT for observation of the staining with formazan crystals (Fig. 2B2 and 2C2). All optical light microscopy images revealed good cell spreading. In all electrodes, exposure of immobilised cells to OA and incubation with MTT showed electrodes surfaces (Fig. 2A3, 2B3 and 2C3) very similar to those of bare electrodes (Fig. 2A1, 2B1 and 2C1), suggesting that OA has killed the cells and they have fallen off. C/PANI and C/PLYS electrodes showed results very similar to those observed with C electrodes, as the surfaces are optically very similar (Fig. S1). According to these results, all electrode materials are biocompatible and allow cell immobilisation.

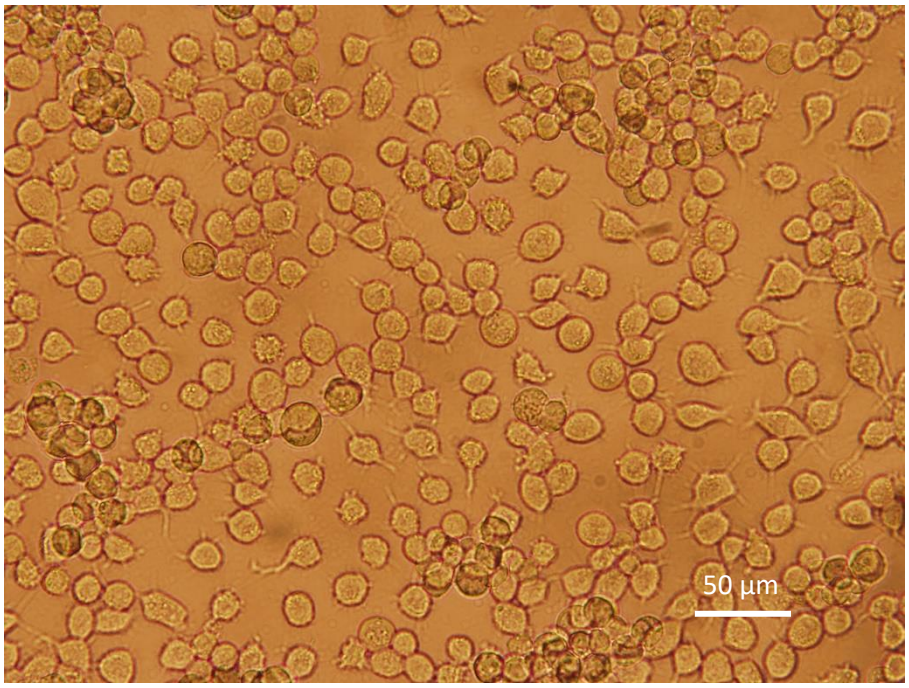
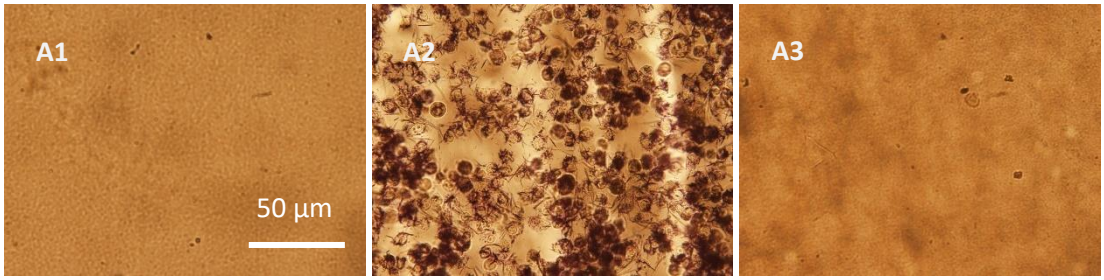


Figure 1. Optical light microscopy image of Neuro-2a cells immobilised on PEDOT electrodes (not incubated with MTT).



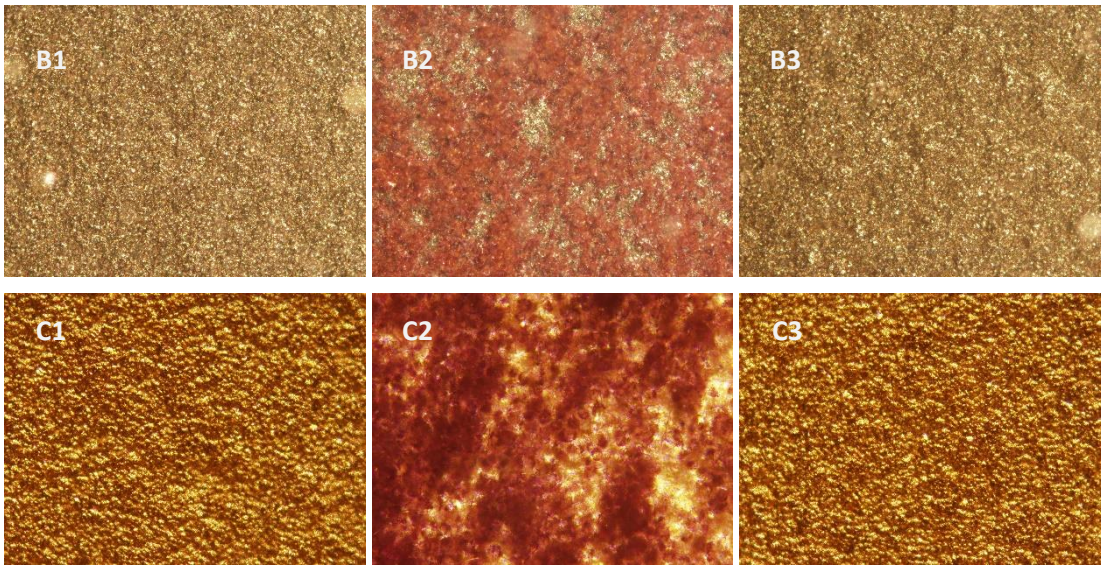
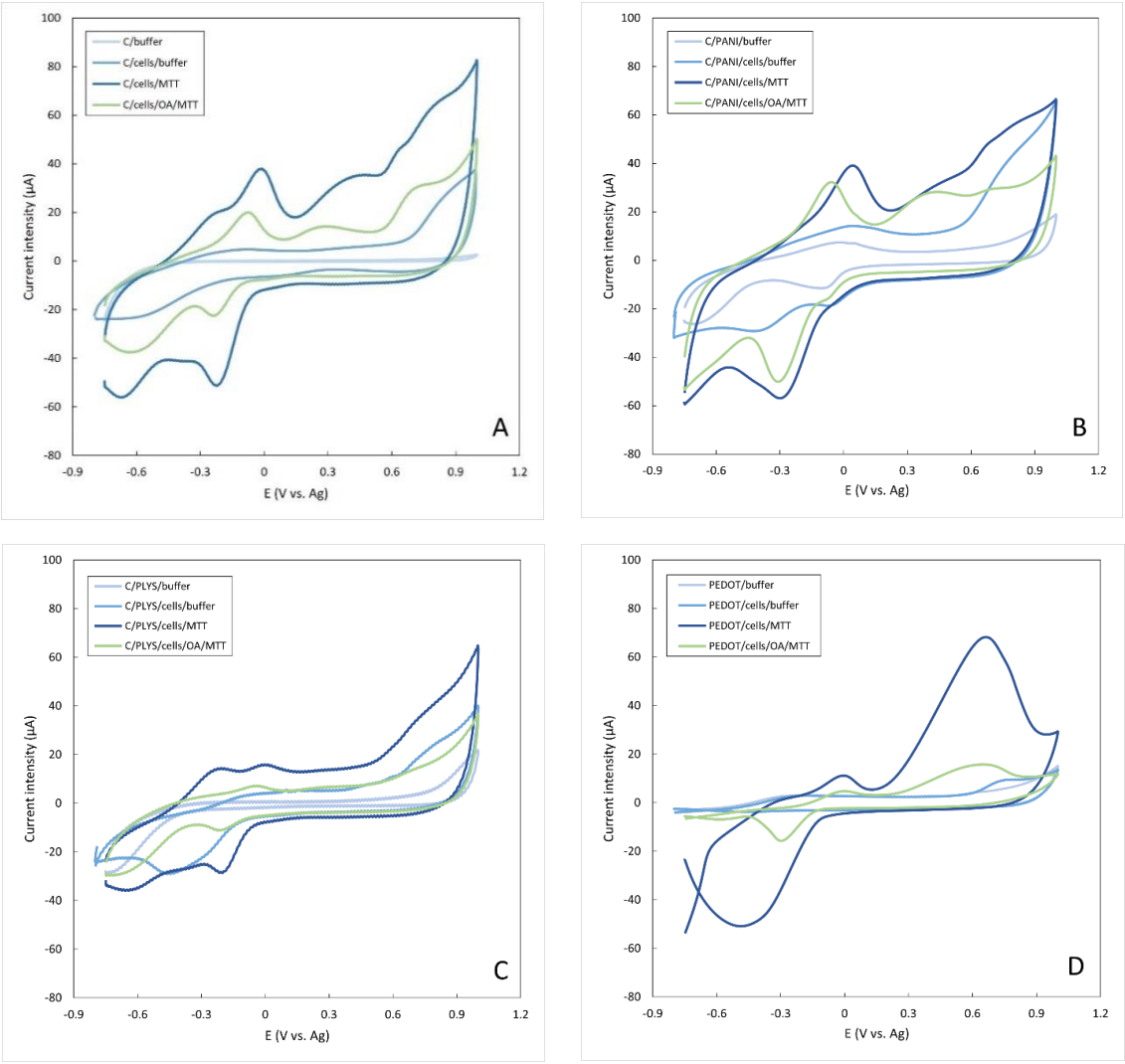


Figure 2. Optical light microscopy images of bare PEDOT (A), C (B) and Au (C) electrodes (1), of Neuro-2a cells immobilised on the electrodes and incubated with MTT (2), and of Neuro-2a cells immobilised on the electrodes, exposed to OA and incubated with MTT (3). Error bar is the same for all pictures.

3.1.2. Characterisation with electrochemistry

Apart from biocompatibility, the electrode material has to be able to transfer electrons between the MTT formazan crystals and the electrode, and the layer of immobilised cells must not inhibit this electron transfer. In the first experiments, cell immobilisation and detection of insoluble MTT formazan crystals were tested on electrodes of different materials after 24-h cell incubation (to not be limited by the incubation time) and 25-min MTT incubation. Regarding C electrodes (Fig. 3A), the CV performed after incubation of cells (C/cells/buffer) showed a higher capacitive current than the CV of bare electrodes (C/buffer). This observation was an indication of the cell immobilisation. The presence of immobilised cells affects the local ionic environment at the electrode/solution interface, leading to an increase of the electrode capacitance. The cell incubation medium, which contains 5% FBS, is also contributing to the increase of the capacitive current (results not shown) but only slightly compared to the cells. Smooth oxidation and reduction peaks were observed at around +0.8 V and -0.55 V (vs. Ag). The oxidation peak has been previously attributed to the oxidation of exogeneous electroactive species commonly existing in cells, such as some enzymes or guanine, which may be able to cross the cell membrane [44-46]. In those works, potential windows were narrower and therefore the reduction peak was not appreciated. In any case, it is necessary to keep in mind that guanine has a characteristic irreversible voltametric response and therefore the reduction peak should be attributed to something else. After MTT incubation (C/cells/MTT), the forward scan showed the oxidation of MTT formazan crystals, with several oxidation peaks, the ones at -0.01 V and +0.40 V (vs. Ag) being the better defined. In the reverse scan, reduction peaks at -0.22 V and -0.66 V (vs. Ag) were observed. This result suggests that the cells have been immobilised and they retain and react with MTT, an indication that they are alive. It also suggests that the cell membranes that are adhered to the electrode surface do not inhibit the electron transfer between the MTT formazan crystals and the electrode. However, the exocytosis of MTT formazan crystals, and therefore direct contact with the electrode, cannot be ruled out [47]. On the other hand, cell-coated electrodes were exposed to OA (1 µg/mL), a toxin that is known to induce membrane damage and therefore cell mortality [48], and afterwards incubated with MTT. In this case, the current intensity of the oxidation and reduction peaks substantially decreased (C/cells/MTT/OA). Since the OA dose was very high, we expected all cells to be dead and no response from MTT formazan crystals at all. However, peaks were still appreciated, indicating

that OA had only partially killed the cells and/or that the system suffers from a certain degree of non-specific adsorption of the MTT on the electrode. In fact, previous works have shown that at high drug concentrations, cytotoxicity values were slightly lower when using cells immobilised on electrodes than when using the conventional colorimetric assay on microtiter plates [46]. Nevertheless, the previous optical light microscopy images showed no cells on electrodes exposed to OA (Fig. 2B3), suggesting that the responses observed with electrochemistry are due to the non-specific adsorption of the MTT.



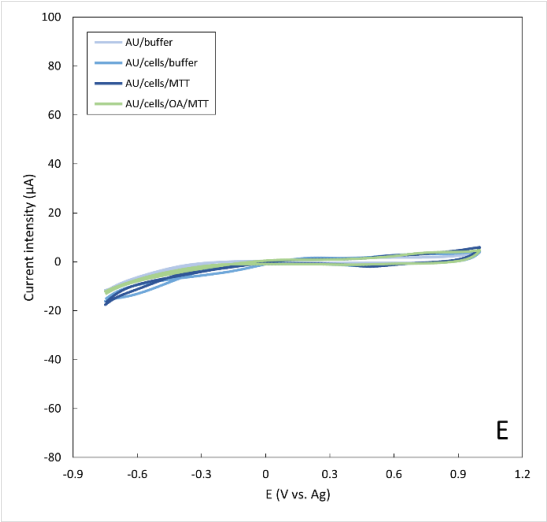


Figure 3. Cyclic voltammetry of bare electrodes, electrodes with immobilised Neuro-2a cells, electrodes with Neuro-2a cells immobilised and incubated with MTT, and electrodes with Neuro-2a cells immobilised, exposed to OA and incubated with MTT (A: C; B: C/PANI; C: C/PLYS; D: PEDOT; and E: AU).

Polyaniline is a conductive polymer, which has been used to immobilise biomolecules such as enzymes [49]. Because of its positive charge and biocompatibility, it has also been used for cell immobilisation, although only adhesion and proliferation (no viability) have been monitored [50]. In our experiments, bare C/PANI electrodes (C/PANI/buffer) showed smooth oxidation and reduction peaks and higher capacitive currents compared to C electrodes, likely due to the polyaniline polymer (Fig. 3B). Nevertheless, the presence of immobilised cells (C/PANI/cells/buffer) also increased the capacitive current and showed some other oxidation and reduction peaks, similar to those observed with C electrodes (C/cells/buffer). After MTT incubation, an oxidation peak at +0.04 V (vs. Ag) and a reduction peak at -0.3 V (vs. Ag) appeared (C/PANI/cells/MTT). The current intensity values of these peaks were quite similar to their homologous peaks in C electrodes. The intensity of the peaks also decreased when cells were exposed to OA (C/PANI/cells/OA/MTT), but less than when using C electrodes, suggesting that cells may be less accessible and are more difficult to be killed and/or MTT is more non-specifically adsorbed. Again, the optical light microscopy image (Fig. S1A3) seems to indicate that the problem is the MTT non-specific adsorption.

Like polyaniline, poly-L-lysine has positively charged sites, which enhance the electrostatic interactions with the negatively charged cell wall. Commercial poly-L-lysine has been used to coat the surface of dishes or microtiter plates for cell culture. Therefore, C/PLYS electrodes were expected to show high cell adhesion. Some works have reported the immobilisation of cells on poly-L-lysine-modified glassy carbon electrodes and the detection of their presence with electrochemical impedance spectroscopy (EIS), but cell viability has not been directly measured electrochemically [44]. In our experiments, although after MTT incubation (C/PLYS/cells/MTT) oxidation and reduction peaks were observed at similar potentials, their current intensity values were much lower than with C or C/PANI electrodes (Fig. 3C). The reason behind could be a lower number of immobilised cells, despite the use of poly-L-lysine, and/or lower electron transfer from the MTT formazan crystals. The staining observed after incubation of the cells with MTT with optical light microscopy (Fig. S1B2) could not be quantified and, therefore, it was not possible to elucidate the reason behind this fact. The difference between living and dead cells (C/PLYS/cells/OA/MTT) was more difficult to appreciate. Although it was reported that poly-L-lysine films have an open and swollen structure that facilitates electron transfer [51], passivation of the electrode has also been observed in some works [44]. Therefore, C/PLYS electrodes were put aside.

PEDOT electrodes were interesting to test because they are optically transparent and because PEDOT polymer is highly conductive and favours biomolecule immobilisation [52-53]. Again, although previous works have reported cell immobilisation on PEDOT-modified electrodes and the detection of their presence with CV, cell viability has not been directly measured [54-55]. In our experiments, the cell immobilisation (PEDOT/cells/buffer) did not significantly change the capacitive current compared to the bare electrode (PEDOT/buffer), but the oxidation peak at around +0.75 V (vs. Ag) was observed (in this case, no reduction peak was observed) (Fig. 3D). After MTT incubation (PEDOT/cells/MTT), two oxidation peaks at 0 V and +0.66 V (vs. Ag) and a reduction peak at -0.48 V (vs. Ag) were observed, which decreased after exposure to OA (PEDOT/cells/OA/MTT). Although the peak at +0.66 V (vs. Ag) showed the highest current intensity values compared to the other electrodes tested, it appears at a potential too high to be used in chronoamperometry. Therefore, PEDOT electrodes were also discarded.

Finally, regarding AU electrodes, no differences were observed among cyclic voltammograms (Fig. 3E). These results suggest that screen-printed gold is not an appropriate cell immobilisation support or a good transducer for the electrochemical detection of MTT formazan crystals. The previous optical light microscopy image showing immobilised cells (Fig. 2C2) indicates that the problem is the lack of electron transfer between the MTT formazan crystals and the Au. Consequently, AU electrodes were also discarded.

Summarising the electrode material study, both C and C/PANI electrodes were biocompatible with cell immobilisation and viability, and showed the best electrochemical responses from MTT in terms of oxidation potential and current intensity. Because of the 1.5-higher price of the C/PANI electrodes and their higher background currents, C electrodes were chosen for the following experiments.

Afterwards, the cell immobilisation on C electrodes was confirmed with another electrochemical method that does not take into account cell viability, but only cell presence. CVs were recorded in the presence of 1 mM ferricyanide/ferrocyanide redox couple at bare, cell-coated electrodes with and without previous exposure to OA (Fig. 4). As expected, well-defined oxidation and reduction peaks of the ferricyanide/ferrocyanide redox couple, at +0.30 V and -0.08 V and (vs. Ag), respectively, were observed when using bare electrodes (C/$[Fe(CN)_6]^{3-}/^{4-}$). In the presence of immobilised cells (C/cells/$[Fe(CN)_6]^{3-}/^{4-}$), the capacitive current increased, as expected from previous results. Additionally, the oxidation and reduction peaks of the ferricyanide/ferrocyanide redox couple lost definition. This observation indicates that the presence of cells inhibits the diffusion of the redox couple to the electrode surface and the electron transfer [44, 56]. Cells not only increase the steric hindrance, but also repel the $[Fe(CN)_6]^{3-}/^{4-}$, because of the negatively charged cell wall [57]. However, when cells were exposed to OA (C/cells/OA/$[Fe(CN)_6]^{3-}/^{4-}$), background currents were not back to those of bare electrodes (C/$[Fe(CN)_6]^{3-}/^{4-}$), but shown similar values than with living cells as well as the oxidation and reduction peaks of the ferricyanide/ferrocyanide redox couple (C/cells/$[Fe(CN)_6]^{3-}/^{4-}$). This result suggests that, although cells should have been killed and are supposed to fall off the electrode, portions/fragments of cell membrane or other cell components (not observable with optical light microscopy) may remain trapped on the electrode due to the surface roughness, contributing to the capacitive response.

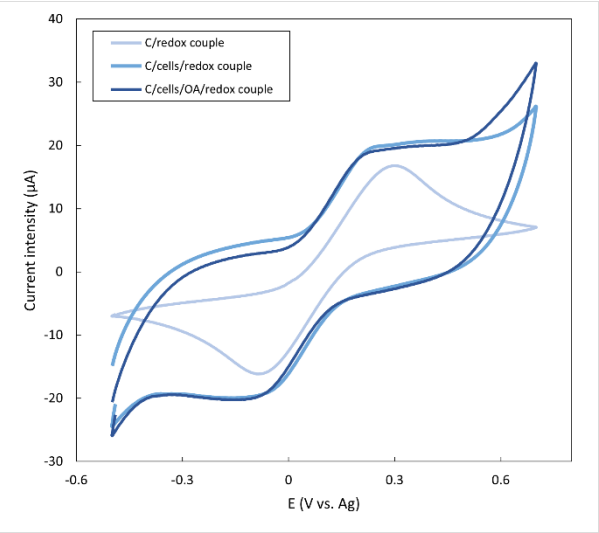


Figure 4. Cyclic voltammetry of $[Fe(CN)_6]^{3-/4-}$ on bare C electrodes (C/redox couple), on C electrodes with immobilised Neuro-2a cells (C/cells/redox couple), and on C electrodes with Neuro-2a cells immobilised and exposed to OA (C/cells/OA/redox couple).

3.2. Optimisation of the assay conditions

Once the type of electrode to be used was chosen, the cell density, cell incubation time and MTT concentration were evaluated.

Cell density and cell incubation time were studied simultaneously with CV. Figure 5 shows the results when incubating different cell densities on the electrodes for 2 h, as an example. Current intensity values increased with increasing amounts of cells immobilised up to 100,000 cells/electrode and no higher values were observed with 200,000 cells/electrode. Additionally, a shift in the formal redox potential towards positive potentials can be observed with increasing cell densities up to 100,000 cells/electrode. This shift could be associated to a pH change, as reported in other works [58-60], which would be due to the formation of lactic acid by the cells. Longer cell incubation times (4, 6 and 24 h) did not result in significantly higher electrochemical signals (Fig. S2). In fact, after 24-h cell incubation, the electrochemical signal slightly decreased. Several reasons may be behind this phenomenon: depletion of one or more of the nutrients, drop in the pH of the medium because of the formation of lactic acid, which is toxic to cells, cell competition for space, and/or formation of clumps and subsequent cell detachment. Therefore, a density of 100,000 cells/electrode and a cell incubation time of 2 h were chosen for the following experiments. It is important to mention that, although higher cell densities provide higher electrochemical responses, this is not always interesting in terms of sensitivity to toxins. In fact, the higher the number of cells, the higher the limit of detection of the assay.

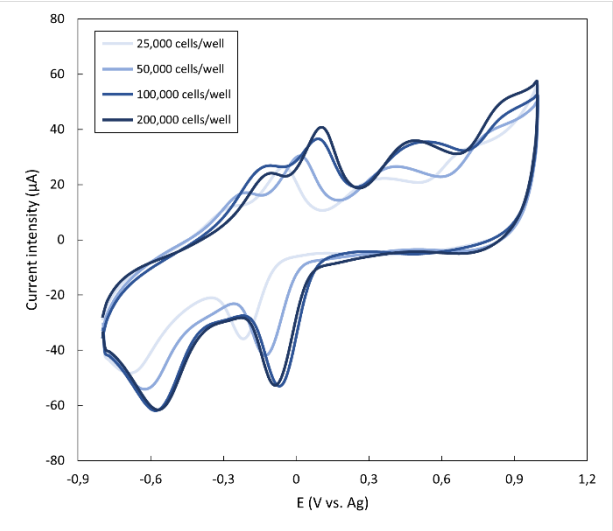


Figure 5. Cyclic voltammetry of C electrodes with Neuro-2a cells immobilised and incubated with MTT using different cell densities.

The MTT concentration was then optimised with CA. Results shown that the current intensity increases with increasing MTT concentration, reaching a maximum value at 0.86 mg/mL MTT (Fig. 6). Therefore, this MTT concentration was chosen for the following experiments.

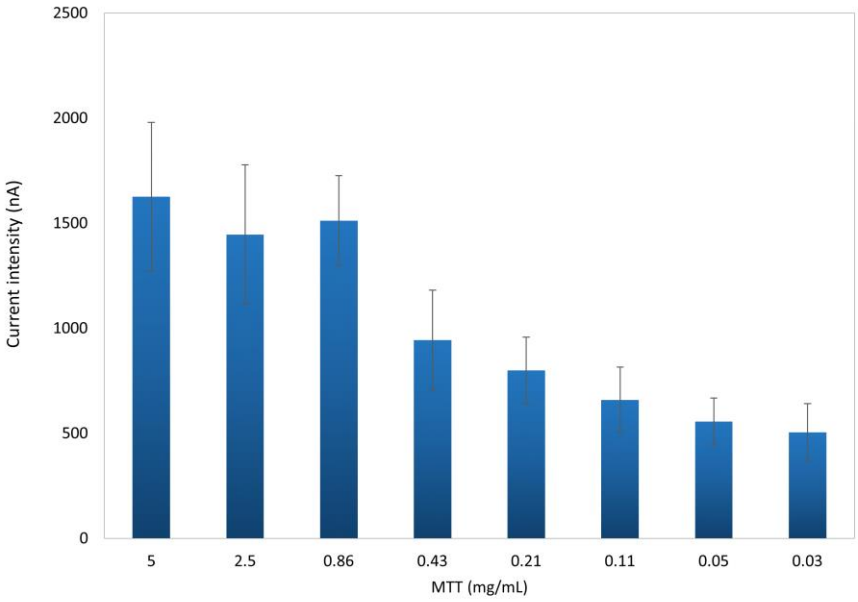


Figure 6. Chronoamperometry of C electrodes with Neuro-2a cells immobilised and incubated with different MTT concentrations.

3.3. Toxin detection – proof of concept

The purpose of this experiment was to evaluate the applicability of the developed method to the detection of toxins. When exposed to toxins acting on VGSCs, cells naturally counteract the sodium influx through the Na^+/K^+-ATPase pump. Thus, in a CBA for CTX, which blocks the VGSCs in an open state, pre-treatment with ouabain, which inhibits the pump and blocks the sodium efflux, is needed. Moreover, veratridine, which enhances the CTX toxicity, is also added. Since the mode of action of TTX is the opposite, i.e., it blocks the VGSCs in a closed state, cells are pre-treated with higher ouabain and veratridine concentrations, and TTX rescues them from this

toxic effect, increasing their viability. Therefore, immobilised cells were exposed to different ouabain/veratridine concentrations and ratios. The low ouabain/veratridine concentration (0.1/0.05 mM), tested for the detection of CTX, decreased the viability to 77%, whereas the high ouabain/veratridine concentration (0.35/0.15 mM), tested for the detection of TTX, decreased the viability to 27% (Fig. 7).

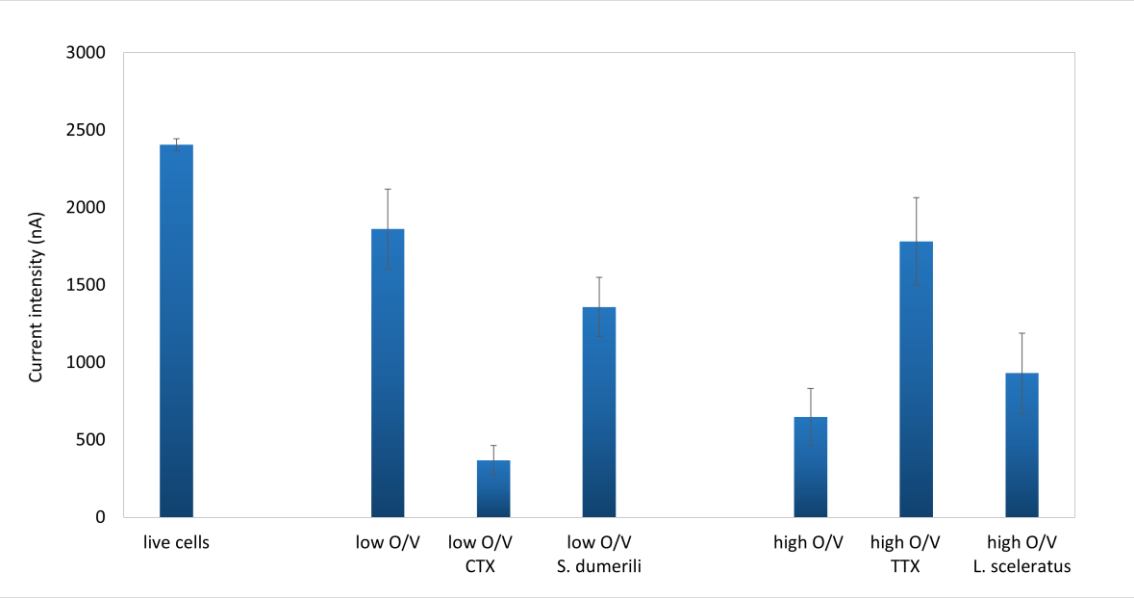


Figure 7. Chronoamperometry of C electrodes with Neuro-2a cells immobilised and incubated with different O/V concentrations, CTX1B and TTX standard solutions and naturally contaminated *S. dumerili* and *L. sceleratus* fish extracts.

Then, the response of the system to CTX1B and TTX standard solutions was evaluated. CA measurements of electrodes with immobilised cells exposed to 0.1/0.05 mM ouabain/veratridine and CTX1B at 2500 pg/mL showed 15% cell viability, therefore cell viability decreased below the 77%. When immobilised cells were exposed to 0.35/0.15 mM ouabain/veratridine and TTX at 2500 ng/mL, CA measurements showed 74% cell viability, higher than the 27%. These results demonstrate that the system is able to detect the presence of both CTX and TTX.

Finally, cells were exposed to naturally contaminated fish samples. Exposure to the CTX-containing *S. dumerili* resulted in 56% cell viability, suggesting that the CTX content in this fish flesh extract is lower than 2500 pg/mL. In fact, as for the colorimetric CBA, the CTX content was 185 pg CTX1B equiv./mL (0.074 pg CTX1B equiv./mg tissue equiv.) [42]. Exposure to the TTX-containing *L. sceleratus* resulted in 39% cell viability, suggesting that the TTX content in this fish gonad extract is lower than 2500 ng/mL. In this case, the analysis of this extract with the colorimetric CBA resulted in 77 ng TTX equiv./mL (0.385 ng TTX equiv./mg tissue equiv.). These results demonstrate that the cells immobilised on the electrodes are sensitive to CTX and TTX present in fish extracts. Even though high toxin standard solution concentrations were used to prove the concept, the analysis of fish extracts has shown that the system is able to detect lower toxin contents.

These results demonstrate that a new method to detect cell viability has been achieved. The proposed strategy shortens the assay time (shorter cell incubation times and no solubilisation of the MTT formazan crystals required) compared to the conventional colorimetric CBA. However, other experimental parameters should be optimised. For example, the surface roughness of the electrodes used in this work, although may be favouring cell adhesion, is affecting the reproducibility of the measurements. Other electrodes, such as photolithographic ones, should be explored. Another issue is the fact that the conventional colorimetric CBA allows

working simultaneously with several microtiter wells, including different samples, samples dilutions, calibration curves, controls, etc. Therefore, design and fabrication of multi-electrode platforms and the corresponding multiplexed potentiostats for high throughput analysis should also be pursued. Despite these weaknesses, our strategy is valid and can be used in the development of electrochemical CBBs for the detection of neurotoxins. Additionally, after proper modifications, it could be applied to the detection of other target analytes.

4. Conclusions

In this work, Neuro-2a cells were successfully immobilised on electrodes and their presence and viability were assessed by the electrochemical detection of MTT formazan produced by mitochondrial dehydrogenases. The detection of the crystals, precipitated and confined on the electrode, has avoided the cell lysis step, necessary for their solubilisation in colorimetric assays. Among the different electrode materials, C and PANI electrodes provided the best electrochemical performance in terms of oxidation potential and current intensity, and the former ones were chosen because of their lower cost. Optical light microscopy also proved the presence of immobilised and living cells. After optimisation of the assay conditions, the strategy was proven to respond to CTX and TTX standard solutions as well as to naturally contaminated fish extracts. Therefore, this work paves the way towards the development of electrochemical CBBs for the detection of marine toxins and could be extended to other target analytes.

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CRediT author statement

MA: Methodology, Formal Analysis, Investigation, Writing – Original Draft, Writing - Review & Editing

SL: Methodology, Formal Analysis, Investigation, Writing - Review & Editing

JD: Methodology, Investigation, Writing - Review & Editing, Supervision

MC: Conceptualisation, formal analysis, investigation, Writing – Original Draft, Writing - Review & Editing, Supervision, Project Administration, Funding Acquisition

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Curriculum vitae

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