Electrochemical Biosensor for the Assessment of Cell Viability Using Methylene Blue. Application to the Detection of Ciguatoxins in Fish from the Canary Islands

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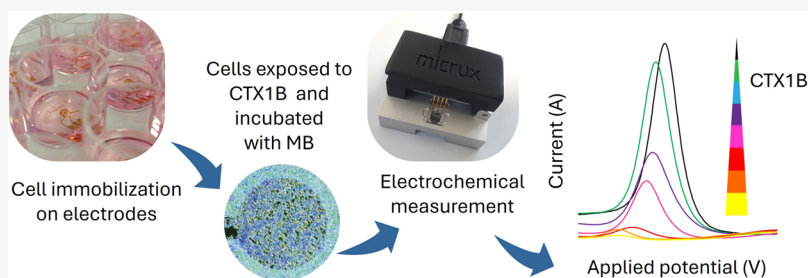
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ABSTRACT: Cell-based biosensors (CBBs) for the detection of marine neurotoxins such as ciguatoxins (CTXs) are of high interest due to the composite toxicological response they can provide and the low limits of quantification (LOQs) they can achieve with the use of sensitive neural cells. However, the development and validation of CBBs are challenging due to the use of living material and the need for appropriate signal transduction strategies. In this work, Neuro-2a cells have been immobilized on thin-film gold electrodes, and their viability after exposure to CTX1B has been evaluated with light optical microscopy as well as cyclic voltammetry (CV) and differential pulse voltammetry (DPV) using methylene blue (MB) as a redox indicator. An LOQ of 0.93 pg CTX1B/mL has been obtained. The CBB has been applied to the analysis of fish samples from the Canary Islands, one of them implicated in a ciguatera poisoning (CP) outbreak, and results have been compared with those obtained with a conventional cell-based assay (CBA), showing a very good agreement. The combination of the benefits of cells with those provided by biosensor platforms in terms of ease of use, miniaturization, automatization, and portability could result in the ideal analytical tool for CP management. Additionally, this is the first time MB is used as a cell viability indicator in a CBB, providing a new versatile approach for multiple applications.

INTRODUCTION

Ciguatoxins (CTXs) are lipophilic marine toxins produced by dinoflagellates of the genera *Gambierdiscus* and *Fukuyoa*.[1] Mainly fish species and rarely marine invertebrates, such as echinoderms, gastropods and bivalve mollusks, along with octopus and crustaceans, accumulate these toxins.[2] This toxin accumulation in fish may reach levels capable of causing illness. The consumption of fish contaminated with CTXs, which are odorless, tasteless, heat stable and resistant to gastric degradation,[3] causes ciguatera poisoning (CP), the most prevalent nonbacterial food-borne disease.[4] CP is suggested to affect between 10,000 and 500,000 people each year worldwide,[4] although these figures would require update and further re-evaluation. CP causes acute digestive, cardiovascular and neurologic effects, but also chronic symptoms that may last for months or even years.[5] Although historically CP was typical of tropical and subtropical regions (e.g., Cuba, Puerto Rico, Florida, US Virgin Islands, the Gulf of Mexico, La Réunion, Mauritius, Rodrigues, Madagascar, French Polynesia, Cook Islands, Kiribati, Hawaii, Queensland, Japan, China, Hong

Kong, the Philippines and Taiwan), in recent years, cases of CP have been reported in more temperate regions (e.g., the Canary Islands, the Madeira Archipelago, New South Wales, Vietnam, Indonesia, Malaysia, Macau, Thailand and South Korea).[1] The US Food and Drug Administration (FDA) proposed a guidance level of 0.01 μg CTX1B equivalents (equiv)/kg.[6] In Europe, the legislation determines that fishery products containing CTXs must not enter the market,[7] and the European Food Safety Authority (EFSA) has also proposed the 0.01 μg CTX1B equiv/kg limit.[8] Spain, in the Canary Islands, conducts a regular monitoring program for CTXs in some species of fish, implementing an assay with Neuro-2a cells.[9] Other countries, such as Australia and New Zealand, provide
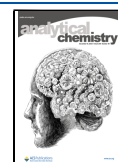
guidelines on possible ciguateric fish species and areas,[10] and in Japan, some fish species are banned to import.[11]

Therefore, several organizations and experts recommend putting efforts into the development and validation of analytical methods for the detection of CTXs, which are a structurally complex family, at very low contents in complex matrices.[12,13] Despite the progress achieved over the past few years, the detection of CTXs is still a challenge. Several analytical methods have been developed, including cell-based assays (CBAs), receptor binding assays, instrumental analysis techniques, immunoassays and immunosensors.[14] Although each method shows advantages and limitations, CBAs for CTXs stand out because of their low limits of detection and the composite toxicological response they provide, which includes the effect of all CTX-like toxic congeners present in a sample, therefore only requiring one CTX standard (e.g., CTX1B, the congener considered as the most toxic) to act as a reference to estimate the toxicity of a sample. The detection principle behind the CBA for CTXs is the following: CTXs act on the voltage-gated sodium channels (VGSCs) of the cell and, in the presence of ouabain (which inhibits the Na^+/K^+-ATPase pump) and veratridine (which increases the Na^+ permeability), cause an influx of Na^+ into the cell, cell depolarisation and subsequent cell mortality.[15] The combination of the benefits of CBAs with those provided by biosensor platforms in terms of ease of use, miniaturization, automatization and portability could result in the ideal analytical tool for CP management.

Cell-based biosensors (CBBs) still are one step behind other types of biosensors, such as enzyme sensors, immunosensors, genosensors or aptasensors. The challenges in the development of CBBs are keeping cells alive, getting measurable signals and obtaining reproducible results. Only one CBB exists in the literature for the detection of CTXs.[16] This system is based on extracellular potential measurements on multielectrode arrays, and it has only been tested with CTX1B standard. Automated patch clamp electrophysiology systems have also been developed for this purpose.[17,18] It is evident that more research efforts should be aimed at addressing the development and applicability of CBBs for neurotoxins. With this purpose, we previously tried to develop an electrochemical CBB for marine toxins acting on the VGSCs, such as CTXs and tetrodotoxins (TTXs), using screen-printed electrodes and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as a cell viability indicator.[19] However, this biosensor suffered from reproducibility problems probably due to the electrode surface roughness and the nonhomogeneous distribution of precipitated MTT formazan crystals on the electrode.

In this work, Neuro-2a cells have been immobilized on thin-film gold electrodes produced by lithography, and their viability after exposure to CTX1B standard has been evaluated with cyclic voltammetry (CV) and differential pulse voltammetry (DPV) using methylene blue (MB) as a redox indicator. The CBB has been used to analyze fish samples from the Canary Islands, one of them involved in a CP outbreak, and results have been compared to those obtained with a conventional CBA. This is the first time MB is used as a cell viability indicator in a CBB, providing a new versatile approach for multiple applications, such as screening of cytotoxic agents, drug discovery and testing, pathogen and toxin sensing.

■ EXPERIMENTAL SECTION

Reagents and Materials. Neuroblastoma murine (Neuro-2a) cells were purchased from ATCC LGC standards (Manassas, VA, USA). Foetal bovine serum (FBS), ouabain (O), veratridine (V), phosphate buffered saline (PBS), penicillin–streptomycin, RPMI-1640 medium, sodium pyruvate and MB 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. Thin-film gold single electrodes (ED-SE1-Au) and a Drop-cell connector were provided by MicruX Technologies (Gijón, Spain). Electrodes (10 × 6 × 0.75 mm) were fabricated on a glass substrate and consist of a gold working electrode of 1 mm in diameter, a gold counter electrode, a gold reference electrode, and a resin protective layer to delimit the electrochemical cell.

Fish Samples. Sample S1F (EFSA-ULPGC-F0178) corresponds to the flesh of an amberjack (*Seriola* sp.) of 70 kg caught in October 2015 in Tenerife (Canary Islands, Spain), which tested positive for CTXs in the frame of the Official Control Program for the Prevention of Ciguatera in the Canary Islands. This specimen was previously used in controlled exposure experiments.[20,21] Samples S2F (CP1–2023 (F)) and S2L (CP1–2023 (L)) correspond to the flesh and liver, respectively, of an amberjack (*Seriola* sp.) of 33 kg caught in May 2023 in Fuerteventura (Canary Islands, Spain) and linked to a CP outbreak. Flesh samples came from muscle tissue without skin.

CTXs Extraction. Fish samples were extracted and purified as in our previous works.[22,23] Briefly, 10 g of fish flesh or liver was heated at 70 °C for 15 min in a water bath. After cooling, 20 mL of acetone was added, the sample mixture was homogenized with an Ultraturrax blender for 2 min and centrifuged at 3000 *g* for 15 min to obtain the supernatant. The pellet was re-extracted with acetone, and supernatants were pooled, kept at −20 °C overnight, passed through a 0.2-μm PTFE filter, rotary evaporated to a small volume, and adjusted to 4 mL with Milli-Q H_2O. The sample was partitioned twice with 16 mL of diethyl ether. The water phase was discarded, and the diethyl ether phases were pooled and evaporated to dryness. The dried extract was resuspended in 2 mL of aqueous MeOH (80%) and partitioned three times with 4 mL of *n*-hexane. The *n*-hexane phases were discarded, and the aqueous MeOH phase was evaporated to dryness with N_2. The dried extract was then resuspended in 4 mL of HPLC-grade MeOH (100%), passed through a 0.2-μm PTFE membrane filter, and stored at −20 °C until analysis. For calculation purposes, 1 mL of extract contains 2.5 g equiv of fish tissue.

Cell Immobilization on Electrodes and MB Incubation. Neuro-2a cells were maintained in RPMI-1640 medium (which also contained 10% FBS (previously heat-inactivated), 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 trypsinized and suspended in the same medium, but with 5% FBS instead of 10% FBS (RPMI-1640−5%). Electrodes were placed in 24-well microtiter plates containing 800 μL of a Neuro-2a cell suspension at 300,000 cells/mL and incubated for 24 h. Then, electrodes were moved to 96-well microtiter plates containing 200 μL of RPMI-1640−5%, 20 μL

O and V at 0.1 and 0.01 mM, respectively, and 10 μL of CTX1B standard or fish extract (previously evaporated and reconstituted in RPMI-1640−5%) at different dilutions and incubated for 24 h. Controls without O/V were always included in the experiments. Afterward, electrodes were moved to 96-well microtiter plates containing 200 μL of MB at 1 mg/mL in RPMI-1640−5% and incubated for 40 min. All incubations were performed at 37 °C in 5% CO_2 humid atmosphere. Finally, electrodes were washed with PBS to remove excess of MB.

Light Optical Microscopy. Cell immobilization on electrodes was characterized 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).

Electrochemical detection. Electrodes with immobilized cells were connected to an Autolab PGSTAT128N potentiostat and data were collected and evaluated with NOVA v2.1 software (Metrohm, Autolab, Utrecht, The Netherlands). For the electrochemical measurements, 5 μL of PBS were placed on the electrodes and two techniques were used to detect the MB present into the cells: cyclic voltammetry (CV) and differential pulse voltammetry (DPV). In both cases, a preconditioning potential of −0.6 V (vs. Au) for CVs and −0.4 V (vs. Au) for DPVs was first applied for 25 s. Then, CVs were recorded between −0.6 V and +0.2 V (vs. Au) at 50 mV/s and DPV measurements were performed from −0.4 V to +0.1 V (vs. Au) with a 1-mV step potential.

CBA. The CBA was performed as previously described.[22,23] Neuro-2a cells were seeded in 96-well microplates containing 200 μL of RPMI-1640−5% at 34,000 cells/well and incubated for 24 h. Then, cells were exposed to 20 μL of O and V at 0.14 and 0.014 mM, respectively, and 10 μL of CTX1B standard or fish extract (previously evaporated and reconstituted in RPMI-1640−5%) at different dilutions and incubated for 24 h. All incubations were performed at 37 °C in 5% CO_2 humid atmosphere. Finally, cell viability was measured using MTT.

■ RESULTS AND DISCUSSION

Cell Immobilization on Thin-Film Gold Electrodes. MB has previously been used as a colorimetric dye for cell counting or cell viability assessment.[24,25] This compound penetrates both alive and dead cells, and whereas living cells are able to reduce MB and remain colorless, the dead cells are stained blue. In the current work, cells have been first successfully immobilized on electrodes, then sensitized with O and V, exposed to CTX1B and finally incubated with MB. Cells killed due to the cytotoxicity caused by CTXs detach from the electrode surface (Figure 1 left), certainly due to the disruption of the actin cytoskeleton,[18] and only living cells remain on the electrode (Figure 1 right). Although, in a first stage, living cells are able to reduce the MB, after a certain exposure time, they are stained blue,[24] probably due to the MB toxicity.

Electrochemical Detection of Cell viability. To measure the total amount of MB inside the cells remaining on the electrode, regardless of its oxidized or reduced state, a negative preconditioning potential was applied before the electrochemical recording. CVs in the absence of CTX1B showed a quasi-reversible redox system, due to the MB incorporated into the cells, with an oxidation peak at −0.10 V
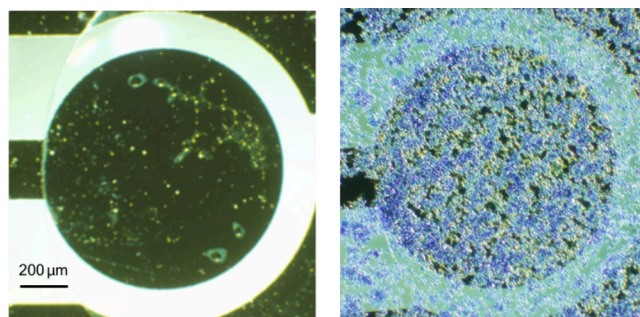

Figure 1. Light optical microscopy images obtained after cell immobilization on electrodes and exposure to O+V+CTX1B/MB at 100 pg CTX1B/mL (left) and O+V+/MB (right).
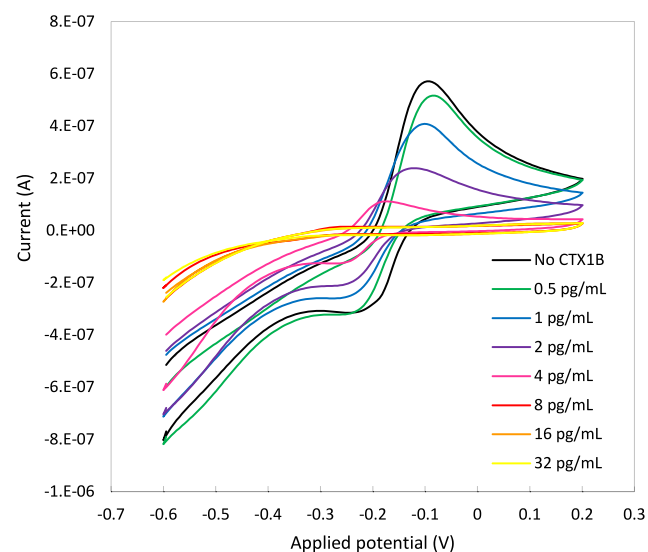

Figure 2. CV recordings obtained after cell immobilization on the electrodes and exposure to O+V+/MB (no CTX1B) and O+V +CTX1B/MB at different CTX1B concentrations.

(vs. Au) and a reduction peak at −0.22 V (vs. Au) (Figure 2). Exposure of cells to CTX1B resulted in redox peaks with lower current intensities, inversely proportional to the CTX1B concentration. Additionally, the oxidation peaks shifted toward more negative potentials, an effect that was barely noticeable in the reduction peaks. DPVs in the absence of CTX1B showed an oxidation peak at −0.18 V (vs. Au), which also decreased in current intensity and shifted toward more negative potentials with increasing CTX1B concentrations (Figure 3). The electrochemical detection of MB indicates presence of cells on the electrodes, which have retained their adhesion and viability after exposure to low CTX concentrations.

The calibration curve for CTX1B was constructed from the oxidation areas of the DPVs recordings (Figure 4) and fit to a sigmoidal 4-parameter logistic equation:

$$y = y_0 + \frac{a}{1 + \left(\frac{x}{x_0}\right)^b}$$

where a (100.8533) and y_0 (−2.6649) are the asymptotic maximum and minimum values, respectively, x_0 (2.3982) is the x value at the inflection point, and b (1.5906) is the slope at the inflection point.
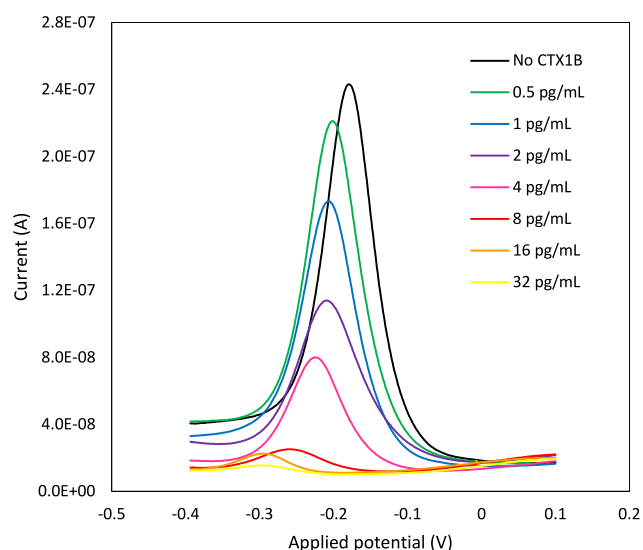
Figure 3. DPV recordings obtained after cell immobilization on the electrodes and exposure to O+V+/MB (no CTX1B) and O+V+CTX1B/MB at different CTX1B concentrations.
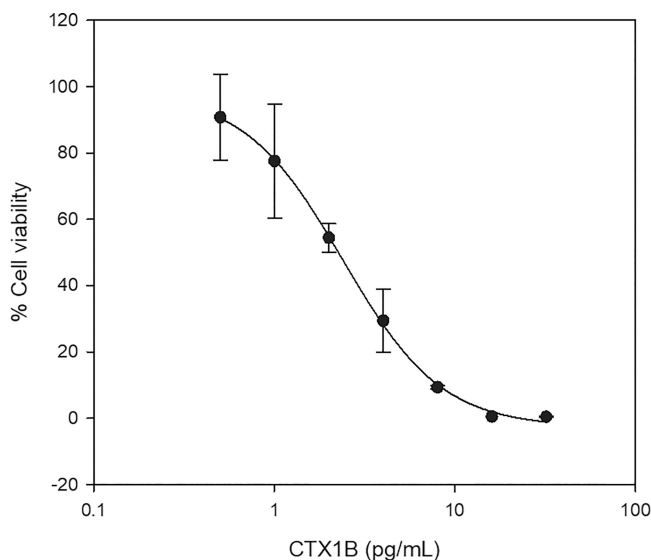

Figure 4. Calibration curve for CTX1B constructed from the oxidation areas of the DPV recordings ($N = 3$).

From the equation, an IC_{50} value of 2.27 pg/mL and a limit of quantification (LOQ, 80% cell viability) of 0.93 pg/mL were obtained. These values are similar to those obtained with the CBA. In fact, both approaches are based on the toxicological mechanism of action of CTXs on Neuro-2a cells, although the detection technique is different: whereas the CBB detects the MB incorporated into cells immobilized on the electrode, the CBA measures the ability of metabolically active cells to reduce the MTT. A method that directly reflects cell number (such as this CBB), rather than biological indicators of cell number (such as mitochondrial dehydrogenase activity in the CBA) may be more advantageous in terms of versatility and broader range of potential applications.

Analysis of Naturally Contaminated Samples. CTX contents obtained from the analysis of the fish extracts with the CBB are shown in Table 1. All fish samples from the Canary Islands, one flesh from the Official Control Program for the

Table 1. CTX Contents (μg CTX1B equiv/kg) in Fish Samples Obtained with CBB and CBA (N = 3)

Sample code	CBB	CBA
S1F	0.7 ± 0.2	1.1 ± 0.1[20]
S2F	3.0 ± 0.9	2.8 ± 0.6
S2L	9.6 ± 1.1	9.3 ± 1.3

Prevention of Ciguatera and the flesh and the liver of a fish involved in a CP outbreak, contained CTXs at contents higher than the guideline value established by the FDA (0.01 μg CTX1B equiv/kg).[6] For the sample involved in the CP outbreak, the CTX contents in the liver were higher than in the flesh, in accordance with other works,[26] probably because it is the most frequently targeted organ in terms of drug toxicity.[27]

The CTX contents obtained with the CBB very similar to those obtained with the CBA (Figure S1). This is not surprising because, as previously mentioned, although the detection technique is different, both approaches are based on the cytotoxic effect of CTXs present in the samples on the Neuro-2a cells. This CBB has clear advantages over the conventional CBA on microtiter plates in terms of miniaturization and portability. However, since manual manipulation of the electrodes one by one is required, which is tedious and time-consuming, the CBB still requires further optimization to meet high-throughput and automatization requirements. Electrode arrays, multiplexer potentiostats and microfluidics systems could contribute to progress in this direction.

Toxicological results may not exactly agree with results obtained from instrumental analysis techniques, as these are based on the structural recognition of the different CTX congeners instead of the composite toxic effect. Previous LC−MS/MS analyses revealed that sample S1F contained 0.27 μg C-CTX1/kg,[20] and sample S2F contained 0.46 μg C-CTX1/kg, 0.22 μg 17-hydroxy-C-CTX1/kg, and trace amounts of their respective 56-methoxy-congeners and C-CTX5.[28] First, it is important to note that the toxicity of C-CTX1 is lower than that of CTX1B and, therefore, is contributing to a lower extent to the global toxicological response. Additionally, other CTX congeners, not detected because of the low contents and/or because they are not targeted by the LC-MS/MS method, could be present. Finally, it is important to highlight that the good agreement of the CBB with the CBA, which is the method commonly used in CP monitoring programs, proves the promising future of this new alternative tool.

■ CONCLUSIONS

MB has been used for the first time as a redox mediator for the electrochemical measurement of the viability of cells immobilized on electrodes. The approach has been applied to the detection of CTXs as a proof-of-concept, providing successful results not only when sensing CTX1B standard, but also when analyzing fish samples with several CTX congeners, some of them involved in CP cases. This CBB, which directly reflects cell number, is a versatile and powerful tool with applicability in research areas where cell counting is required, such as drug discovery, bioactive compounds and natural products.

■ ASSOCIATED CONTENT

ⓢ Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.analchem.4c05174.

Analysis of samples with CBA (PDF)

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Author Contributions

M.C.: conceptualization, methodology, writing - original draft, writing - review and editing, supervision, project administration, funding acquisition. S.L.: investigation, writing - review and editing, visualization. A.S.-H.: investigation writing - review and editing. F.R.: resources writing - review and editing, project administration, funding acquisition. J.D.: writing - review and editing, project administration, funding acquisition.

Notes

The authors declare no competing financial interest.

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