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# Detection of diarrhetic shellfish poisoning toxins using high-sensitivity human cancer cell-based impedance biosensor

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ABSTRACT

Diarrhetic shellfish poisoning (DSP) toxin is a dangerous contamination in seafood worldwide that can threaten human health and fishing. A large number of animal bioassays and chemical analytical methods are employed for DSP toxin detection. However, these toxin detection methods are low-throughput and high-cost which hamper their wide applications. In this study, HeLa and HepG2 cell lines were selected as the sensitive elements to establish the CIBs for monitoring the cytotoxicity induced by a representative DSP toxin, okadaic acid (OA). The limit of detection (LOD) of HeLa- and HepG2-based biosensors are 10.2 μg/L and 3.3 μg/L, respectively, which are both lower than the conventional cell-based assay (21.2 μg/L of HeLa cells and 9.8 μg/L of HepG2 cells). The half maximal inhibitory concentration (IC50) values of OA in HeLa and HepG2 cells which were obtained from CIB (49.9 ± 4.9 and 39.2 ± 4.3 μg/L) are both lower than Cell Counting Kit-8 assay (CCK8) (62.7 ± 7.1 and 45.8 ± 6.7 μg/L). Besides, CIB measurement presents good correlation with mouse bioassay (MBA) and liquid chromatography–tandem mass spectrometry (LC–MS/MS). In summary, all the results indicate that the CIB technology had great potential to be an effective complement in DSP toxins detection.

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## 1. Introduction

Diarrhetic shellfish poisoning (DSP) toxin is one of the five recognized symptom types of shellfish poisoning toxins causing diarrhea, vomiting and abdominal pain [1,2]. DSP toxins pose a threat to public health and mariculture industry due to their wide distribution and frequent occurrence. For example, hundreds of people were poisoned by DSP toxins in China in 2011 following consumption of *Mytilus galloprovincialis*. In the same year, some DSP illnesses were also reported in the US Pacific Northwest [3,4].

Current detection methods of marine toxins include the mouse bioassay (MBA), chemical analysis method, immunoassay and cell-based assay (CBA) [5,6]. MBA method is the common and official recommended method in EU, Japan and some other countries since 1990 [7]. Although this method is easy to operate, it is protested by animal protectors for killing many animals [8]. In the chemical analysis method, liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) has attracted more and more attention in recent years for high performance of qualitative and quantitative analysis. The European Commission set the LC–MS/MS method as the standard method in DSP toxins detection recently [9]. However, it cannot evaluate toxicity of unidentified toxins in shellfish [10]. Moreover, LC–MS/MS needs expensive reference substance, maintains cost of instrument and well-trained operators, hampering its wide application around the world. Immunoassay based on the antigen–antibody reaction has high specificity and sensitivity, but the specific monoclonal antibodies are relatively high-cost and difficult to obtain [11].

To solve the current problems, cell-based assays are introduced to detect DSP toxins for several years with high throughput, low cost and good reproducibility. Detection principle of cell-based assays is DSP toxins-induced cytotoxicity. Cell status can reflect DSP toxin cytotoxicity by morphological change, cell viability and F-actin levels, which could be detected by microscopy, spectrophotometer and fluorimetric microplate reader [12–14]. However, cell-based assays are still tedious and cannot provide some real-time and dynamic information with regard to cell actives induced by toxins. Electrical cell-substrate impedance sensing (ECIS) becomes a powerful tool in cell analysis, such as cell

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adhesion, proliferation and cytotoxicity since it was pioneered by Giaever and Keese [15–18]. Taking the advantage of ECIS into consideration, cell-based impedance biosensor (CIB) is widely used in the field of cellular physiology [19], pharmacology [20], and toxicology [21].

Based on the detection of paralytic shellfish poisoning (PSP) toxin in the former research [22], two human cancer CIBs were employed to dynamically detect the cytotoxicity of OA toxin at different concentrations. The sensitivity of both CIBs is compared with the conventional cell-based assay, Cell Counting Kit-8 (CCK8), and the correlation is studied between CIBs and other methods. Finally, the specificity of CIBs is tested by saxitoxin (STX) and brevetoxin (PbTx-2). All the details will be discussed in following sections.

## 2. Material and methods

### 2.1. Sensor fabrication and detection system design

The sensor chip is fabricated on a sterilized Pyrex glass 7740 (corning). A 20 nm thick Cr layer is sputtered on glass matrix to enhance the strength adhesion between Au layer and glass, and then 300 nm Au is sputtered on the Cr layer as the electrode material. Subsequently, the interdigitated electrodes (IDEs) array, leads and pads are fabricated with UV lithography and etching technology. Sensor chip is then fixed on a printed circuit board (PCB), and multi-well chamber made by PET material is sealed on the sensor chip to form cell culture chamber. Several studies have shown that the sensitivity of impedance biosensor is inversely proportional to the electrode length and width, while the distance between the electrode has little effect on the sensitivity [23–25]. In cell-based experiment, the signal detected was determined by cells attached on the electrode surface. Therefore, both the sensitivity and the cell number need to be considered in balance in sensor design. If the width and length of electrode are reduced to obtain a high sensitivity, the number of cells will be reduced and a large electrical potential difference along the electrode length will happen. Cells attached at different positions on the electrode will produce significantly different impedance signals to the total impedance, therefore, the electrode width should not be too narrow and the electrode length should not be too long can solve this problem to some degree.

Application of IDEs can significantly shorten the electrode branches, which can provide a more uniform distribution of the electrical field. On the whole, the electrode configuration of impedance detection should meet the following requirements: IDEs structure, short electrode branch length and high branch number. In our CIB system, we used IDEs structure to shorten the electrodes branches, circular electrode design to enlarge the cells attached area. As shown in Fig. 1(A), each sensor chip consists of 16 round-shape interdigitated electrodes (IDEs), and 5 mm in diameter. The IDEs has 32 single electrodes and the distance between the adjacent electrode is 120 μm. The total base area of each well is 0.2 $mm^2$ and the electrodes account for 60%.

CIB employed the ECIS technology to measure the impedance. An electric field between the IDEs is created by applying a low voltage alternating current (AC) signal voltage. A baseline impedance $Z_0$ is appeared when there are no cells on the electrodes, which mainly induced by the ionic environment of culture medium in the wells. After cells added, the impedance $Z_{cell}$ is determined by cellular number, cellular morphology and cellular adhesion. And then impedance signal is measured by CIB system and converted into cell index (CI) value by the equation: $CI = (Z_{cell} - Z_0)/Z_0$. $Z_0$ is the baseline impedance which is measured without cells on the sensor chip in each experiment. Hence, CI value is mainly determined by the physiological state of cells on the IDEs in real time, such as cell number, cell morphology and cell adhesion. As shown in Fig. 1(B), CI value fluctuation of CIB system without cells was ±0.05, which is very small compared with the impedance change induced by cells. Therefore, the system can satisfy the requirement of cell-based assay.

Cell growth (e.g., adhesion, proliferation, spreading) is a relatively slow behavior and the sensor's response will be smooth. So the CIB sensor system was usually designed with a low data acquisition rate (1 sample/min). As shown in Fig. 1(C), CI value increased dramatically in 0–2 h due to cell adhesion on the IDEs. Fig. 1(D) provided 0–400 s data which was derived from Fig. 1(C). Therefore, CI curves showed the real-time information of impedance change in whole cell-based experiment.

### 2.2. Chemical reagents and shellfish extracts preparation

OA (Sigma Aldrich, USA) stock solution is dissolved in dimethyl sulfoxide (DMSO, Sigma Aldrich, USA) and filtered with 0.22 μm membrane filter unit (MILLEX-GV, Millipore, Bedford, USA). Different OA concentrations are diluted by Dulbecco's Minimum Essential Medium (DMEM) and stocked in the darkness at 4 °C for several months. PbTx-2 (Sigma Aldrich, USA) is diluted in sterile distilled water at a stock concentration of 100 μM. Other concentrations were diluted by cell culture medium before used. STX stock solution (66.3 μM) is purchased from the National Research Council of Canada, Halifax, NS, Canada. Other concentrations were diluted by 0.005 M acetic acid. Fetal calf serum (FCS) and DMEM are obtained from Gibico, USA. CCK8 is purchased from Dojindo Lab., Kumamoto, Japan.

*M. galloprovincialis* is collected from nine shellfish beds in Gouqi Island in the spring and summer of 2014. The preparation of shellfish extracts used in MBA and CBA are carried out according to the protocol offered by Ledreux et al. [26]. Then the residue of 20 g shellfish extracts is resuspended in 1 mL 1% Tween 60 in saline for MBA and CBA. For LC–MS/MS analysis, 2 g of the shellfish homogenate is extracted for 2 min with 9 mL methanol twice. Combining the extracts and dried under nitrogen at 40 °C, the residue is redissolved in 5 mL methanol. Finally, all the extracts are filtered with 0.22 μm membrane filter and stored at −20 °C before CBA and LC–MS/MS.

### 2.3. Cell culture

HeLa (human epithelial carcinoma cells) and HepG2 (human hepatoma cells) cell lines are purchased from the American Type Culture Collection and cultivated in 25 $cm^2$ plastic flasks (Corning, USA) with DMEM containing 10% FCS and 1% penicillin and streptomycin in an atmosphere containing a humidified 5% $CO_2$ at 37 °C. The cell culture medium is renewed every second or third day.

### 2.4. Impedance measurement with the CIB system

100 μL culture medium without cells is firstly added to each well of sensor chip in order to obtained $Z_0$. Then additional 200 μL suspensions which contain a certain number of cells are added and the impedance signals of CIB are checked every 10 min for 24 h. The cells in different wells of sensor chip are treated with toxins after 24 h of cell culture. The signals are measured again every 10 min for another 24 h or 48 h. The CIB system is placed in the cell incubator throughout the experiments.

### 2.5. Cell viability assay using CCK8

The detection principle of CCK8 assay is based on WST-8 which can be reduced by dehydrogenases in living cells to an orange-colored Formazan. WST-8 is a water soluble Tetrazolium-salt which is similar to MTT, therefore, CCK8 assay could be considered as

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