Biosensors for the Detection of Emerging Marine Toxins

Sandra Leonardo, Laia Reverté, Jorge Diogène and Mònica Campàs

Abstract Emerging marine toxins present in the environment are relevant for food safety issues. Researchers are currently putting special emphasis on the development of biosensors for their detection. Due to their structural complexity and the difficulty to produce the corresponding biorecognition molecules, the development of assays and biosensors for their detection has become a challenge. Compared to traditional detection techniques, biosensors can provide advantages in terms of sensitivity, specificity, design versatility, portability and multiplexed configurations. This chapter provides a critical overview of the immunosensors, receptor-based biosensors, cell-based biosensors and aptasensors that have been developed for the detection of palytoxins (PlTXs), brevetoxins (PbTXs) and tetrodotoxins (TTXs). Although only few biosensors for these emerging marine toxins have been described to date, the chapter reflects the promising advances made in this field.

Keywords Emerging marine toxins · Palytoxins · Brevetoxins · Tetrodotoxins · Immunosensors · Receptor-based biosensors · Cell-based biosensors · Aptasensors

1 Introduction

Marine toxins are secondary metabolites some of which can be produced by microalgae of the groups of dinoflagellates and diatoms. The specific role these toxins may play in the microalgae that produce them is not clear. These toxins often enter the food webs and may ultimately reach humans through food consumption or direct exposure to marine water, causing different illnesses.

As some virus, bacteria, fungi and protozoa, some marine toxins, such as saxitoxins (STXs) and tetrodotoxins (TTXs), can be considered as potential chemical warfare agents [1, 2]. Nevertheless, reported poisoning incidents related with

S. Leonardo · L. Reverté · J. Diogène · M. Campàs (✉)
IRTA, Carretera Poble Nou Km. 5.5, 43540 Sant Carles de La Ràpita, Spain
e-mail: monica.campas@irta.cat

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marine toxins have only been due, to the best of our knowledge, to accidental ingestion of contaminated seafood or direct exposure to marine water. In a "fictive world" (movies, books) everything is plausible, including the use of marine toxins as potential weapons. In the "real world", STX, which is a potent neurotoxin produced by several species of marine dinoflagellates (e.g. *Alexandrium minutum, A. catenella, Gymnodynium catenatum*), is included in the list of chemical weapons described by the Organisation for the Prohibition of Chemical Weapons [3]. However, although being extremely potent, marine toxins seem not to be presently considered as practical weapons, probably due to the difficulty or cost to produce them in comparison with other chemical weapons. Still, as one may understand, this analysis may only be speculative since probably very little information would be available in case these toxins were presently being used as chemical weapons.

Some marine toxins are quite well described according to their structure, mechanism of action, potency and geographical distribution. This is the case for Amnesic Shellfish Poisoning (ASP, e.g. domoic acid, DA), Diarrheic Shellfish Poisoning (DSP, e.g. okadaic acid, OA) and Paralytic Shellfish Poisoning (PSP, e.g. STX) toxins for which international regulations exist that set up maximum permitted levels in food and define the official methodologies to detect them.

Nonetheless, many marine toxins are presently considered, with a certain degree of subjectivity, as "emerging" toxins including those recently discovered (e.g. pinnatoxins), those that may have recently appeared in certain areas (e.g. ciguatoxins—CTXs recently identified in fish from the Canary Islands), or those that are not yet regulated because not enough information is available regarding their toxicity or distribution (e.g. palytoxins—PlTXs, brevetoxins—PbTXs and cyclic imines—CIs), and those for which regulation exists but additional toxicological information is required (e.g. azaspiracids, AZAs) [4].

The little information and data available for some emerging marine toxins, their structural complexity and the scarcity of standards have compromised the development of methodologies for their detection. The development of biosensors requires stable biorecognition molecules such as enzymes, receptors or antibodies that will unequivocally detect the analyte. In some cases, biorecognition molecules for marine toxins have not been produced and this limits the number of biosensors developed. Nevertheless, marine toxins are nowadays awaking interest in the biosensors world, possibly due to their importance in food safety and environmental monitoring and this opens new applicability fields for biosensors.

In this chapter, we present a detailed overview of the biosensors that have been developed for the detection of PlTXs, PbTXs and TTXs. AZAs, CTXs and CIs (spirolides, gymnodimines, pinnatoxins and pteriatoxins), although also of concern as reflected in the Scientific Opinions on marine biotoxins in shellfish of the European Food Safety Authority (EFSA) Panel on Contaminants in the Food Chain [5–7], have not been included since no biosensors have been reported to date.

2 Biosensors for Palytoxins

Palytoxin (PlTX)-group toxins are complex polyhydroxylated compounds with lipophilic and hydrophilic areas (Fig. 1). They have been isolated from marine zoanthids (soft corals) of the genus *Palythoa* and are also found in benthic dinoflagellates of the genus *Ostreopsis*. PlTX-group toxins have been identified as the toxins responsible for clupeotoxism, a form of ichthyosarchotoxism caused by sardines, and are also found in seafood (fish, shellfish, gastropods or echinoderms) from Japan, Vietnam, Philippines, Malaysia, Singapore, Indonesia, Micronesia, Australia, New Zealand, Hawaii, Cook Islands, French Polynesia, Brazil, Mexico, the Caribbean sea, Madagascar, Reunion Island and the Mediterranean sea [4, 8]. Apart from the contamination with PlTX-group toxins of seafood intended for human consumption, outbreaks of *Ostreopsis* spp. have been implicated in respiratory, dermatological and ophthalmologic symptoms in coastal human populations in the Mediterranean [9, 10]. However, the available toxicological information is limited and the signs and symptoms are not well-defined. Currently there are no regulations on PlTX-group toxins in shellfish, either in the European Union (EU) [11] or in other regions of the world. Nevertheless, the National Reference Laboratories for Marine Biotoxins (NRLMB) have proposed a provisional limit of 250 μg/kg in shellfish [12].

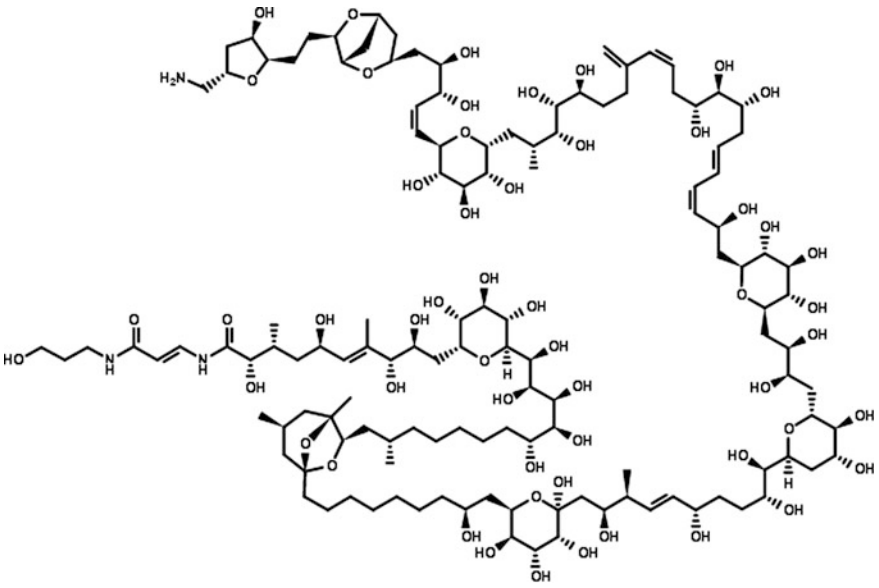

Fig. 1 Palytoxin structure

2.1 *Immunosensors for Palytoxins*

The relatively large size of PlTX makes easy the antibody (Ab) production via animal immunization, thereby simplifying the development of immunoassays and immunosensors. Optical surface plasmon resonance (SPR) biosensors offer the capability of Ab characterization and then incorporation of these bioreceptors into rapid, sensitive assays on the same platform. An SPR biosensor based on a direct format has been developed for the detection of PlTX [13]. After characterization of monoclonal antibody (MAb) kinetics and optimisation of the experimental parameters, the appropriate performance of the optical immunosensor was demonstrated by performing standard curves for PlTX in buffer as well as in seafood matrices. The limits of detection (LODs) obtained were 0.52, 2.8 and 1.4 ng/mL for PlTX in buffer, grouper and clam samples, respectively, which highlight the need of matrix matching for accurate determination of toxin concentration.

A substantial matrix effect was also observed in the development of a multiplexed SPR immunosensor for the detection of PlTX, STX, OA and DA [14]. Regarding PlTX, the toxin was immobilised onto the chip surface of a flow cell using the carbodiimide reaction to covalently link the amine group of the PlTX molecule to activated carboxylic groups of the chip. The low amount of antibody and toxin available—note that there are no commercial antibodies and that PlTX-group toxins standards are not always available—did not allow to perform exhaustive optimisation studies and validate the assay. Nevertheless, the work clearly demonstrated the applicability of SPR multichannel systems to multiple toxin detection on a single bioanalytical sensing platform. Additionally, the analysis was fast (it required less than 1 h to analyse four toxin classes per sample) and simple (a single multi-toxin extraction procedure was used). Multiplexed and miniaturised devices pave the way towards the development of compact and automated tools for high-throughput sample analysis in a fast and expensive way, and additionally facilitate to move analyses to places away from laboratories, such as harvesting sites.

Regarding electrochemical biosensors, a highly sensitive biosensor for the detection of PlTX-group toxins based on a sandwich format and electrochemiluminescence detection has been described [15] (Fig. 2). The immunosensor

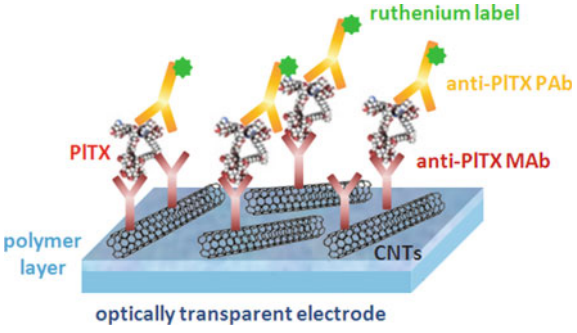

Fig. 2 Scheme of the electrochemiluminescence immunosensor for the detection of palytoxin

incorporated doubly carboxyl-functionalised multi-walled carbon nanotubes (MWCNTs), the groups on the sidewalls being used to conjugate capture anti-PlTX MAb and those on the tips being used to immobilize it on the surface of an optically transparent electrode coated by an electrochemical polymer layer. The electrochemiluminescent detection was performed by labelling the detection anti-PlTX polyclonal antibody (PAb) with a ruthenium complex. The use of CNTs increased the amount of immobilised MAb and favoured the electron transfer, providing specific and sensitive electrochemiluminescent immunosensors with an LOD as low as 0.07 ng/mL of PlTX. The applicability of the immunosensor was demonstrated by the analysis of PlTX-spiked mussels and microalgae samples. Low matrix effects were observed due to the modification of the electrode with CNTs, which minimised the non-specific adsorption, and the electrochemiluminescent transduction strategy.

2.2 Receptor-Based Biosensor for Palytoxins

Other biosensors for PlTX-group toxins are based on their mechanism of action. PlTX binds to the Na^+/K^+-ATPase in the cell membrane, inhibiting its activity and converting it into a permanently open ion channel [16]. As a consequence, a rapid sodium influx and potassium efflux from cell is produced, triggering with this a plethora of secondary effects. An optical SPR biosensor for the detection of PlTX based on its affinity towards Na^+/K^+-ATPase has been reported [17]. In this work, the Na^+/K^+-ATPase pump was immobilised on the SPR chip via thiol coupling, and the PlTX binding was afterwards recorded in real time. The developed biosensor showed a very low LOD (3.73 pg) and it was applied to the analysis of PlTX-group toxins in *Ostreopsis siamensis* cultures, which demonstrated the viability of the approach. It is interesting to mention that a previous work had tried to immobilise the Na^+/K^+-ATPase pump on the SPR sensor by amine coupling, but this kind of immobilisation prevented PlTX-group toxin from binding. Amine coupling is usually easy and effective because most macromolecules contain many groups that can participate in the reaction. However, in some cases, these reactive groups can be located near or on the active site of the macromolecule and the immobilisation process can result in conformational changes and loss of biological activity, as it seemed to occur when immobilising the Na^+/K^+-ATPase pump.

2.3 Cell-Based Electrochemical Assay for Palytoxins

Haemolysis assays for the detection of PlTX-group toxins are also based on the capacity of the toxins to interact with the Na^+/K^+-ATPase of erythrocytes and its conversion into a non-specific cation channel. Ion imbalance in red blood cells results in their haemolysis. Samples pre-treated with ouabain, a glycoside which inhibits the Na^+/K^+-ATPase pump, are used as a control to ensure the specificity of

the assay towards PlTX; the presence of ouabain will reduce the haemolytic activity of PlTXs, while it will have no effect on the haemolytic effect of other haemolytic compounds. Haemolysis is usually detected by spectrophotometry, but it is interesting to describe the work performed by Volpe and co-workers [18]. These authors combined the haemolysis of sheep erythrocytes by PlTX-group toxins with the electrochemical measurement of the lactate dehydrogenase (LDH) released in the culture supernatant after cell disruption. The LDH activity was measured by adding NADH/pyruvate as enzyme substrates and PMS^+. The later reacted with the remaining NADH to produce PMSH, which reacted with the redox mediator hexacyanoferrate(III) to produce hexacyanoferrate(II). Oxidation of hexacyanoferrate(II) was then performed on the electrode surface of a strip of eight screen-printed electrodes (SPEs). The LOD depended on the haemolysis time, being 0.007 and 0.16 ng/mL for 24 and 4 h, respectively. The necessity to use matrix-standard calibration curves for accurate analysis of PlTX was observed when applying the electrochemical assay to the analysis of PlTX-spiked samples.

3 Biosensors for Brevetoxins

Brevetoxin (PbTX)-group toxins are lipid-soluble cyclic polyether compounds (Fig. 3). They are primarily produced by the dinoflagellate *Karenia brevis* (formerly *Ptychodiscus brevis*, Pb giving name to the acronym PbTX) and can accumulate in shellfish and fish. PbTX-group toxins seem to be limited to the Gulf of Mexico, the east coast of USA, and the New Zealand Hauraki Gulf region. These toxins cause neurotoxic shellfish poisoning (NSP), with symptoms such as nausea, vomiting, diarrhoea, paraesthesia, cramps, bronchoconstriction, paralysis, seizures and coma, and dermal or inhalation exposure can result in irritant effects. Moreover, they are potentially carcinogenic. However, the toxicological database for PbTX-group toxins

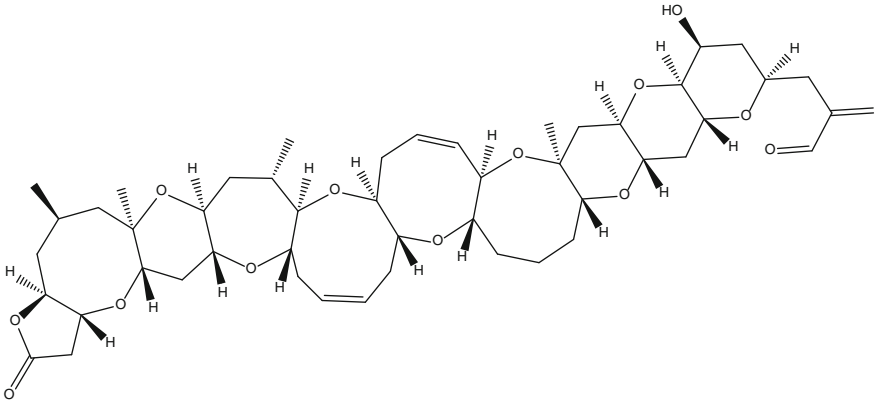

Fig. 3 Brevetoxin-1 structure

is limited. As a consequence, currently there are no regulatory limits for PbTX-group toxins in shellfish or fish in Europe [19]. Nevertheless, maximum permitted levels have been established in USA (20 mouse units (MUs)/100 g or 0.8 mg PbTX-2 equivalents/kg fish) [20], New Zealand and Australia (20 MUs/100 g, analogue not specified) [21, 22]. The discovery of new PbTX-group toxin producing algae and the apparent trend towards expansion of algal bloom distribution, suggest that PbTX-group toxins could emerge in Europe and their analysis in shellfish and fish should be considered. Further information is needed to better characterize the oral toxicity of PbTX-group toxins and their relative potencies.

3.1 Immunosensors for Brevetoxins

PbTX is a low-molecular weight toxin with very few functional groups available for cross-linking, and this usually hinders its immobilisation to develop immunosensors. The first biosensor for PbTX was reported in 1993 [23]. PbTX-3-bovine serum albumin (PbTX-3-BSA) conjugates were prepared to immobilise the toxin on a membrane through the lysine moiety of BSA. Free and immobilised PbTX-3 competed for glucose oxidase (GOx)-labelled anti-PbTX-3 Ab. Finally, the H_20_2 produced by the addition of β-D-glucose was detected amperometrically. Some years after, Kreuzer and co-workers [24] developed electrochemical immunosensors to detect different marine toxins (PbTX, OA, DA and TTX). PbTX-3-BSA conjugates were prepared via carbodiimide reaction to immobilise the toxin on the SPE surface. Nevertheless, the hydroxyl group of PbTX-3 (the only functional group available) had to be modified to make the molecule more active for coupling to BSA via carbodiimide chemistry. Once the conjugates were immobilised on the electrode surface, a competition step between immobilised and free toxin in solution for the labelled primary Ab was performed. Amperometric detection of *p*-aminophenyl phosphate produced by the enzyme label, alkaline phosphatase (ALP), was used to measure the recognition event. Sensitivity problems were observed, which were attributed to the difficulties in the conjugate synthesis and the low amount of toxin available.

The immobilisation of biomolecules on the electrode is a crucial step in the development of electrochemical immunosensors. Usually, the sensitivity of immunosensors can be improved by the control of the amount and orientation of antigens/Abs immobilised on the electrode, which have to be able to retain their biological activity. Dendrimers are hyperbranched polymers precisely engineered to carry molecules encapsulated in their interior void spaces or attached to their surface. A sensitive electrochemical immunosensor for PbTX was developed exploiting dendrimers as toxin immobilisation nanostructured supports [25]. Au NPs were simultaneously synthesised and encapsulated into amine-terminated poly (amidoamine) dendrimers, providing AuNP-PAADS conjugates that were afterwards absorbed on ester-modified gold electrodes. PbTX-2-BSA conjugates were then immobilised on the nanostructured surface. Free and immobilised PbTX-2 competed for the horseradish peroxidase (HRP)-labelled anti-PbTX-2 MAb and the

immunosensor response was measured using *o*-phenylendiamine and H_2O_2. An LOD of 0.01 ng/mL was obtained, which is much lower than the LOD for the immunosensor without Au NPs or dendrimers (0.5 and 0.1 ng/mL, respectively). As demonstrated, the use of the 3D-newtwork of AuNP-PDAAs greatly increased the amount of immobilised PbTX-2-BSA and improved the conductivity of the immunosensing interface, therefore enhancing the sensitivity of the electrochemical immunosensor.

Enzyme labels, usually HRP or ALP, are widely used and well-explored in the development of immunosensors. Nevertheless, these strategies usually require an enzyme substrate and its bioactivity can decrease when the biomolecules are exposed to reactive groups and harsh reaction conditions. To tackle this issue, other electroactive species such as thionine or guanine/adenine nucleobases can be used as indicators, as well as various nanomaterials, including quantum dots (QDs), metal nanoparticles and metal ions. Chen's research group explored the use of magnetic beads to covalently immobilise anti-PbTX-2 MAb and use them as immunosensing probes for the capture of PbTX-2 [26] (Fig. 4). The recognition element was prepared by chemical modification of PbTX-2-BSA conjugates with guanine-assembled graphene nanoribbons (GGNRs). Guanine was used as label, since it can be oxidised in the presence of $Ru(bpy)_2^{3+}$. The catalytic oxidation was electrochemically detected after the competition step and entrapment of the magnetic immunocomplex on a carbon paste electrode by a magnet. The LOD of the magneto-controlled electrochemical immunoassay was 1 pg/mL of PbTX-2 and its applicability was demonstrated by the analysis of spiked mussel, clam and cockle samples, providing similar results than those obtained with a commercial ELISA kit for PbTX-2 determination. The same research group also explored the use of metal nanoclusters as labels [27]. In this work, magnetic beads were used to co-immobilise anti-PbTX-2 and anti-dinophysistoxin-1 (anti-DTX-1) MAbs by

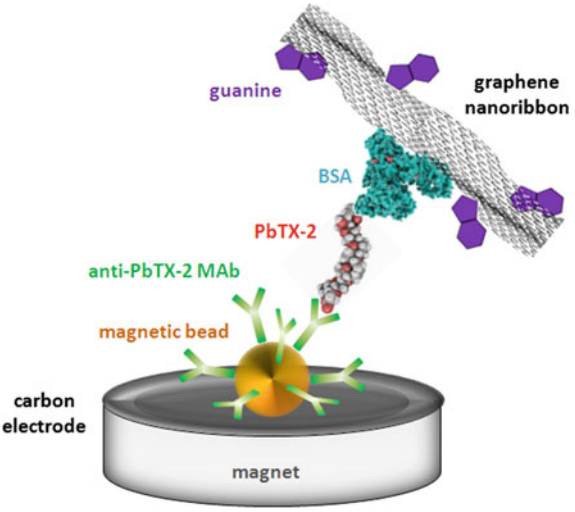

Fig. 4 Scheme of the magneto-controlled electrochemical immunosensor for the detection of brevetoxin-2

epoxy-amine reaction. Cadmium nanoclusters (CdNC) and copper nanoclusters (CuNC) were linked to PbTX-2-BSA and DTX-1-BSA, respectively, and used as distinguishable signal tags. On the basis of the competitive-type immunoassay format, the magnetic immunocomplexes were collected onto a magnetic detection cell and the electrochemical signals were simultaneously recorded at different peak potentials using square wave anodic stripping voltammetry (SWASV). The multiplexed immunosensor was able to discriminate between PbTX-2 and DTX-1 toxins without any interference. The LODs obtained were 1.8 ng/mL and 2.2 ng/mL for PbTX-2 and DTX-1, respectively. The method featured unbiased identification of negative and positive samples, as was demonstrated by the analysis of 12 spiked mussel, clam and cockle samples containing both marine toxins and the comparison with the commercial PbTX-2 ELISA kit.

Another enzyme-free electrochemical immunoassay for PbTX-2 has been recently developed using a mesoporous carbon-enriched palladium nanostructure (MSC-PdNS) as a label due to its peroxidase mimic activity [28]. In this configuration, PbTX-2-BSA was immobilised onto a nanogold-functionalised carbon electrode through the affinity between cysteine or lysine residue of BSA and gold. Afterwards, free PbTX-2 present in the sample competed with immobilised PbTX-2-BSA for the MSC-PdNS-labelled MAb, and the resulting catalytic current in the presence of H_20_2 and thionine mediator was recorded. The electrochemical immunosensor showed an LOD of 5 pg/mL and was successfully applied to the analysis of spiked mussel samples, providing results in good correlation with the PbTX-2 ELISA kit.

Quartz crystal microbalance (QCM) has also been used for the detection of small molecules such as PbTX-2 [29]. The QCM sensors measure the resonant frequency by the standard oscillation technique. The immunosensor is based on the immobilisation of dextran onto the quartz crystal coated with graphene and the following binding of the capture anti-PbTX-2 MAb to concanavalin A (ConA) via biotin-streptavidin interaction. The anti-PbTX-2 MAb-ConA immunocomplex is bound to dextran through the affinity between dextran and ConA. Gold nanoparticles functionalised with glucoamylase and PbTX-2-BSA conjugates compete with free PbTX-2 for the binding to the immobilised MAb. In the absence of free PbTX-2, amylopectin is hydrolysed by the glucoamylase to glucose, which displaces the anti-PbTX-2 MAb-ConA immunocomplex from the graphene-coated crystal surface, leading to a large change in the frequency of the immunosensor. The presence of free PbTX-2 decreased this effect. An LOD as low as 0.6 pg/mL of PbTX-2 was attained and the applicability of the QCM immunosensor was demonstrated by the analysis of a large number of spiked mussel, clam and cockle samples. Results were in good agreement with those obtained by the commercial ELISA kit.

3.2 Aptasensor for Brevetoxins

Aptamers are single stranded DNA (ssDNA) or RNA oligonucleotides that were proposed two decades ago as stable, reproducible and low-cost bioreceptors to replace antibodies in assays and biosensors. In this regard, an electrochemical biosensor for the detection of PbTX-2 using aptamers has been developed [30]. After obtaining several aptamers, the aptamer with the highest binding activity was selected using fluorescence and electrochemical impedance spectroscopy. Then, the corresponding impedimetric label-free competitive biosensor for PbTX-2 was developed. The competition was established between PbTX-2 immobilised on the gold surface and free PbTX-2 in solution in the presence of a fixed amount of aptamer. PbTX-2 had been covalently linked to the gold surfaced through a cysteamine self-assembled monolayer. An LOD of 106 pg/mL was achieved, and a high degree of cross reactivity of the aptamer towards other PbTX-group toxins was observed. The applicability of the aptasensor was demonstrated by the detection of PbTX-2 in spiked shellfish samples, which showed high recovery percentages.

3.3 Phosphodiesterase Inhibition-based Sensor for Brevetoxins

As previously mentioned, SPR is used either to study interactions or to quantify relevant molecules. However, SPR systems show low sensitivity to small molecules. Inhibition detection and sandwich protocols can be implemented in this case to solve this inconvenient. To this purpose, a SPR-based method for the detection of ladder-shaped polyether compounds (PbTX-2 among them) has been reported [31]. The ability of these molecules to inhibit the interaction of desulfo-yessotoxin to phosphodiesterase II was used to design an indirect assay able to detect several toxins. In the case of PbTX-2, inhibition was achieved in the μM range. However, this assay was not tested in shellfish matrixes and the data point to a lack of specificity, since toxins from different groups can be detected, including the marine toxins yessotoxins.

3.4 Cell-based Sensors for Brevetoxins

PbTX-group toxins bind with high affinity to receptor site 5 of the α subunit within voltage-gated sodium channels (VGSCs) present in cell membranes. Binding of PbTX to VGSCs leads to channel activation, uncontrolled Na^+ influx into the cells and depolarization phase of action potential in excitable cells, such as cardiomyocytes and neurons. With this in mind, some biosensors based on the use of cardiomyocytes [32] or neuronal networks [33] for the detection of PbTX-group toxins have been described. Since STX also binds to VGSCs on a different site of the α

subunit, both biosensors have been used to detect these two marine toxins. In contrast to PbTX, STX is a potent and selective inhibitor of VGSCs, which produces a blockage of action potentials. In both works, the excitable cells have been cultured on the surface of a microelectrode array (MEA) and the changes in the electrophysiologic parameters in the presence of the toxins have been recorded. The cardiomyocyte-based biosensor consisted of a label-free and real-time wireless 8-channel recording system, which dynamically monitor the multi-site electrical activity of the cardiomyocyte network [32]. This biosensor attained an LOD of 1.55 ng/mL of PbTX-2 within 5 min and it was able to discriminate between STX and PbTX-2. This biosensor is a clear example that the development of portable and remote devices with a real-time detection is possible but still incipient. On the other hand, the work reported by Kulagina and co-workers [33] demonstrated the utility to use a neuronal network biosensor for the detection of important neurotoxins in algal samples, with only a minimum sample preparation being required. This biosensor provided an LOD for PbTX-3 of 0.30 ng/mL in buffer and 0.43 ng/mL in the presence of 25-fold-diluted seawater. Although the two biosensors described could be used to classify potential neurotoxins due to their signature effects on electrophysiological parameters, it is important to note that this generic detection approach will neither fully identify nor quantify the individual toxins. Nevertheless, it is a complementary tool to other structure-based assays able to detect biologically active mixtures and provides an integrative overview.

4 Biosensors for Tetrodotoxins

Tetrodotoxin (TTX)-group toxins are low-molecular-weight compounds consisting of a guanidinium moiety connected to a highly oxygenated carbon skeleton that possesses a 2,4-dioxaadamantane portion (Fig. 5). TTX-group toxins are usually found in puffer fish, and are produced by endo-symbiotic bacteria that naturally inhabit the gut of the animal. These toxins have also been found in gastropods, newts crabs, frogs, sea slugs, star fishes, blue-ringed octopuses and ribbon worms. Previously they were reported only in Japan, but later on they have also been found in Korea, Taiwan, China, Thailand, Bangladesh, India, Australia, New Zealand, Hawaii, USA, Madagascar, Norway, Israel, Egypt, Greece and Spain (gastropod caught in Portugal). TTX is a potent neurotoxin, which acts as a sodium channel blocker. Some symptoms of poisoning are tingling of the tongue and lips, headache,

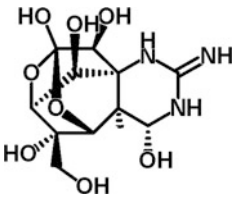

Fig. 5 Tetrodotoxin structure

vomiting, muscle weakness, ataxia and even death [34]. In Japan, the regulatory limit for TTX in food is 2 mg/kg, while in the USA a zero level has been established. No regulation specific for this toxin exists in Europe, although the commercialisation of tetraodontidae is forbidden.

4.1 Immunosensors for Tetrodotoxins

Most of the existing biosensors for the detection of TTX are based on the use of specific Abs as biorecognition molecules. However, the production of Abs has been hindered by the small size of this toxin. In regard to this, TTX needs to be conjugated to protein carriers to immunize the animals but also to develop the corresponding immunoassays and/or immunosensors.

A few works exploit the use of TTX conjugates in direct competitive immunoassays for the subsequent transfer to SPEs to develop the corresponding electrochemical immunosensors [24, 35]. Following the same procedure used to develop the electrochemical immunosensor for PbTX in Sect. 3.1. [24], TTX has been conjugated to BSA, and the conjugates have been then immobilised on SPEs for the subsequent competition step. In the other work, TTX has been conjugated to the ALP enzyme, the conjugate being used in this case as a tracer [35]. The approach using the tracer provided an LOD of 1 ng/mL. In the work of Kreuzer and collaborators [24], the use of TTX-BSA coater together with a primary Ab labelled with the ALP enzyme, allowed to obtain the lowest LOD ever reported for TTX (0.016 ng/mL). In this case, it is important to mention that the use of a labelled primary Ab is an advantage compared to the use of a secondary Ab, since it improves the performance of the biosensor and decreases the analysis time.

Several optical SPR immunosensors have been reported for TTX. Due to the small size of TTX, most of them are based on the immobilization of the toxin on gold SPR chips and the subsequent competition step. In the first works, the immobilization of TTX was performed through mixed self-assembled monolayers (SAMs) of hydroxy- and amino-terminated oligo-ethylene glycol alkanethiols (OEG-ATs) [36–39]. While the amino-terminated OEG-ATs were used to covalently link the TTX to the surface through formaldehyde, hydroxy-OEG-ATs were used as spacer molecules to avoid cross-linking between amino-OEG-ATs. The use of SAMs provides an oriented TTX immobilisation and the ethylene glycol molecules minimise the non-specific adsorption of proteins onto the surface of the chip. All these immunosensors attained similar LODs, ranging from 0.3 to 3.4 ng/mL of TTX in buffer. Recently, Campbell and co-workers [40] have proposed the direct TTX immobilisation on carboxymethylated chips. Although in this approach TTX is not oriented as properly as when using SAMs, it simplifies and shortens the experimental protocol, still retaining the performance of the biosensor (LOD ≤ 0.2 ng/mL) (Fig. 6). The applicability of the SPR immunosensors to the analysis of naturally-contaminated samples has been demonstrated in several matrixes: puffer fish [37–39], sea snail [40], human urine [37], milk and apple juice [39].

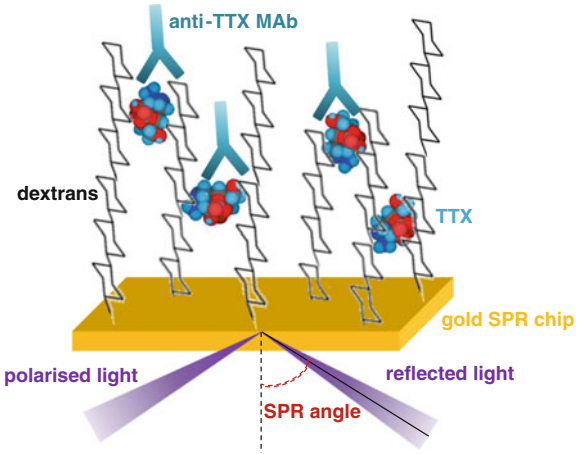

Fig. 6 Scheme of the optical SPR immunosensor for the detection of tetrodotoxin

Although, as previously mentioned, the detection of small molecules by SPR usually requires the immobilisation of the antigen, Yakes and co-workers [41] have proposed the immobilisation of the Ab on the chip and the subsequent non-competitive detection of TTX. This new configuration provided an LOD of 0.09 ng/mL, even lower than in the previous SPR immunosensors. This has been possible thanks to the advances in SPR instrumentation, including higher signal-to-noise ratio, improved fluidics with stronger vacuum pumps and higher number of antibody sites on the chips, advantages that could benefit the detection of other small analytes.

4.2 Aptasensor for Tetrodotoxins

An electrochemical aptasensor for the detection of TTX has been recently proposed [42]. In this work, glassy carbon electrodes were modified with poly (4-styrenesolfonic acid)-doped polyaniline films (PSSA/PANI) by electropolymerisation. Afterwards, an amino-terminated aptamer against TTX was covalently immobilised on the PANI/PSSA films via glutaraldehyde cross-linking. The detection of TTX was assessed by electrochemical impedance according to the charge transfer resistance of the PSSA/PANI film, providing an LOD of 0.199 ng/mL. This low LOD was attained thanks to the ordered monolayer of conductive PANI/PSSA films which improved the electron transfer.

4.3 Cell-based Sensors for Tetrodotoxins

Apart from the antibody-based methods, cell-based biosensors also deserve to be mentioned since they provide realistic models mimicking the original tissue, information which is usually complementary to that obtained from immunosensors.

These cell-based methods are based on the mechanism of action of TTX, which is able to selectively inhibit sodium channels, blocking both nerve and muscular action potentials. Following this principle, some biosensors have been reported [43–46]. In the simplest approach, Cheun and co-workers [43] developed a biosensor consisting of a sodium electrode covered with frog bladder membrane integrated within a flow cell. TTX concentration was measured from the inhibition ratio of the sensor peak output by patch clam recording, the lowest amount detected being 86 fg. The application of this biosensor to the analysis of puffer fish was successfully achieved and results were in agreement with those obtained by MBA.

Aiming at the development of compact and multiplexed devices, portable microelectrode arrays incorporating cultured neuronal networks for the detection of TTX have been reported [44, 45]. In these works, spinal cord cells cultured on gold electrodes were exposed to TTX and extracellular potentials from these cells were recorded, providing an IC_{50} of 0.95 ng/mL of TTX. Following the same biorecognition principle but a different measurement technique, a cardiomyocyte-based impedance biosensor has been recently developed for both TTX and STX, because of their common mechanism of action [46]. In this case, cardiomyocyte cells growth and beating status after TTX treatment were measured on 96-well gold electrodes plates by impedance recording. Although the LOD is higher than in other methods described for the detection of TTX (89 ng/mL vs 0.087 ng/mL for TTX and STX, respectively), this novel real-time and label-free impedimetric biosensor platform is versatile and could be applied to the detection of other neurotoxic molecules.

5 Conclusions and Perspectives

In the past years, a few biosensors for the detection of PlTXs, PbTXs and TTXs have been described. Most of them are immunosensors, but some cell-based sensors, a receptor-based biosensor for PlTX, and even aptasensors for PbTXs and TTXs and an inhibition-based sensor for PbTXs have also been recently reported, showing a breakthrough in the development of biosensors for the detection of emerging marine toxins. This is not the case for other emerging toxins such as AZAs, CTXs and CIs, for which no biosensors have been described yet. Nevertheless, given the numerous possibilities in the development of biosensors (e.g. different transduction strategies, biorecognition molecules and configurations) and the multiple advantages they offer (e.g. simplicity, rapidity, low-cost, sensitivity, multiple toxin detection and portability), it is safe to say that the future of biosensors for the detection of most marine toxins is assured.

In the field of immunosensors for the detection of emerging marine toxins, it is important to keep in mind the difficulty in some cases to obtain the recognition antibodies, since they are not always commercially available and they can be difficult to produce due to the small size of some marine toxins. It is important to mention that the interaction of toxins with antibodies is based on a structural

recognition, thus not necessarily related to their toxicity. However, antibodies are robust biorecognition molecules, with high affinity and sensitivity towards their analytes, sometimes being able to detect different analogues of the same group of toxins. Additionally, their easy handling and manipulation allow their integration into different assay formats, offering a wide range of possibilities and configurations using several detection methods.

Aptasensors have been proposed to avoid some of the drawbacks associated with the development of immunosensors, such as the complicated in vivo production of antibodies. Aptasensors for PbTX and TTX have been described, demonstrating that the aptamer-based detection methods are promising. Nonetheless, it is necessary to note that the development of aptamers still require the use of toxin standards, which are not always available.

Receptor-based and cell-based biosensors provide a signal related with the toxicity of the toxins. In that sense, these biosensors have the advantage to better reflect the toxic effects that these toxins may cause to in vivo models, contrarily to structural-based sensors. However, these approaches do not allow identifying or discriminating compounds different from the target toxins but that share the same mechanism of action. Moreover, cell-based biosensors imply the use of "live" material, a factor that may increase variability in the response.

Biosensors can be considered as effective screening tools to be used in combination with confirmatory instrumental analysis methods to achieve highly specific, sensitive and fast routine monitoring of emerging marine toxins. Research on biosensor development for marine toxins should be focused on elucidating or better describing the mechanisms of action of emerging marine toxins, producing biorecognition molecules, and validating the biosensing systems with naturally-contaminated samples in order to promote their implementation. To really foster the use of biosensors in food safety and environmental monitoring, compact analysis devices, sensitive, robust, reliable and easy to handle even by non-trained personal are desired. The use of biosensors to detect and quantify emerging marine toxins is being achieved, but the implementation of such devices in daily life still requires a lot of effort. Nevertheless, progress in this area is very fast and, provided the scientific community will focus not only on the development of the bioanalytical systems but also on their validation, biosensors for emerging marine toxins could soon be implemented in routine analysis.

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