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Immunosensor array platforms based on self-assembled dithiols for the electrochemical detection of tetrodotoxins in puffer fish

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Abstract

The recent detection of tetrodotoxins (TTXs) in European fish and shellfish has emphasized the urgent need to develop specific, selective, rapid and easy-to-use methods for their detection to assess the potential risk posed to human health. For this purpose, a dithiol self-assembled monolayer (SAM)-based immunoassay previously performed on maleimide plates (mELISA) has been adapted to gold electrode arrays for the development of an electrochemical immunosensor for TTX. The electrochemical SAM-based immunosensor designed herein, provided an oriented, stable and spaced sensing platform for the determination of TTX, attaining a limit of detection of 2.6 ng mL^{-1}. The applicability of the biosensor array was demonstrated by the accurate quantifications obtained in the analysis of different tissues of several puffer fish species (*Lagocephalus lagocephalus, L. sceleratus and Sphoeroides pachygaster*) caught along the Mediterranean coast of Spain. The good agreements found between the TTX concentrations determined by the

immunosensor array platforms and those determined by mELISA, surface Plasmon resonance (SPR) immunosensor and liquid chromatography-high resolution mass spectrometry (LC-HRMS) analysis, proved the feasibility of the approach. The electrochemical immunosensor enables the determination of TTXs at levels as low as 0.07 mg TTX equiv. kg^{-1} tissue, thus, well below the Japanese value of 2 mg TTX equiv. kg^{-1} tissue used as a criterion to consider puffer fish safe for consumption. Compared to the colorimetric SAM-based approach, the immunosensor array described herein shows promise towards the development of disposable, portable and compact analysis tools applicable in monitoring programs for the surveillance of fishery products.

Keywords: electrochemical immunosensor array, self-assembled monolayer, dithiol, tetrodotoxin, puffer fish, food safety

1. Introduction

Tetrodotoxin (TTX) is a low-molecular-weight potent marine neurotoxin believed to be primarily produced by certain marine endosymbiotic bacteria [1]. Once exogenously produced, it may accumulate through the food webs and enter into other organisms [2], eventually reaching humans. Whilst this toxin was originally discovered in the organs of fish from the *Tetraodontidae* family, especially puffer fish [3], its distribution has expanded to a wide range of marine organisms including amphibians, echinoderms, cephalopods and bivalve mollusks [4]. Structurally, TTX and several TTX analogues have been described as heterocyclic, heat-stable and hydrophilic compounds [5, 6]. They are well-known selective sodium channel blockers [7], which obstruct neural and muscular transmission with a similar mechanism of action to saxitoxin (STX), a potent Paralytic Shellfish Poisoning (PSP) toxin.

Tetrodotoxin is responsible for numerous human intoxications worldwide in most cases with poisoning occurring following the ingestion of contaminated seafood. Typical poisoning symptoms include mild gastrointestinal effects, respiratory paralysis, numbness and even death [8]. Food poisoning incidents were first restricted to warm water regions, particularly in the Pacific and Indian Oceans, affecting mainly Japan and China [9], where puffer fish (*fugu)* is legally consumed. Although fatalities and intoxications do still occur in Japan, this risk was reduced by the value of 2 mg TTX equiv. kg^{-1} of edible portion used as a criterion to consider puffer fish safe for consumption [10]. Although there is no regulatory limit for TTX in Europe, EU regulation establishes that fishery products derived from poisonous fish of the families *Tetraodontidae*, *Molidae*, *Diodontidae* and *Canthigasteridae* must not be placed in the market [11, 12]. However, given that

fugu is sometimes offered in some private events, the risk may not be completely discarded [13]. TTX poisoning episodes have also been reported following the consumption of mislabeled fish products [14] and, additionally, may be consumed by accident due to their similarity to other non-poisonous fish.

Recently, puffer fish from the species *Lagocephalus sceleratus* have been found migrating from the Red Sea to the Mediterranean Sea through the Suez Channel (Lessepsian migration) [15]. Additionally, several reports confirm the increasing occurrence of TTX in European seafood. A first toxic episode was described in 2007 in Málaga (Spain), caused by the consumption of trumpet shells caught in Portugal [16]. Afterwards, two episodes were reported along the Mediterranean coast, following the ingestion of *L. sceleratus* [17, 18]. In the last two years (2014-2015), TTXs have been found in bivalve mollusk shellfish grown in the south of England [19], along the Greek coast [20] and in the Netherlands [21].

Currently, there is no official method of analysis for TTXs in Europe. Nevertheless, different methodologies have been developed for their detection, being LC-MS/MS analysis [22] and the mouse bioassay (MBA) [23] the most widely employed. Although proven useful techniques, the need of skilled personnel, expensive equipment, standards of TTXs and the establishment of toxicity equivalency factors (TEFs) for LC-MS/MS have hampered their appropriate performance. Additionally, the lack of specificity of MBA in discriminating between other coexisting PSP toxins and the non-availability of certain TTX standards, have restricted its use. Due to these limiting factors and given the increasing occurrence of TTX in Europe, the development of specific, rapid and cost-effective methods as support tools in monitoring programs to ensure human safety is highly required. To date, alternative methods based on antibodies that have been developed for TTX include several colorimetric immunoassays [24-30], two electrochemical immunosensors [31, 32] and optical surface Plasmon resonance (SPR) immunosensors [33-36]. Biosensors for other marine toxins have also been reported in the recent years, showing the increasing interest in the field [37-40].

Recently, a special configuration of a colorimetric immunoassay was used by our group for the development of an ELISA for TTX [27]. This assay was based on the immobilization of TTX through dithiols self-assembled on maleimide plates (mELISA) and provided an ordered and oriented antigen immobilization, which proved to be an efficient, reliable and powerful tool for the precise quantification of TTXs. With the aim of moving towards miniaturized and compact devices, this self-assembled monolayer (SAM)-based strategy for the development of an electrochemical immunosensor for TTX detection was exploited herein. The conversion of the colorimetric immunoassay to the electrochemical immunosensor was achieved by transferring the SAM-based strategy from microtiter plates to arrays of gold electrodes, and by recording the electrochemical signal after selecting the appropriate redox mediator for the horseradish peroxidase (HRP)

label (**Figure 1**). Compared to the previous reports of electrochemical immunosensors [31, 32], the dithiol-based SAM strategy provides not only an oriented and stable antigen-modified sensing platform, but also decreases the non-specific adsorption. Additionally, whereas the previous reports were proofs of concept, the present work goes a step further, as demonstrated by the application of the immunosensor array platform to the analysis of natural puffer fish samples. Different tissues of several puffer fish species (*L. lagocephalus, L. sceleratus and Sphoeroides pachygaster*) were analysed for the accurate determination of TTX equivalent contents and the results were compared to those provided by the previously developed mELISA, by the SPR immunosensor and by liquid chromatography-high resolution mass spectrometry (LC-HRMS) analysis [41].

2. Materials and methods

2.1. Reagents and materials

The TTX standard was obtained from Tocris Bioscience (Bristol, UK). TTX standard solution was prepared at 1 mg mL^{-1} in 10 mM acetic acid. The anti-TTX monoclonal antibody (mAb) TX-7F was produced as previously described [25, 36]. DithiolalkanearomaticPEG6-COOH (carboxylate-dithiol) was purchased from Sensopath Technologies (Bozeman, USA). Anti-mouse IgG (whole molecule)-horseradish peroxidase antibody produced in rabbit (IgG-HRP), bovine serum albumin (BSA), ethanolamine, ethylendiamine, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), ethylenediaminetetraacetic acid (EDTA), formaldehyde solution (37%), 2-(N-morpholino) ethanesulfonic acid hydrate (MES), *N*-hydroxysuccinimide (NHS), potassium chloride, potassium hexacyanoferrate II, potassium hexacyanoferrate III, potassium phosphate dibasic, potassium phosphate monobasic, thioctic acid, TMB liquid substrate, TMB enhanced one component HRP membrane substrate and Tween-20 were all supplied by Sigma-Aldrich (Tres Cantos, Spain). HBS-EP buffer (0.01 M HEPES, 0.15 M NaCl, 3 mM EDTA, and 0.005% Surfactant P20, pH 7.4) was provided by GE Healthcare (Little Chalfont, UK). Maleimide-activated plates were obtained from Thermo Fisher Scientific (Madrid, Spain).

2.2. Equipment, electrodes and software

Cyclic voltammetry (CV) measurements were performed with a PGSTAT128N potentiostat purchased from AUTOLAB (Massó Analitica S.A., Barcelona, Spain). Data was collected and evaluated by General Purpose Electrochemical System (GPES) software version 4.9. Disposable screen-printed gold electrodes (SPGE), with gold as working and counter electrodes and silver as a reference electrode (220AT), were purchased from Dropsens S.L. (Oviedo, Spain).

Chronoamperometries (CA) were recorded with a Palmsens multiplexer potentiostat (Palmsens BV, The Netherlands) and a DRP-CAC8x connector from Dropsens S.L. (Oviedo, Spain). Data were collected and evaluated by PSTrace version 4.7.2 software. The 8x screen-printed gold electrode arrays, with gold as working and counter electrodes and silver as reference electrode (8X220AT), were purchased from Dropsens S.L. (Oviedo, Spain).

Colorimetric measurements for mELISA experiments were performed with a Microplate Reader, GEN5 2.09 software from BIO-TEK Instruments, Inc. (Vermont, USA).

An SPR device (Biacore Q) with Control Software (Version 3.0.1), BIAevaluation software version 4.1, and CM5 sensor chips were obtained from GE Healthcare Bio-Sciences (Uppsala, Sweden).

LC-HRMS analysis was carried out with an Orbitrap-Exactive HCD (Thermo Fisher Scientific, Bremen, Germany) and data was processed with Xcalibur 3.1 software (ThermoFisher Scientific, Bremen, Germany).

The adjustment of calibration curves to sigmoidal logistic 4-parameter equations was performed using SigmaPlot 12.0 (Systat Software Inc., California, USA).

2.3. Puffer fish samples and toxin extraction

A total of 6 female puffer fish from NW Mediterranean Sea were used in this study, 3 of them belonging to the species *L. lagocephalus* (1 from Blanes, 1 from Altafulla and 1 from Denia, Spain), 1 to the species *L. sceleratus* (from Denia, Spain) and 2 to the species *S. pachygaster* (1 from LLançà and 1 from Denia, Spain). One of the *L. lagocephalus* (determined as non-containing TTX by LC-MS/MS; “blank puffer fish sample”) was used for the evaluation of matrix effects and spiking experiments.

Toxin extraction from puffer fish tissues (muscle, skin, liver and gonads) was performed as described previously [27]. Briefly, a double extraction was performed with 0.1% acetic acid for each tissue. In the case of liver, an additional liquid-liquid partition with hexane was required. The analysis by the mELISA, SPR immunosensor and electrochemical immunosensor was performed with the aqueous extracts, but for the LC-HRMS analyses, extracts were evaporated, re-dissolved in methanol and filtered through 0.2-µm nylon filters.

2.4. Electrochemical immunosensor array platforms

The titration assay protocol used for the immobilization of TTX through carboxylate-dithiol self-assembled on SPGEs was similar to that used for the mELISA previously described [27], with some modifications of volumes and immobilization supports. The steps taken were as follows: (1) 15 µL of 0.1 and 1 mM

carboxylate-dithiol in 0.1 M potassium phosphate, 10 mM EDTA, pH 7.2 (PBS) were placed on the SPGEs of the array (8x220AT) and self-assembled for 3 h. (2) Carboxylic groups of dithiols were activated by the addition of 15 µL of 0.1 M NHS and 0.4 M EDC (1/1, v/v) in 25 mM MES, pH 5.5 for 30 min. (3) Activated carboxylic groups reacted with primary amines of ethylenediamine (15 µL of 0.1 M ethylenediamine in PBS) for 30 min, forming amide bonds (4) The remaining carboxylic groups were deactivated by adding 15 µL of 1 M ethanolamine in PBS for 30 min. (5) TTX was then immobilized on ethylenediamine through formaldehyde cross-linking following an amino−amino reaction, adding 14.5 µL of 0.2 and 2 µg mL^{-1} of TTX in PBS and 0.5 µL of formaldehyde (37%) for 15 h. Following TTX immobilization, (6) 15 µL of 1/800, 1/1,600 and 1/3,200 mAb dilutions in 1% BSA-PBS were incubated for 30 min. (7) Remaining sites were blocked with 1% BSA-PBS for 30 min. (8) 15 µL of IgG-HRP at 1/1,000 dilution were incubated onto the electrodes for 30 min. (9) Finally, TMB enhanced liquid substrate was allowed to react for 10 min and chronoamperometries (CA) were recorded at -0.11 V for 5 s (current intensities were taken at 0.5 s). After each step, electrodes were rinsed with washing buffer (0.1 M potassium phosphate, 0.15 M NaCl, 0.05% Tween-20, pH 7.2) and air-dried.

Once the optimum concentrations of carboxylate-dithiol and TTX were selected, three competition assays were performed, the protocol differing from the checkerboard only in step (6). Thus, 7.5 µL of 1:800, 1/1,600 and 1/3,200 mAb dilutions were mixed with 7.5 µL of TTX standard solution or “sample” (blank puffer fish tissue extract, TTX-spiked puffer fish tissue extract or naturally-contaminated puffer fish tissue extract) and pre-incubated in tubes for 15 min before placing the mixture on the electrodes for 30 min. Several competitive electrochemical assays were performed differing in the sample used in the competition step: 1) A TTX standard calibration curve was constructed from 0.625 to 80 ng mL^{-1} in PBS. The background was corrected with respect to the controls without mAb and the curve was fitted to a sigmoidal logistic 4-parameter equation. From the equation, inhibitory concentrations (ICs) were calculated. Specifically, the midpoint (IC_{50}), the limit of detection (LOD) established as the IC_{20}, and the working range, considering the linear interval of the curve (IC_{20}-IC_{60}), were determined. 2) Puffer fish matrix effects were evaluated using different tissues of a blank puffer fish sample (muscle, skin, liver, and gonads) with no presence of TTXs as determined by LC-MS/MS analysis, using a matrix concentration of 40 mg mL^{-1}. 3) Different puffer fish tissue extracts at a matrix concentration of 40 mg mL^{-1} were spiked with TTX at 2.5, 5 and 10 ng mL^{-1} to determine the percentage of toxin observed in spiked-puffer fish tissue extracts with respect to the spiked TTX levels. 4) Finally, extracts of different tissues from 5 puffer fish individuals were analyzed. Toxin standard solution and samples were prepared in PBS, diluted by half and assayed in quadruplicate.

2.5. SAM characterization by reductive desorption

The electrochemical reduction of the thiol-gold bond between the carboxylate-dithiol/thioctic acid SAMs and the SPGEs was evaluated through CVs. Bare, 1 mM carboxylate-dithiol and 100 mM thioctic acid SAM-modified individual SPGEs (220AT) were immersed in thoroughly degassed PBS and two consecutive CVs were recorded from -0.50 to -0.90 V at a scan rate of 10 mV s^{-1}.

2.6. TTX immobilization characterization

The immobilization of TTX on carboxylate-dithiol SAM was performed following the protocol used for the development of the electrochemical immunosensor from steps 1 to 5, but using individual SPGEs (220 AT) and 40 µL of working volume instead of 15 µL. For the immobilization of TTX on thioctic acid, step 1 was performed using 40 µL of 100 mM thioctic acid in MeOH:H_20 (1:1).

The immobilization of TTX on carboxylate-dithiol and thioctic acid SAMs was characterized by CV using 40 µL of 2.5 mM potassium hexacyanoferrate redox couple (1:1, 5 mM potassium hexacyanoferrate (II):5 mM potassium hexacyanoferrate (III)) in 0.1 M phosphate buffer solution with 0.1 M KCl, pH 7.2. CVs were recorded from -0.25 to +0.45 V at scan rate of 10 mV s^{-1} on bare SPGEs, 1 mM carboxylate-dithiol and 100 mM thioctic acid SAM-modified electrodes, after ethylendiamine addition, and after TTX immobilization (5,000 ng/mL). All steps were carried out using the same electrode, rinsing thoroughly with wash buffer between steps.

2.7. mELISA

Briefly, the 5 puffer fish samples analyzed by the electrochemical immunosensor developed herein were also analyzed by the mELISA as previously reported [27]. All puffer fish tissue extracts were analyzed at a matrix concentration of 40 mg mL^{-1}. Puffer fish extracts of *L. sceleratus,* containing high levels of TTX, were further diluted to allow for quantification within the working range (IC_{20}-IC_{80}). All samples, mAb and toxin standard solutions were prepared in PBS, diluted by half and assayed in triplicate. Quantifications were corrected by applying the mELISA correction factors (CFs) established for each tissue [27].

2.8. SPR immunosensor

The protocol used for the analysis of puffer fish samples was carried out as described in previous works [27, 36]. A TTX standard calibration curve was constructed from 0.06 to 1,000 ng mL^{-1}. As for mELISA, all puffer fish extracts were analyzed at a matrix concentration of 40 mg mL^{-1}. Puffer fish tissue extracts of *L.*

sceleratus, containing high levels of TTX, were further diluted to allow for quantification within the working range (IC_{20}-IC_{60}). All samples, mAb and toxin standard solutions were prepared in HBS-EP buffer, diluted by half and assayed in duplicate. As for mELISA quantifications, TTX contents obtained by the SPR immunosensor were corrected by applying the corresponding SPR CFs established for each tissue [27].

2.9. LC-HRMS analysis

The quantification of TTXs contents in five samples of puffer fish extracts of the species *L. lagocephalus, L. sceleratus and S. pachygaster* was performed following the protocol reported in Rambla-Alegre et al. [41]. The sum of total TTX as well as the individual TTX analogues concentrations were calculated for muscle, skin, liver and gonads tissues of five samples. To facilitate the comparison of the results with the other techniques used herein, only the sums of TTX contents are shown.

2.10. Statistical analysis

Correlations between TTX contents obtained by the electrochemical immunosensor and those obtained by mELISA, SPR immunosensor and LC-HRMS analysis were evaluated using linear regression. To evaluate if differences were significant between techniques, data were tested for normality using the Shapiro-Wilk test. For data following a normal distribution, t-test was performed; otherwise, Mann-Whitney Rank Sum Test was performed. The level of significance was set at $p<0.05$. All statistics were performed using Sigmastat 3.1 software (Systat Software Inc. California, US).

3. Results and discussion

Due to the similar affinity of maleimide and gold for thiol groups, the SAM-based strategy used for the development of the mELISA reported previously [27] was followed for the development of the present electrochemical immunosensor, whereby maleimide plates were replaced by SPGEs and the electrochemical signal was recorded instead of the colorimetric one. Prior to the development of the electrochemical immunosensor, the SAM formation as well as the different steps for the immobilization of TTX on SAMs were characterized.

3.1. SAM characterization by reductive desorption

Since thiolated compounds have been proven to experience desorption from gold surfaces at negative potentials under highly alkaline or neutral pH solutions, reductive desorption experiments were carried out to

characterize the self-assembling of carboxylate-dithiol and thioctic acid on individual SPGEs (corresponding structures are shown in **Figure 2**). **Figure 3** shows the forward scans of the two consecutive CVs carried out on bare SPGEs and carboxylate-dithiol SAM-modified SPGEs. As expected, no reduction peaks were observed when using bare SPGEs (dotted line) in any of the scans. On the contrary, a peak at -0.8 V was obtained in the first scan on SAM-modified electrodes (solid line), which corresponds to the reduction of thiol groups [42, 43]. In the second scan, no peak was obtained, suggesting that the negative potential applied to the electrodes caused the complete thiol bond break in the first scan, and demonstrating the previous presence of SAM on the modified electrode. Although being destructive, this technique has been useful to characterize the carboxylate-dithiol SAM formation. Unfortunately, thioctic acid SAM-modified SPGEs did not provide a suitable response (no reduction peak was observed), but the strategy was retained for further evaluation.

3.2. Characterization of TTX immobilization on SAMs

The different steps for the immobilization of TTX on carboxylate-dithiol and thioctic acid SAMs were monitored by CV. The CVs of the $[Fe(CN)_6]^{3-/4-}$ at bare, SAM, ethylendiamine and TTX-immobilized SPGEs were recorded (**Figure 4**). Both SAMs resulted in a decrease of the electrochemical signal due to the lower permeability of the electrode, being drastic in the case of thioctic acid and only slight in the case of carboxylate-dithiol. As described in the work performed by Fragoso and co-workers [44], the length and structure of thiolated molecules as well as the corresponding orientation and SAM packaging could influence the charge transfer in the ferrocyanide solution. In this case, despite being longer, carboxylate-dithiol provides a monolayer with the long chains of polyethylene glycol and spaced slightly titled from the perpendicular to the electrode surface, which favors the electron transfer. On the contrary, the shorter thioctic acid-based SAM was more packed and the short alkyl chain obliquely oriented to the electrode, resulting in a higher electron transfer blocking. When the positively charged ethylendiamine was added to the thioctic acid SAM-based SPGEs, oxidation and reduction peaks did not substantially change, probably due to the high blocking effect from the thioctic acid. On the contrary, the ethylendiamine addition significantly counteracted the blocking effect caused by the carboxylate-dithiol, as it is demonstrated by the reappearance of the well-defined oxidation and reduction peaks. Finally, an appropriate CV response after TTX immobilization was obtained for carboxylate-dithiol SAM-modified SPGEs (only a mild improvement was observed when using thioctic acid). Indeed, this result together with the unsatisfactory results obtained in the SAM reductive experiment for thioctic acid SAM reinforce the hypothesis that carboxylate-dithiol SAMs was

the only choice for the immunosensor development. The orientation provided by carboxylate-dithiol probably favors the TTX immobilization and, consequently, the subsequent antigen-antibody affinity interaction. Furthermore, the spacing effect of carboxylate-dithiol SAM may not only favor the antigen-antibody affinity interaction, but also eases the electrochemical redox mediator to reach the electrode surface, thus, promoting the electron transfer. Given the improved orientation and permeability displayed by the carboxylate-dithiol SAM strategy, thioctic acid was no longer used in the development of the electrochemical biosensor.

3.3. Electrochemical immunosensor development

In order to select the optimal concentrations of carboxylate-dithiol, TTX and mAb, electrochemical titrations were performed using conventional TMB as redox mediator. On the evaluation of the carboxylate-dithiol concentration although 0.1 mM of carboxylate-dithiol provided a proper layer in the mELISA configuration, this concentration did not provide sufficient current intensities (<1 μA) in the electrochemical approach, probably due to the different surface properties of the SPGEs. Therefore, the following experiments were performed using 1 mM of carboxylate-dithiol, which provided higher current intensities (>2 μA). Afterwards, immobilization of TTX on SAMs was assessed using the concentration of TTX required for the mELISA configuration of 0.5 μg mL^{-1} [27]. This TTX concentration was examined with different mAb dilutions: 1/800, 1/1,600 and 1//3,200. Controls without mAb were included in the checkerboard to have an indication of the non-specific binding of the HRP-labelled secondary antibody on the system. At this concentration, similar reduction current intensities were attained regardless of the antibody concentration (around 2 μA), suggesting that at 0.5 μg mL^{-1} of TTX, the SAM was completely saturated of mAb even when using the 1/3,200 dilution. As the high non-specific adsorption values (between 60 and 70%) seem to indicate, this amount of TTX would not be enough to fully cover the specific binding sites of the SAM and whereby these sites could be non-specifically occupied by the secondary antibody. In order to obtain higher current intensities, a higher TTX concentration (2 μg mL^{-1}) was used in the immobilization step. In this case, higher current intensities were attained and a trend was observed according to the different mAb dilutions (4.2, 3.4 and 2.8 μA for 1/800, 1/1,600 and 1/3,200 dilutions, respectively). As expected, the non-specific binding from the secondary antibody followed the opposite trend (35, 44 and 52%, respectively). Therefore, the use of a higher TTX concentration for the immobilization step increased the specific response and lowered the non-specific binding.

In order to evaluate the sensitivity of the immunosensor, electrochemical competition assays were carried out with 1 mM of carboxylate-dithiol, 2 μg mL^{-1} of TTX and several free TTX concentrations to choose between the three mAb dilutions. While the signal provided by the highest antibody dilution (1/3,200) was not sufficient to discriminate between different TTX concentrations, significant differences in the current intensity were observed when using 1/800 and 1/1,1600 mAb dilutions. As expected, slightly better sensitivity was achieved with 1/1,600 than with 1/800 mAb dilutions (50% of mAb binding corresponding to 40 and 80 ng mL^{-1} of TTX, respectively). Therefore, conditions selected to perform further competitive assays were: 1 mM of carboxylate-dithiol, 2 μg mL^{-1} of TTX in the immobilization step and 1/1,600 mAb dilution. The shift from the colorimetric to the electrochemical approach required using 10-fold higher concentration of carboxylate-dithiol, 40-fold higher of TTX, and double mAb concentration, due to the different surface characteristics of maleimide-coated microtiter wells and SPGEs.

With the aim of improving the electron transfer, thus, increasing further the current intensities, a different redox mediator (enhanced TMB) was used at the same conditions. Compared to conventional TMB, enhanced TMB provided current intensities 4-fold higher, as well as 5% less of non-specific binding. These differences are probably due to the different nature of the final product resulting from the enzyme reaction. While the product of the reaction with conventional TMB is in liquid form, an insoluble precipitate is produced when the enzyme reacts with enhanced TMB. This solid precipitate concentrates at the electrode surface, enhancing the electrochemical response. Given the higher intensities and lower non-specific binding values obtained with enhanced TMB, the following experiments were performed with this mediator.

A calibration curve in buffer was then constructed using enhanced TMB (**Figure 5**) and, from the adjustment, a regression factor (R) of 0.992 was obtained. Standard deviation (SD) for 4 replicates (n=4) was ≤15% for all concentrations. Overall, the use of this mediator allows the improvement in sensitivity of the immunosensor, by lowering the IC_{50} from 40 to 7 ng mL^{-1}. The LOD was found to be 2.6 ng mL^{-1}, and the working range (IC_{20}-IC_{60}) was 2.6-10.2 ng mL^{-1}.

The sensitivity achieved by the present electrochemical immunosensor was in good agreement with the two electrochemical immunosensors reported for TTX [31,32]. In comparison with the previous works, this electrochemical immunosensor enables for the first time the detection of TTX by immobilizing the toxin on specific locations of a SAM through stable chemical bonds. This SAM-based approach provides an oriented, specific and spaced TTX sensing platform, avoiding the need to conjugate the toxin to protein carriers (e.g. BSA), which may block the electron transfer, and decreasing the non-specific binding thanks to the carboxylate-dithiol chemical structure. In addition, the new electrochemical platform has been characterized

in detail, demonstrating the toxin immobilization as well as the enhanced electron transfer favored by the carboxylate-dithiol SAM. The shift from the colorimetric to the electrochemical SAM-based approach represents an advance towards disposable, portable and compact devices for the detection of TTX. In addition to these benefits, the multiplexed configuration permits reducing the reagent volumes (only 15 μL per electrode) as well as the consecutive measurement of 8 samples, decreasing the cost and analysis time.

3.4. Evaluation of puffer fish matrix effects

The applicability of the biosensor was first studied by evaluating matrix effects of blank puffer fish tissue extracts at a matrix concentration of 40 mg mL^{-1} (from a sample determined as non-containing TTX by LC-MS/MS). The analysis of negative puffer fish extracts at 40 mg mL^{-1} of tissue resulted in mAb binding percentages of 91, 106, 95 and 103% for muscle, skin, liver, and gonads, respectively. Taking into account that the highest SD in the calibration curve was of 15%, a response between 85 and 115% of mAb binding indicates no effect from the extract. Thus, matrix effects can be considered negligible at 40 mg mL^{-1} of puffer fish matrix.

Further matrix effects were then evaluated through the spiking of 2.5, 5 and 10 ng mL^{-1} of TTX into blank puffer fish tissue extracts at a matrix concentration of 40 mg mL^{-1} (corresponding to 0.06, 0.13 and 0.25 mg TTX kg^{-1} of tissue, respectively). Percentages of toxin were calculated with reference to the concentrations of TTX spiked (**Table 1**). Again, taking into account the 15% of SD, similar TTX concentrations were determined in buffer and in spiked puffer fish tissue extracts, reaffirming that matrix does not interfere with the immunosensor performance.

Given the negligible matrix effects and the good toxin percentages observed in spiked-extracts, the analysis of puffer fish tissue extracts can be performed using 40 mg mL^{-1} of matrix, which means that the immunosensor should be able to detect as low as 0.07 mg TTX equiv. kg^{-1} tissue. These results proven the reliability and feasibility of this biosensor to be applied to the analysis of samples with complex matrices such as puffer fish extracts.

3.5. Analysis of naturally contaminated puffer fish samples and comparison with mELISA, SPR immunosensor and LC-HRMS analysis

In order to demonstrate the applicability of the electrochemical immunosensor, *L. lagocephalus, L. sceleratus and S. pachygaster* samples were analyzed. Quantifications obtained by this immunosensor were compared with those determined by mELISA, SPR immunosensor and LC-HRMS. Whereas no TTX was found in either

L. lagocephalus or *S. pachygaster* puffer fish samples (mAb binding percentages between 85 and 115%), high TTX contents were determined in *L. sceleratus* tissues by all techniques. **Table 2** shows the TTX equivalent contents determined in *L. sceleratus* extracts by the electrochemical immunosensor, mELISA and SPR immunosensor, in addition to the sum of the total TTX content obtained by LC-HRMS (the individual content of TTX and each analogue provided by LC-HRMS [41].

Among the species of puffer fish analyzed in this work, *L. sceleratus* is considered one of the most toxic Lessepsian invasive species of the Mediterranean [45]. *L. lagocephalus* is a native species of the Mediterranean for which there are no known specific toxin threats and, consequently, has been listed as least concern by a European Regional Assessment [46]. Finally, *S. pachygaster* is a well-known alien species of the Mediterranean [47], considered weakly toxic by Noguchi et al. [48], but non-toxic in the Mediterranean by Ragonese and co-workers [49]. Therefore, the toxin contents found in this work for different puffer fish species were in accordance with the levels of TTX described in the literature for these species of puffer fish caught along the Mediterranean coast.

With regard to the distribution of TTX in *L. sceleratus* tissues, the same trend was obtained with all the techniques used in this study, whereby the highest to the lowest TTX content was found in: gonads>liver>skin>muscle. Distribution of TTX into tissues was in accordance with that described for other female *L. sceleratus* specimens captured along the Mediterranean [17, 27, 50, 51]. Notably, levels of TTX found in gonads and liver tissues were up to 10-fold above the Japanese value of 2 mg TTX eq. kg^{-1} used as criterion to judge the acceptability of puffer fish as safe for human consumption [10].

When comparing the techniques used for the analysis of *L. sceleratus* extracts, a good correlation is obtained between the TTX contents determined by the electrochemical immunosensor and those provided by mELISA (R^2=0.933), as well as equivalent quantifications (y=0.95x-1.81; t=0.245, P=0.815). The similarity between results obtained with both techniques is due to the fact that both approaches are based on the antigen-antibody affinity principle and, additionally, TTX immobilization was achieved using the same strategy. Even though the transducer and the detection method are different, the recognition of antibody antigen binding and the competition step are similar.

When comparing the TTX contents determined by the electrochemical method with those provided by the SPR immunosensor, results correlate well (R^2=0.907), and equivalent TTX contents are reported by SPR immunosensor (y=1.05x-3.05; t=0.221, P=0.833). Despite the different approach used in the TTX immobilization (oriented through dithiols self-assembled on gold electrodes *vs.* randomly on dextran chips), the different detection principles of these techniques (electrochemical *vs.* optical) and the measurement of

the biorecognition event (end-point *vs.* real-time), comparable toxin contents were obtained, with no significant differences.

Finally, in order to compare the quantifications obtained by this immunosensor with those provided by LC-HRMS, the known cross-reactivity factors (CRFs) [27] were applied to the individual contents of TTX and analogues. As the similar recognition event occurs between immunosensor and immunoassay, and due to the lack of available TTX analogues, CRFs established by mELISA [27] were assumed for this immunosensor. Good correlation was obtained between both techniques (R^2=0.902) and, as observed in the previous study [27], slightly lower contents were determined by LC-HRMS, although differences were statistically not significant (y=1.28x+2.78; t=0.538, P=0.610). As mentioned in the previous work [41], within the different tissues analyzed, the greatest disparity between the quantifications provided by mELISA and LC-HRMS was observed in case of the liver. The lack of CRFs for some analogues determined by LC-HRMS, the possible presence of unknown analogues as well as other matrix components could be responsible for this disagreement between techniques.

Overall, TTX contents determined by the electrochemical immunosensor were in good agreement with those obtained by mELISA, SPR immunosensor and LC-HRMS. **Table 3** reviews and compares the main analytical parameters of each technique. The good agreements found between the quantifications provided by this immunosensor and those provided by the other techniques proved the reliability and usefulness of the biosensor in the analysis of puffer fish samples. Certainly, this biosensor enabled the screening of TTX in several puffer fish species and tissues, from which *S. pachygaster* and *L. lagocephalus* individuals were considered safe for human consumption, whereas *L. sceleratus* was determined risky for human safety because of the high TTX contents determined, according to the Japanese criterion.

4. Conclusions

Encouraged by the necessity to develop alternative methods to MBA and LC-MS/MS analysis for the detection of the emerging TTX in European fish and shellfish, an immunosensing platform based on the TTX immobilization through dithiols self-assembled on 8-gold electrode arrays is described. The spaced and oriented TTX immobilization on the carboxylate-dithiol SAM as well as the choice of a precipitating redox mediator favored the electron transfer. Good sensitivity was attained by the immunosensor, with an LOD of 2.6 ng mL^{-1}.

The immunosensor allowed working with the high loading tissue concentration of 40 mg mL^{-1} and showed no matrix effects under these conditions. Therefore, taking into account the LOD in buffer and the tolerable

matrix concentration, the immunosensor was able to detect as low as 0.07 mg equiv. TTX kg^{-1} tissue. TTX contents determined in puffer fish samples by the electrochemical immunosensor were in good agreement with those obtained by mELISA, SPR immunosensor and LC-HRMS analysis. Thus, the analytical performance of the SAM-based electrochemical immunosensor shows feasibility for its implementation in food safety programs, since it enables the determination of TTX in puffer fish well below the Japanese value of 2 mg equiv. TTX kg^{-1} tissue and provides TTX quantifications comparable to other techniques. The format of the immunosensor described in this work makes it promising as a screening tool, with advantages such as the possible integration in miniaturized devices, the use of low reagent volumes, and the short time required for the consecutive analysis of 8 samples. In conclusion, given the improved sensitivity, the high accuracy and the good agreement of the immunosensor with other methods, this work constitutes a breakthrough in the development of rapid, compact, robust, reliable and easy-to-use analysis devices for the detection of the emerging TTX in puffer fish samples.

The authors declare that there is no conflict of interests regarding the publication of this paper.

Figure legends

Figure 1. Schematic representation of the electrochemical immunosensor array platform for TTX detection.

Figure 2. Carboxylate-dithiol and thioctic acid molecules self-assembled on SPGEs.

Figure 3. Forward scan voltammograms obtained in degassed PBS for bare SPGE (dashed line), scan 1 (solid line) and scan 2 (dotted line) of carboxylate-dithiol SAM-modified SPGE at a scan rate of 10 mV s^{-1}.

Figure 4. Cyclic voltammograms in 2.5 mM $[Fe(CN)_6]^{3-/4-}$ at 10 mV s^{-1} obtained at **(a)** bare, **(b)** SAM, **(c)** ethylendiamine and **(d)** TTX-immobilised SPGEs for carboxylate-dithiol and thioctic acid approaches.

Figure 5. Calibration curve obtained by the electrochemical immunosensor. mAb binding is expressed as percentage of the control (without free toxin). X values refer to initial toxin concentrations and error bars show standard deviation (SD) for 4 replicates (n=4).

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Table 1. Toxin percentages measured by the electrochemical immunosensor array platforms in spiked-muscle, skin, liver and gonads tissue extracts of *L. lagocephalus* with reference to the TTX spiked levels (0.06, 0.13 and 0.25 mg TTX kg^{-1} of tissue).

	TTX spiked level (mg TTX kg^{-1} tissue)		
Puffer fish tissue	**0.06**	**0.13**	**0.25**
Muscle	104%	112%	113%
Skin	89%	87%	97%
Liver	93%	89%	106%
Gonads	96%	114%	87%

Table 2. TTX equivalent contents (mg TTX equiv. kg^{-1} tissue) in *L. sceleratus* extracts obtained by the electrochemical immunosensor, mELISA and SPR immunosensor, and the sum of total TTX determined by LC-HRMS analysis. LODs were 0.23, 0.43 and 0.05 mg kg^{-1} for mELISA, SPR immunosensor and LC-HRMS, respectively.

Puffer fish tissue	Electrochemical immunosensor	mELISA [41]	SPR immunosensor	Σ LC-HRMS [41]
Muscle	1.45	2.53	3.51	0.98
Skin	2.11	3.50	4.42	2.08
Liver	16.67	25.30	24.82	5.36
Gonads	33.90	33.55	30.50	25.22

Table 3. Comparison of the performance parameters: LOD, high throughput sample analysis, ease of sample preparation, ease of use, cost and portability provided by the different analytical techniques used in this work.

Technique	LOD (mg TTX kg^{-1} puffer fish)	High-throughput sample analysis	Ease of sample preparation*	Ease of use	Cost	Portability
Electrochemical immunosensor	0.07	medium	yes	medium	medium	high
mELISA	0.23	high	no	high	medium	medium
SPR immunosensor	0.43	high	no	medium	high	low
LC-HRMS	0.05	high	no	low	high	low

* According to the requirement of solvent evaporation for solvent exchange.

FIGURE 1

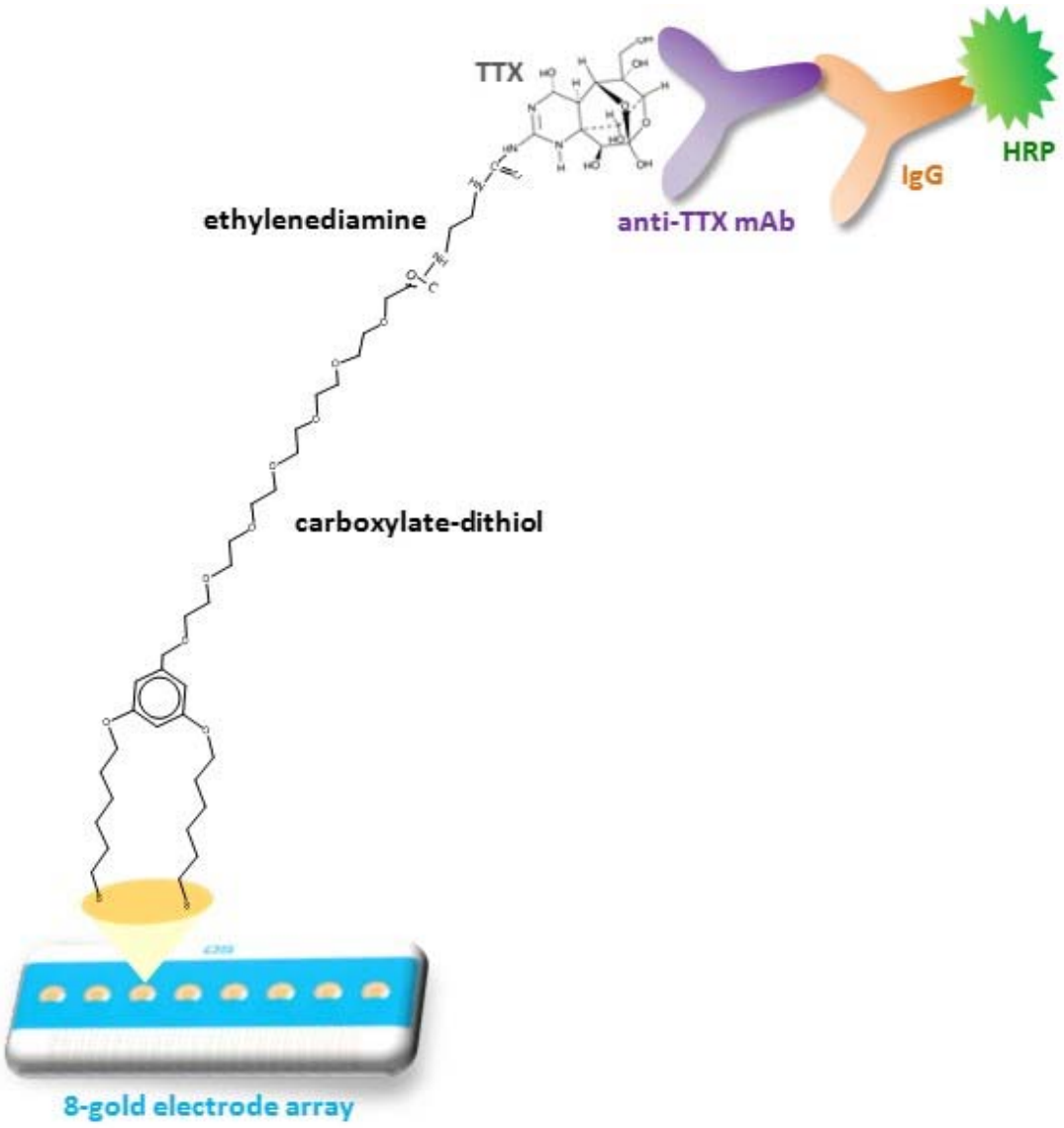
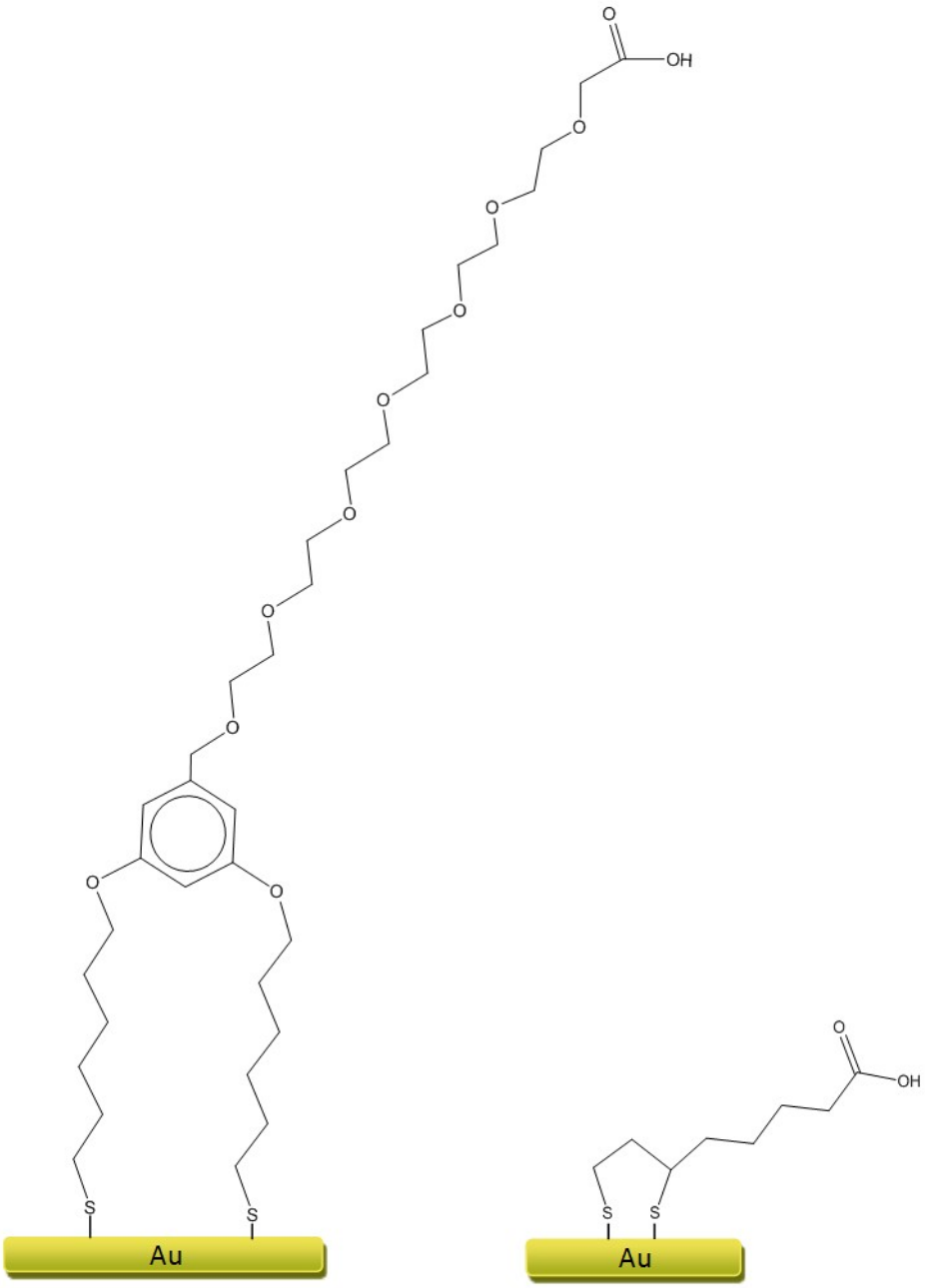

TTX
HRP
IgG
anti-TTX mAb
ethylenediamine
carboxylate-dithiol
8-gold electrode array

FIGURE 2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Thioctic acid

FIGURE 3

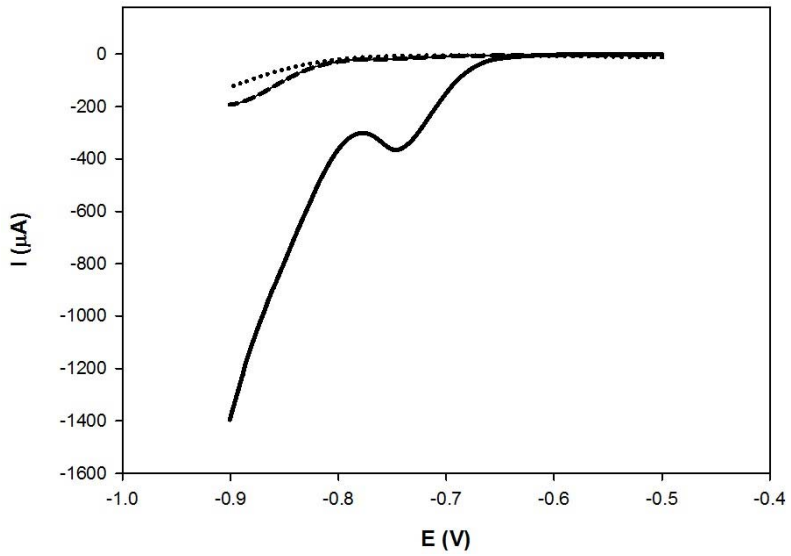

FIGURE 4

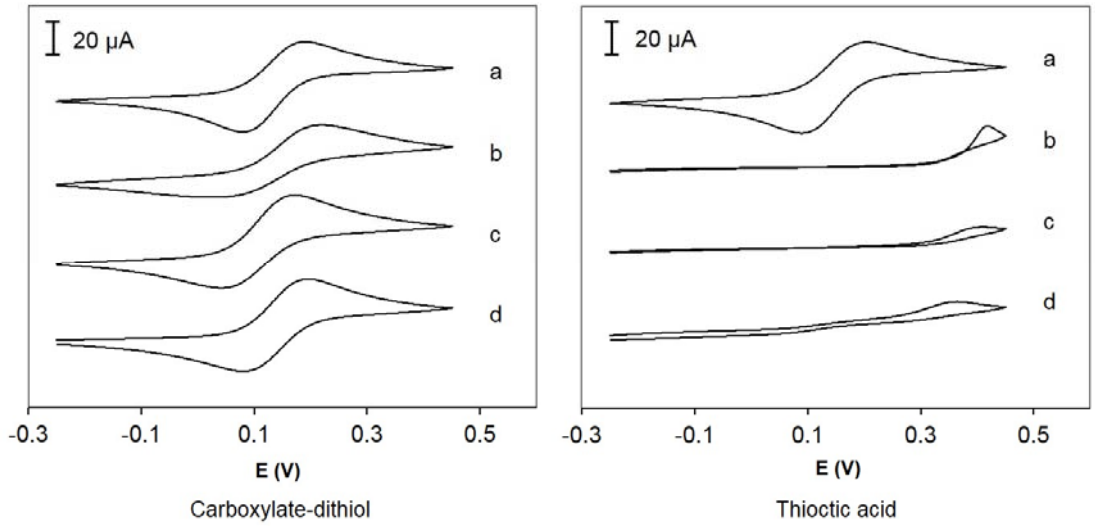

FIGURE 5