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Review

Detection of the marine toxin okadaic acid: Assessing seafood safety

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ABSTRACT

Diarrheic Shellfish Poisoning (DSP) is a gastrointestinal illness caused by consumption of shellfish contaminated with DSP toxins such as okadaic acid (OA) and dinophysistoxins (DTX). The occurrences of OA in bivalves induce not only public health problems but also economic damages to shellfish farming. Consequently, the development of fast, reliable and sensitive detection methods is an evident necessity. The mouse bioassay has been the reference and most commonly used analysis method. However, this technique suffers from low accuracy, specificity and ethical problems due to the animal experimentation. Thus, the development of alternative and efficient detection systems is required. Several biological, chemical, and immunological methods have been developed to evaluate the presence of DSP toxins in seafood. This review gives an overview of different analytical methods and new trends for the detection of OA. Over the past decade, considerable attention has been given to the development of biosensors for the efficient detection of marine toxin. Recent advances in the field of aptamers and nanomaterial offer exciting new opportunities to develop improved and more reliable devices allowing the detection of OA.

Contents

1. Introduction

Okadaic acid (OA) and dinophysistoxins (DTX) (Fig. 1) are produced by some unicellular algae from plankton and benthic microalgae. These phycotoxins accumulate in the digestive glands

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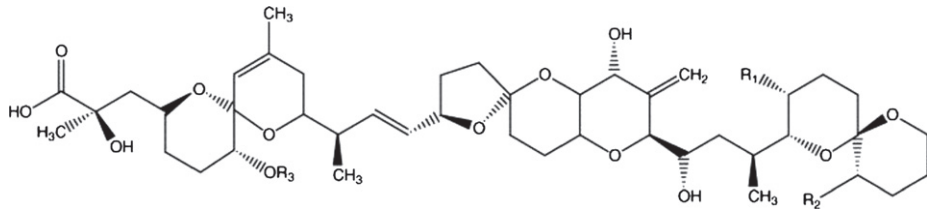

	R1	R2	R3
OA	CH3	H	H
DTX1	CH3	CH3	H
DTX2	H	CH3	H
DTX3	CH3	CH3	Acyl

Fig. 1. Structure of okadaic acid and dinophysistoxins.

of shellfish without causing any toxic effect on the bivalves. However, when humans consume a sufficient amount of contaminated seafood, gastrointestinal troubles known as DSP (Diarrheic Shellfish Poisoning) occur [1]. Studies carried out on animals have identified OA as a tumour promotor and also proved its mutagenic and immunotoxic effects.

Toxins causing DSP were first reported in Japan in 1978. Yasumoto was the first to study the causes of intoxication after eating cooked shellfish [2–4]. OA was finally identified by Murata and co-workers [5] as the main bioactive compound for DSP. Since then, occurrences of OA-group toxins in shellfish have been reported in Europe [6–10], North and South America [11–14], Asia [15–19] and Oceania [20–22].

Their mechanism of action is based on the inhibition of protein phosphatases (PPs) [23,24] which play an important role in protein dephosphorylation in cells. Consequently, hyperphosphorylation of the proteins that control sodium secretion by intestinal cells and of cytoskeleton or junctional moieties that regulate solute permeability is favored, causing a sodium release and a subsequent passive loss of fluids, responsible for the diarrheic symptoms [25].

To guarantee the seafood safety, and to minimize the potential risk to human health, it is necessary to develop fast, sensitive and reliable methods to detect OA. In the European Union, the regulation (CE) No. 853/2004 establishes a maximum permitted level of 160 μg of OA equivalent to kg^{-1}. The European Food Safety Authority (EFSA) has proposed to decrease the maximum limit of 160 μg of OA equivalent to kg^{-1} to 45 μg of OA equivalent to kg^{-1} in bivalve mollusks [1].

The simplest screening method is the mouse bioassay, which suffers from low sensitivity, specificity and ethical problems due to animal experimentation. Due to its drawbacks, the European Union has decided to use alternative methods for the analysis of contaminated shellfish [26]. This paper reviews the bio/analytical techniques for the detection of OA.

2. Bioassays

2.1. In vivo assays

The first screening method to detect marine toxin was the mouse bioassay. This test consists in administration of shellfish extracts to laboratory mice and monitoring the time until death. Three mice have to be used for each test. A sample is considered as positive for the presence of marine toxin when 2 out of 3 mice die within 24 h of inoculation with an extract equivalent to 5 g hepatopancreas or 25 g whole body. The mouse bioassay gives an indication of the overall toxicity of the sample.

The mouse bioassay has several drawbacks:

- The results show high variability because they depend on strain, gender, sex, state of the health and weight of the animals.
- The mouse bioassay did not show good reproducibility between laboratories.
- It suffers from controversial ethical problems since animals are sacrificed.
- It is expensive due to the animal maintenance.
- The mouse bioassay may give false positives because of interferences by lipids, notably free fatty acids [27].
- The mouse bioassay shows a low specificity (no differentiation between the various DSP toxins). Even if the mouse bioassay suffers from low specificity, the fact that this bioassay gives an indication about overall toxicity of the sample, can also be considered as an advantage in term of health protection.

The Commission European (EC) No 15/2011 authorized the use of the mouse bioassay until 31st January 2014. Thus, it is required to develop alternative and efficient detection systems.

The rat bioassay is also used for the detection of DSP toxins [1]. The test is used routinely in the Netherlands and is an officially allowed procedure in EU legislation. In the procedure currently applied in Netherlands, 10 g shellfish hepatopancreas is collected and fed to female rats that have been starved for 24 h. After a 16 h-period, the consistency of the faeces is observed along with the quantity of food eaten.

2.2. In vitro assays

Cytotoxicity assays are based on morphological changes in cells, such as neuroblastoma cells, rat hepatocytes, Buffalo green monkey kidney cells, mammalian fibroblasts, neuro-2a neuroblastoma cells, neuroblastoma *x* glioma hybrid cells (NG108-15), KB cells and cerebellar neurons [25,28]. The potency of the cytotoxic effect of OA depends on the type of cells used. The morphological changes seem to be due to an alteration in the cytoskeleton which can be related to the PP2A-inhibiting property of OA [29]. Suitable results can be obtained using stabilized and standard cell lines that ensure a reduced variability of the response. For this purpose, the KB cell line, a human

epidermoid carcinoma of the mouth, is a good candidate since this cell line is sensitive to OA and has good temporal stability [29]. OA induces morphological changes in KB cells within a few hours. Toxin-induced changes in cell morphology have been detected by microscopy [30]. A loss of the characteristic polygonal shape to a rounded form and an alteration of the cytoplasmic content were observed. Cytotoxicity assay based on direct microscopic observation of toxin-induced morphological changes in Buffalo green monkey kidney cells was also described [31]. The method was highly sensitive since very low OA concentrations (0.005 μg/mL) induced toxic effects after 5–6 h exposure. A high correlation ($r=0.95$) was found between the mouse bioassay and the cytotoxicity test conducted on naturally contaminated samples of *Mytilusgallo provincialis*.

Cytotoxicity assays based on MTT colorimetric method have also been developed for the detection of OA in mussels [29,32,33]. This work was based on the ability of cells to reduce MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium) to a blue formazan product. Tubaro and co-workers [29] developed a MTT-based cytotoxicity assay using KB cells. The assay was suitable for detecting OA concentrations in mussel samples as low as 50 ng/g in the digestive glands. The results were compared with those obtained by ELISA test and good correlations between the two methods were shown. This method required only minimal processing and the results were read on a standard multiwell scanning spectrophotometer. The main disadvantage was the relatively long time required for the assay, which was more than 24 h. A decrease in the contact time would probably result in a loss of sensitivity of the method.

A fluorimetric microplate assay for quantitative measurements of F-actin levels in living cells was developed [34,35]. This method was based on fluorimetric determination of F-actin depolymerization by DSP toxins in the BE(2)-M17 neuroblastoma cell line. No interference was observed with other marine toxin such as saxitoxin, domoic acid or yessotoxin, indicating a good specificity of the assay.

The cytotoxicity assays are subjective, time-consuming and confusing results may appear in the presence of toxin mixtures [25]. These methods require the use of living cells. The main drawback of cell-based assays is the need to maintain cell cultures [28].

3. Chemical techniques

Chromatographic techniques have been widely developed for the analysis of contaminated shellfish. They allow selective identification and sensitive quantification of the different toxins present in a sample. However, these techniques require expensive equipment and high trained personnel. They are laborious and time-consuming [25].

3.1. Liquid chromatography-fluorescence detection (LC-FD)

The method developed by Lee and co-workers [36] is commonly used for the identification and quantification of OA, DTX1 and DTX2. Due to the lack of natural fluorescence of the marine toxins, a derivatization step is necessary to convert these compounds into the corresponding fluorescent derivatives [37]. The protocol is as follows: from shellfish digestive glands, methanolic extraction and purification of OA was performed. Then, the extract was derivatized in chloroform with a fluorophore (9-anthryldiazomethane, ADAM). The derivatized compound was purified on a silica gel mini-column and analyzed using high performance liquid chromatography-fluorimetric detection. Several improvements to Lee's protocol have been proposed [38,39].

Numerous derivatization compounds that react with the carboxyl group of OA and DTX 1 and 2 have been described in the literature [40–44]. The most widely used, because of its selectivity and sensitivity, is ADAM. However, this reagent is unstable and must be stored at low temperatures. Decomposition of ADAM can induce incomplete derivatization and these products can interfere in the analysis.

3.2. Liquid chromatography–mass spectrometry (LC–MS)

The use of chromatography coupled with mass spectrometry (LC–MS) is a standard analytical tool to identify marine toxins. This method does not require the complex derivatization and purification steps that are necessary for LC-FD approach. LC/MS can provide relevant information in the presence of closely related compounds of a known structure. There are different types of commercially available mass spectrometers (e.g., single quadruple, triple quadruple ion traps) that differ mainly in sensitivity and capability of providing ion fragmentation for structural information and selective detection. Two basic types of atmospheric pressure ionization are available: electrospray ionization (ESI) and atmospheric pressure chemical ionization. The ESI method has been frequently used for marine toxins because of its suitability for ionization of all known toxins. Several LC–MS methods differing in mobile phase, type of buffer, pH, ionic strength, stationary phase, electrospray mode have been described for the detection of OA, DTX1 and DTX2 [45]. Current LC–MS methods used for the detection of lipophilic shellfish toxins and the influence of different parameters on the LC–MS performances have been reviewed [46]. An important issue to be taken into account in case of LC–MS is ionization efficiency of the analytes which may be significantly affected by matric components accumulated on the LC column after repeated injections [45].

Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) has been validated and approved by the European Regulation as the new official control method for lipophilic toxins in shellfish [26]. The use of a tandem MS provides structural information.

3.3. Capillary electrophoresis

Capillary electrophoresis is a technique that provides high resolution separation and requires a very small amount of sample [45]. To our knowledge, the only described application of capillary electrophoresis for OA detection was a micellarelectrokinetic chromatography [47]. Detection of non-derivatized toxin was performed by ultraviolet absorption at 200 nm. The detection limit and specificity are limited due to the poor UV extinction coefficient of the analyte. OA was detected in mussel spiked with 0.01 $\mu g\, g^{-1}$ whole tissue and the presence of OA and DTX2 was observed in crude extract of the dinoflagellate *Prorocentrum lima*.

4. Biochemical methods

4.1. Enzyme inhibition-based methods

4.1.1. Protein phosphatases

Enzyme inhibition-based methods have been widely used for the detection of OA. The main enzymatic method is based on the inhibition of protein phosphatases by the toxin. The enzyme inhibition can be detected by several methods such as colorimetry, fluorescence or electrochemistry. Radioisotopic assays [48] allow low detection limits but they required complex labeling procedures and hazardous problems.

The commercially availability of PP2As which avoids laborious purification makes the approach very attractive. Several assays and biosensors have been developed using PP2As purchased from Millipore (New York, USA) [49–51]. This enzyme is isolated as the

heterodimer of 60 kDa (A) and 36 kDa (C) subunits from red blood cells. A French company called GTP Technology produced (by genetic engineering) PP2A that consists of a 39 kDa (tag included) human catalytic (C) subunit of the α-isophorm isolated from SF9 insect cells infected by baculovirus [49,50]. Recently, PP2A from ZEU Immunotec (isolated from red blood cells) was also used for the development of colorimetric PP inhibition assay [49]. However, the sensitivity of enzymes to OA can be drastically different. Thus, the choice of the enzyme is crucial to the performance of the system. A comparative study demonstrated that PP2A from ZEU Immunotec is more sensitive to OA than the other enzymes [52].

Numerous enzymatic assays for OA detection have been developed using protein phosphatase in solution. One of the main drawbacks of this approach is the poor enzymatic stability. To overcome this problem, enzymes can be immobilized. The choice of the immobilization technique is crucial for the performance of assays and biosensors [52].

4.1.2. Enzyme inhibition-based assays

4.1.2.1. Colorimetry. Numerous colorimetric protein phosphatase inhibition assays have been described (Table 1). These tests are based on the following principle: protein phosphatase reacts with the colorless *p*-nitrophenyl phosphate (*p*-NPP) substrate to produce the yellow *p*-nitrophenol (*p*-NP) by the enzymatic reaction. OA inhibits enzyme causing the loss of production of the yellow *p*-NP. After incubation, the absorbance was measured at $\lambda = 405$ nm.

Most colorimetric enzymatic assays for OA detection have been developed using protein phosphatase in solution [50,51,53–55] (Table 1). Two companies, called ZEU Immunotec and Abraxis, sell colorimetric protein phosphatase inhibition assays (Table 2). In both cases, PP2A isolated from red blood cells are used in solution.

Recently, colorimetric PP inhibition tests have been developed by entrapping enzyme within a porous membrane. Several matrices have been tested: photopolymers, silica and agarose gels [49,56]. Sol gel process was widely used to entrap enzymes. Nevertheless, this immobilization technique presents an important drawback as the obtained brittle membrane suffers from cracking. In optimal conditions, the colorimetric tests based on PP2A from ZEU Immunotec immobilized within a photopolymer or an agarose gel showed excellent sensitivity to OA. In both cases, the detection limit was 0.18 $\mu g\,L^{-1}$. To evaluate the efficiency of the assays, PP inhibition tests were successfully used for OA detection in contaminated mussels. Although this

Table 1
Performances of protein phosphatase inhibition assays.

Enzyme	PP in solution or immobilized	Detection limit ($\mu g\,L^{-1}$)	Linearity range ($\mu g\,L^{-1}$)	IC_{50} ($\mu g\,L^{-1}$)	Reference
Colorimetric protein phosphatase inhibition assays					
Catalytic subunit of recombinant human PP2A from Invitrogen	In solution	ND	ND	0.095	[55]
Catalytic subunit of PP2A prepared from rabbit skeletal muscle	In solution	4	ND	ND	[54]
PP2A from Millipore	In solution	0.063	0.125–0.75	0.26	[51]
	In solution	0.19	0.19–5.97	1.25	[50]
	In solution	0.47	ND	2.7	[49]
	In solution	1.8	1.8–9.9	4	[57]
	Immobilized within a silica gel	1.14	ND	2.6	[56]
PP2A from GTP	In solution	0.96	0.75–5.97	2.94	[50]
	In solution	0.123	ND	0.97	[49]
	In solution	5.3	2.3–12.9	ND	[57]
	Conjugation to magnetic beads via metal coordination chemistry	30.1	30.1–111.2	64.4	[57]
	Immobilized within a silica gel	0.29	ND	1.68	[56]
PP2A from ZEU	In solution	0.0124	ND	0.72	[49]
	Immobilized within a photopolymer	1	ND	8.24	[49]
	Immobilized within an agarose gel	0.23	ND	3.11	[49]
Fluorescent protein phosphatase inhibition assays					
PP2A from Millipore	In solution	0.0032	$3.2.10^{-3}$–3.2	0.08	[59]
PP2A from Millipore	In solution	ND	ND	0.97	[58]
PP2A from Millipore	Immobilized onto CNBr-activated Sepharose beads	3.5	ND	10	[60]
Electrochemical sensor system and biosensor					
PP2A from Millipore	PP2A: in solution ALP and GOD immobilized on a nylon net membrane	ND	0.03–0.25	0.07	[64]
PP2A from Millipore	Entrapment within a photopolymer	6.42	2.69–171.9	22.19	[50]

Table 2
Commercial kits.

Company	Product name	Required time	LOD	Price
Colorimetric PP2A inhibition assay (enzyme in solution)				
ZEU Immunotec [58]	Oka-Test	< 1 h	44 μg/kg	252 € for 48 tests
Abraxis	OA (PP2A), microtiter plate	< 1 h	ND	ND
ELISA test				
Abraxis	OA (DSP) ELISA, microtiter plate	< 2 h	0.1 μg/g of whole tissue	488 € for 40 tests (in duplicate)
Europroxima	Okadaic acid ELISA	1 h	50 μg/kg	498 € for 40 tests (in duplicate)
DSP lateral flow test				
Jellett Rapid Testing Ltd. [59]	DSP Rapid test	1–2 h	0.1 μg/g of whole tissue	27.50 $/test
Neogen	Reveal for DSP	25 min	160 ppb	295 € for 24 tests

immobilization technique maintains PP2A in a flexible conformation and substantially retains the stability of the enzyme activity, the entrapment within a porous membrane creates a barrier which limits the accessibility to the enzyme by both the substrate and the toxin. To eliminate this diffusion barrier, other immobilization techniques could be used. One strategy is the conjugation of genetically engineered PP2A (from GTP Technology) with hexa-His tail to Ni-modified magnetic particles via coordination chemistry [57].

4.1.2.2. Fluorescence. Protein phosphatase inhibition assays have been adapted for fluorescence measurements using the substrates fluorescein diphosphate (FDP) [58,59] and methylumbelliferyl phosphate (MUP) [58–62] (Table 1). In most cases, PP2A was used in solution [58,61,62]. To our knowledge, only, one fluorescent protein phosphatase inhibition assay was developed by immobilizing PP2A [60]. In this work, the enzymes were bound to CNBr-activated Sepahrose beads placed in microfilter plate wells. In optimal conditions, the detection limit was 3.5 μg L^{-1}.

4.1.3. Electrochemical enzyme inhibition-based sensor systems and biosensors

Hamada-Sato and co-workers [63] were the first to exploit the PP2A inhibition for the OA detection by electrochemical methods. The authors measured the amount of oxygen consumed by pyruvate oxidase, which was proportional to the phosphate ion released from *p*-NPP and PP2A (Fig. 2). The inhibition step was performed in a microtube with PP2A in solution. Only pyruvate oxidase was immobilized on a membrane that covered the oxygen electrode. Consequently, this approach could not be considered as a biosensor but as a sensor system. In 2009, another sensor system was developed taking advantage of the particular characteristics of a biochemical pathway in which PP2A was involved [64] (Fig. 3). PP2A also has significant activity toward glycogen phosphorylase (PHOS a), which catalyzes the conversion of glycogen to glucose-1-phosphate [65]. In this sensor system, alkaline phosphatase (ALP) converted glucose-1-phosphate into glucose, which was then converted by GOD into H_2O_2. This final product was electrochemically oxidized at a platinum electrode. PP2A enzyme was able to block this reaction chain because it converted the PHOS a (active form) into PHOS b (inactive form) that was unable to catalyze the conversion of glycogen to glucose-1-phosphate. Thus, the absence of substrates for ALP and GOD resulted in negligible production of H_2O_2. In the presence of OA, PP2A was inhibited and the PHOS a led to an increase in the current signal proportional to the OA concentration. In this work, PP2A and PHOS a were in solution in a microtube whereas ALP and GOD were co-immobilized on a nylon net membrane that was placed over an H_2O_2 platinum probe inserted into a flow injection analysis system.

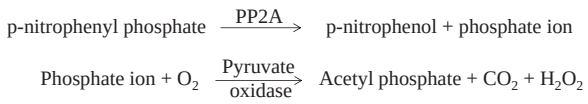

Fig. 2. Summary of enzymatic reactions involved in the method proposed by Hamada-Sato and co-workers [65].

Our group worked on the development of electrochemical PP2A inhibition-based biosensors for the detection of OA. The strategy was based on the entrapment of PP2A within a photopolymer formed onto a screen-printed electrode [50]. PP2A inhibition was detected directly using appropriate substrate electrochemically active only after the dephosphorylation by the enzyme. The enzymatic activity of immobilized PP2A (Millipore) towards *p*-aminophenyl phosphate (*p*-APP) and catechol monophosphate (CMP) was studied by chronoamperometry. In optimal conditions, the detection limit was 6.42 μg L^{-1}. This strategy is much simpler than the enzymatic approach previously mentioned [63,64], since only one immobilized enzyme is used. However, several drawbacks have to be noted. A low reproducibility, most likely due to the manual enzyme deposition on the electrode surface and/or the fouling of the electrode by electropolymerization of the phenoxy radicals that appeared during the enzymatic reaction, was observed [66]. Although the authors demonstrated the feasibility of that electrochemical biosensor and applied it to the analysis of toxicogenic dinoflagellate extracts, the detection limit was not low enough for the routine determination of okadaic acid in shellfish. Work is now in progress to improve the performance and the reproducibility of this electrochemical biosensor. For instance, other types of PP2As and immobilization techniques are being investigated.

4.2. Immunology-based methods

4.2.1. Enzyme-linked immunosorbent assay (ELISA)

ELISA tests based on different formats have been described for rapid measurement of OA and its isomers [67] (Table 3). Due its small size (MW 900–1100 g mol^{-1}), sandwich assays are not possible for OA detection. ELISA tests are based on colorimetric detection. Colorimetry is the most commonly used detection method due to its simplicity, cost effectiveness and sufficient sensitivity.

A direct competitive immunoassay was developed using enzyme conjugated OA (OA-ALP) [67]. The toxin present in a sample and OA-ALP conjugate competed for binding to the anti-OA Ab. After incubation and a washing step, the amount of bound OA-ALP was visualized by the addition of *p*-nitrophenyl

Table 3
Performances of ELISA tests for OA detection.

ELISA test format	Detection limit (μg L^{-1})	IC50 (μg L^{-1})	References
Direct competitive test	1.7	ND	[69]
Indirect competitive test	0.5	ND	[69]
Indirect competitive test (ALP-labeled secondary Ab)	3.73	9.23	[70]
Indirect competitive test (HRP-labeled secondary Ab)	4.49	8.38	[70]
Indirect competitive test	0.8	3.92	[71]

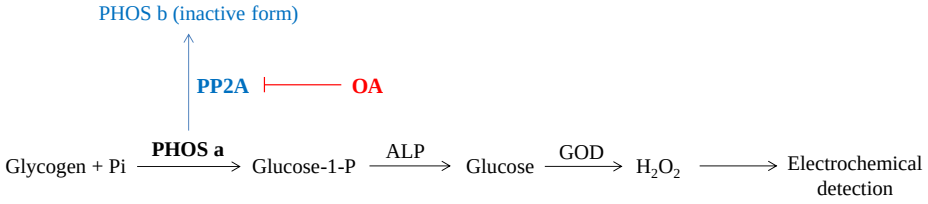

Fig. 3. Summary of enzymatic reactions involved in the method proposed by Volpe and co-workers involved [66].

phosphate. The colorless chromogen was converted by the enzymeinto a yellow reaction product. Then, the obtained color intensity was measured using an ELISA reader at 405 nm. The more OA present in the sample, the less the color developed. Thus, the color intensity was inversely proportional to the OA concentration in the sample. An indirect competitive ELISA test was also developed using anti-OA Ab labeled to ALP [67]. OA-bovin serum albumin (BSA) conjugate was coated to the surface of a microwell plate. Afterwards, a mixture of labeled anti-OA Ab and toxin present in a sample were added to the well. The bound and free toxin competed for the anti-OA Ab. The colorimetric detection was achieved as previously described. Recently, our laboratory described an indirect competitive ELISA test [68]. In this work, anti-OA monoclonal Ab was not labeled and the use of enzyme-labeled secondary antibody was required for the colorimetric detection. Two enzyme labels, ALP and horseradish peroxidase (HRP), were used for comparison (Table 3). A competitive indirect ELISA test based on super paramagnetic beads was also described [69]. Streptavidin-coated magnetic beads were used as support to immobilize biotinylated OA.

Classically, an indirect competitive ELISA test is based on an indirect labeling: the unlabeled anti-OA Ab binds to the toxin and a labeled secondary Ab is used for the signal generation. Alternatively, a direct labeling can be envisaged. In this case, the label is attached via a covalent bond to the primary antibody. However, many immunoassays employ the principle of indirect labeling since the conjugaison of primary Ab is relatively complicated. Recently, our group developed a colorimetric competitive indirect ELISA test based on a direct labeling [70]. In this work, anti-OA Ab was conjugated with HRP through a periodate activation process. Performances of optimized ELISA tests based on direct and indirect labeling were compared. The detection limit obtained with the test based on direct labeling (0.006 $\mu g\ L^{-1}$) was 10-fold lower than that obtained with the conventional immunoassay based on indirect labeling (0.06 $\mu g\ L^{-1}$).

ELISA tests have been commercialized by Abraxis and Europroxima. These immunoassays consist of a microtiter plate containing anti-rabbit Ab pre-coated wells. Toxin present in a sample and an enzyme conjugated OA (OA-HRP) compete to bind with the anti-OA Ab. After addition of a substrate solution, a color signal is produced. The intensity of the blue color is inversely proportional to the OA concentration present in a sample. The tests are based on the same principle but the kit purchased from Europroxima is the most sensitive.

4.2.2. Immunosensors

The different formats of ELISA tests have been adapted for the development of immunosensors. ELISA test rely on immobilization of the analyte or Ab on a surface that is not a transducer. Contrary to the immunoassay principle, a biosensor requires the immobilization of the recognition element directly onto a transducer surface such as a working electrode.

For the design of an immunosensor, the key points are the choice of the transducer and the immobilization technique of the Ab or analyte (or analyte derivative, e.g., OA-ovalbulin) on the transducer surface.

4.2.2.1. Electrochemical immunosensors. A wide variety of electrodes can be used to develop electrochemical immunosensors. For the last few years, screen-printed electrodes (SPEs) have been extensively employed in the fabrication of electrochemical biosensors. These electrodes can combine ease-of-use and portability with simple, inexpensive fabrication techniques. The modest cost of SPEs has further enhanced their use because it allows the devices to become disposable. Several SPE-based immunosensors have been developed for the detection of OA (Table 4). Various linkers such as ovalbumin [68], BSA [71] or magnetic beads [69] have been used to immobilize OA on the electrode surface.

Our group developed an electrochemical immunosensor based on a competitive indirect ELISA by immobilizing an OA-ovalbumin conjugate on screen-printed electrodes [68]. Competition of an anti-OA monoclonal Ab for free and immobilized toxin was subsequently performed. Secondary Ab labeled with ALP or HRP were used for signal generation. Each enzyme label required a specific electrochemical transduction system, Whereas +300 mV vs. Ag/AgCl were applied for the detection of *p*-aminophenol produced by the reaction of *p*-aminophenyl phosphate with ALP, −200 mV vs Ag/AgCl were used for the detection of 5-methyl-phenazinium methyl sulphate, HRP mediator in HRP bioelectrocatalysis. An electrochemical signal amplification system based on diaphorase (DI) recycling was also integrated into the ALP-based immunosensor (Fig. 4), decreasing the detection limit to 0.03 $\mu g\ L^{-1}$ and enlarging the working range by two orders of magnitude. Mussel and oyster extracts from the Ebre Delta embrayments were analyzed with the immunosensor. The results were compared with those obtained by colorimetric immunoassay, the PP inhibition and LC–MS/MS and correlations demonstrated the viability of the approach.

Recently, Marty and co-workers [69] combined SPEs to the advantages of magnetic beads in order to develop an efficient competitive indirect enzyme-linked electrochemical immunosensor for the detection of OA. Magnetic beads are known to be powerful and versatile tools in a variety of analytical and biotechnology applications. The use of magnetic beads presents many advantages [72–74]:

(i) An increase in the surface area [73].
(ii) The magnetic beads can be easily magnetically manipulated by using permanent magnets or electromagnets. Magnetic separation can be used for an easy removal of unbound binding molecules.
(iii) According to their properties as well as the improved washing and separation steps, the matrix effect is minimized despite this increased surface area.

Table 4
Performances of electrochemical immunosensors for OA detection.

Immobilization of Ag or Ab	Detection limit ($\mu g\ L^{-1}$)	IC50 ($\mu g\ L^{-1}$)	References
Competitive indirect immunosensor			
OA-BSA conjugate adsorbed on electrode	1.5	32	[71]
OA-ovalbumine conjugate adsorbed on electrode—diaphorase recycling	0.03	0.22	[68]
OA immobilized on magnetic beads—batch mode	0.38	3.15	[69]
OA immobilized on magnetic beads—flow system	0.15	ND	[75]
OA immobilized on electrode via diazonium salt	0.00144	0.01658	[78]
Competitive direct immunosensor			
Ab immobilized on magnetic beads	0.5	ND	[79]

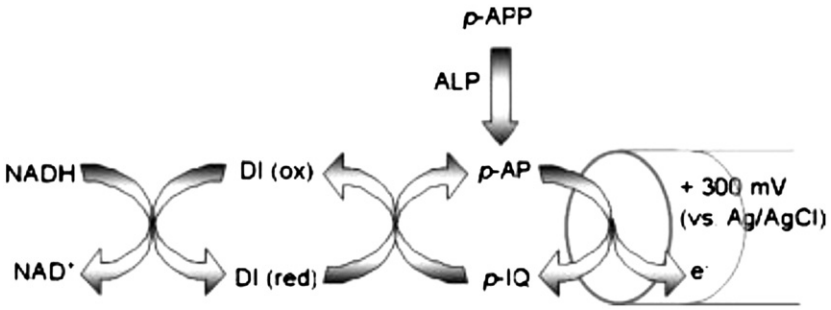

Fig. 4. Scheme of the reaction involved in the enzymatic signal amplification for the detection of the ALP activity [70]. The detection principle is based on the dephosphorylation of non-electroactive *p*-APP by ALP, the oxidation of corresponding electroactive *p*-AP to *p*-iminoquinone (*p*-IQ) on the electrode surface, and the regeneration of *p*-IQ by DI, which requires NADH as a substrate.

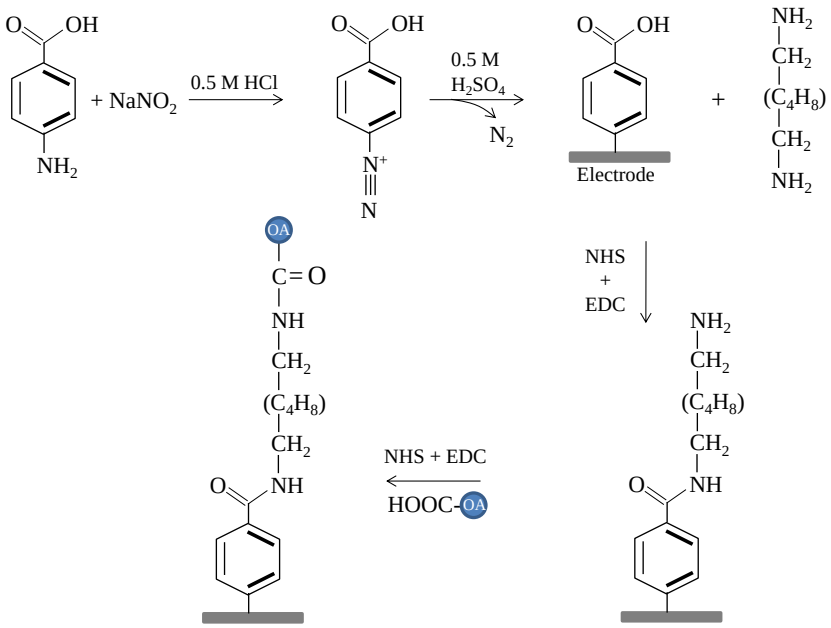

Fig. 5. Schematic representation of OA immobilization via diazonium salt [80].

(iv) Less time consuming due to decreased coating, competition and blocking times.
(v) Numerous commercial magnetic particles are available. The microparticles are functionalized with different groups (e.g., carboxyl, amino).

Recently, our group used streptavidin-coated magnetic beads to immobilize OA. The beads were successfully used in a batch mode for low level determination of OA in mussel samples [69]. Recently, an automated flow-through amperometric immunosensor was developed for the analysis of okadaic acid in mussel sample [75]. The fully automated flow system was based on the incorporation of OA modified MBs into the central flow cell, which were retained there by the application of an external magnet. The device was connected with a flow injection system and amperometric detection based on an indirect competitive immunoassay was performed for the sensitive and on-line detection of OA. The incorporation of the modified magnetic beads into flow device with SPCE electrode to measure an electrochemical signal has increased the ability of the system to determine OA at low concentration (0.15 $\mu g\,L^{-1}$) as compared with previous studies using immunosensor under batch conditions (0.38 $\mu g\,L^{-1}$). The decreased detection limit could be attributed to decreased diffusion distance and increased reactive surface in the flow system.

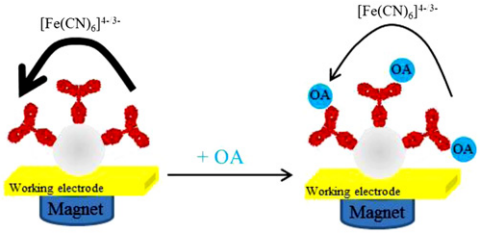

Fig. 6. Principle of the electrochemical immunosensor based on modified protein G-magnetic beads [81].

This automated flow-through amperometric immunosensor was used for the analysis of real mussel samples. The potential advantages of the flow injection analysis system are rapidity and accuracy due to the high degree of control and constancy of analytical parameters. Furthermore, automation makes routine tasks easier and less cumbersome [76,77]. The use of magnetic beads in flow injection analysis system is the first step towards miniaturized and portable analysis devices.

Alternatively, the direct immobilization of OA via covalent coupling on the electrode surface has been used to improve the sensitivity of the electrochemical immunosensors. Recently, a novel method of OA immobilization on SPE via diazonium salt

was described (Fig. 4) [78]. First, electrochemical reduction of diazonium species via one electron process lead to the formation of 4-carboxyphenyl (4-CP) radicals that covalently attached to the electrode surface. The terminal carboxylic group of 4-CP was activated to bind hexamethyldiamine. Then, OA was immobilized to the second terminal amine group of hexamethyldiamine via its activated carboxylic group. After immobilization, an indirect competitive immunosensor was developed for the detection of toxin. The method was validated with certified reference mussel sample, demonstrating the efficiency of the approach Fig. 5.

Recently, a label-free amperometric immunosensor based on a competitive direct ELISA was developed (Fig. 6) [79]. In this case, protein G-magnetic beads were modified with anti-OA Ab. A magnet allowed to maintain the magnetic beads on a gold working electrode. The electrochemical detection was achieved using a redox couple to monitor the behavior of the electrode interface. When the anti-OA Ab modified electrode was incubated with a solution containing OA, the specific interaction Ag/Ab led to the formation of layers on the electrode surface. The Ab/Ag complex increased the steric hindrance and hindered the charge transfer. Therefore, a decrease in redox reaction of ferri/ferrocyanide was observed. Electrochemical detection was carried out by differential pulse voltammetry in ferri/ferrocyanide solution. The detection limit was 0.5 $\mu g\ L^{-1}$. The experiments with mussel samples validated the suitability of the immunosensor for OA detection.

4.2.2.2. Optical immunosensors

- Surface plasmon resonance (SPR)
  SPR is an optical technique that allows to analyze macromolecular interactions in real-time and label-free [80,81]. Elliott and co-workers developed SPR-based immunosensors for the rapid detection of OA contamination in shellfish extracts [82,83]. First, OA was covalently immobilized on CM5 chip. When anti-OA Ab binds to the immobilized ligands, the mass of biomolecules on the chip surface and thus the refractive index increase. Originally, the immunosensor was not able to detect DTX 1 and DTX-2 [82]. Recently, the authors improved its performance using a new monoclonal Ab able to detect OA and DTX toxins [83].
- Chemiluminescence
  The peroxidase-catalyzed chemiluminescent oxidation of luminol was used to develop an optical immunosensor for the detection of OA [84]. OA-BSA conjugate was immobilized on a membrane. Anti-OA Ab was labeled with HRP through a periodate activation process. The immunosensor was used in a semi-automated analysis procedure in which the free OA containing sample was injected in the flow system concomitantly with the labeled anti-OA Ab. The bound and free toxin competed for the anti-OA Ab. The chemiluminescent reaction was catalyzed by HRP in the presence of luminol, hydrogen peroxide and *p*-iodophenol. The generated light was measured using an optical fiber connected to the photomultiplier tube of a luminometer. The immunosensor had a detection limit of 0.2 μg OA/100 g mussel homogenate and measurements could be performed over three decades. Finally, the operational stability and the reproducibility of the sensor were also demonstrated.

4.2.2.3. Piezoelectric immunosensors. A piezoelectric immunosensor was developed for the detection of OA [85]. A quartz crystal microbalance sensor is a mass-sensitive sensor capable of measuring very small mass changes [86]. A competitive format incorporating an immobilization method using polyethyleneimine with glutaraldehyde cross-linking was adopted. The crystal surface was modified with polymer polyethyleneimine and the free amino groups were activated by glutaraldehyde. OA-BSA conjugate was bound to the activated derivatives to form a cross-linked complex, which strongly attached to the gold surface of the crystal. The polyethyleneimine immobilization with glutaraldehyde cross-linking was a successful coupling method that produced a piezoelectric immunosensor with a good long-term storage lifetime of 38 days. However, the sensitivity and the detection limit of the sensor for OA were not satisfactory. For this reason, an antibody-BSA hydrogel was used to improve the performance of the piezoelectric immunosensor. Hydrogel can suddenly modify their volume in response to environmental change, such as solvent composition, pH, or temperature. The oscillation frequency of crystal with hydrogel decreased due to a volume change of the gel resulting from the binding of OA to antibody recognition sites. The use of the antigen-sensitive hydrogel improved the detection limit and the sensitivity by 524- and 80-fold, respectively.

4.2.3. Lateral flow immunochromatrographic test strips

Lateral flow immuchromatographic assays are similar in format to the home pregnancy tests. These tests provide a qualitative (yes/no result) indication to the presence of OA and its analogue. The lateral flow test is based on a competitive immunoassay format. Recently, Lu and co-workers described a lateral flow immunochromatrographic test strip which can be used as a food safety screening tool on-site [87]. Fig. 7 shows that the OA-BSA conjugate and goat anti-mouse IgG are separately placed onto the test and control regions in the detection region. When there was no toxin in the sample solution, the colloidal gold-labeled anti-OA McAb was captured by the immobilized OA-BSA on the test line and goat anti-mouse IgG on the control line. Thus, two red bands appeared due to the accumulation of red-colored colloidal gold–McAb (Fig. 7B, strip 1). When the target toxin presents in the sample, it bound to the colloidal gold-labeled anti-OA McAb. The more analyte that was present in the sample, the less colloidal gold-labeled anti-OA McAb was bound to the immobilized OA-BSA on the test line, resulting in a weaker test signal. Therefore, a positive signal was demonstrated when the test line was barely visible and only the control line was visible (Fig. 7B strips 2–4).

Two lateral flow immunochromatrographic test strips are commercialized. For Jellett Rapid Testing Ltd., the absence of a colored test line on the strip indicates that the sample contains the toxin at a concentration around half the regulatory limit. Reveal for DSP is designed to be clearly positive at 160 ppb, the maximum permitted level established by the European Union. Since most samples tested by regulatory agencies are negative, dipstick tests can be used to screen a wide range of samples quickly and only those presenting a positive result need to be tested further, thereby speeding throughput and reducing costs.

5. Perspectives: The aptamers

In 1990, Ellington's group [88], Gold's group [89] and Robertson's group [90] independently reported the development of an in vitro selection technique which allowed the discovery of specific nucleic acid sequences that bind non-nucleic acid targets with high affinity and specificity. The technique was called SELEX (Selection Evolution of Ligands by EXponential enrichment) and the resulting DNA or RNA oligonucleotides are referred ~~to~~ as aptamers [91–96]. Aptamers show high affinity towards a wide range of target analytes, including proteins, metal ions, toxins and pathogenic microorganisms. Aptamers offer many advantages compared to antibodies [96] such as small size, their chemical production, their stability (for DNA aptamers), the possibility of repeated cycles of denaturation and regeneration, easy chemical modification without affecting their affinity.

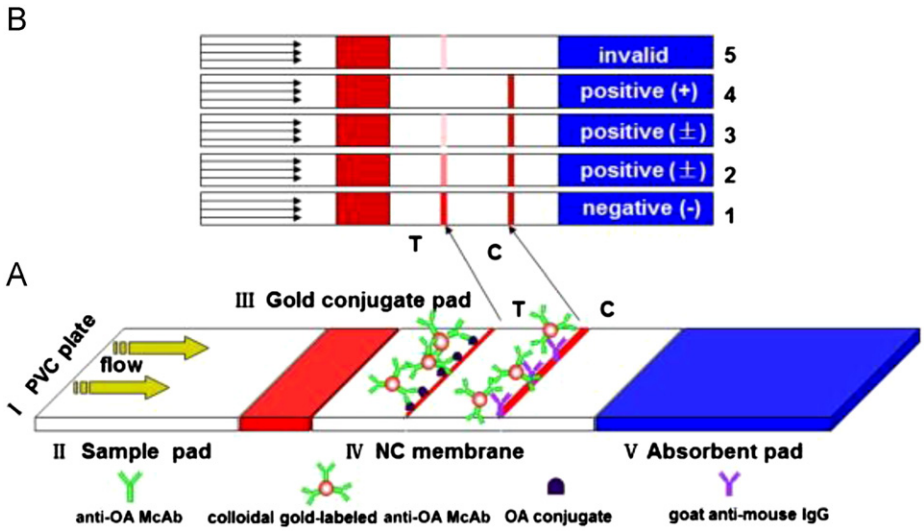

Fig. 7. (A) Schematic diagram of the test principle of Lateral flow immuchromatographic flow test. (B) Estimate of the LFIC test. C, control line; T, test line. Strip 1 indicates negative results when the color density of the test line is the same as that of the control line. Strips 2 to 4 indicate positive results when the color density of the test line is weaker than that of the control line (strips 2 and 3) or when the concentration of the toxin is high enough to neutralize all colloidal gold-conjugated McAb, resulting in no color (strip 4). Strip 5 indicates an invalid test result when no lines appear in the control line [89]. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 5
Advantages and disadvantages of the current analysis methods.

Methods	Advantages	Disadvantages
Mouse bioassay	✓ Does not require complex analytical equipment ✓ Indication about the overall toxicity.	✓ Lack of specificity ✓ No quantitative determination ✓ High potential for variability ✓ Expensive ✓ Time-consuming ✓ Ethical problems
In vitro bioassays	✓ No ethical conside-ration ✓ Cheaper than the mouse bioassay	✓ Lack of specificity: potential occurrence of false positives due to the interference by other lipids and/or matrix components). ✓ Variability due to the cell line that is used and method of toxicity evaluation. ✓ The use of maintenance of cell lines requires technical qualification. ✓ Needs interlaboratory validation and standard protocols ✓ Confusing results can appear in the presence of toxin mixtures. ✓ Time-consuming ✓ Require laboratory equipment
Chemical analysis	✓ High sensibility and specificity ✓ Rapidity ✓ No ethical considera-tion ✓ Possibility of automa-tion	✓ Require costly equipment and highly trained personnel. ✓ Require reference standards for identification and quantification. ✓ Needs interlaboratory validation and standard protocols
Enzyme inhibition-based methods	✓ No ethical considera-tion ✓ High sensibility ✓ Short analysis time	✓ Poor enzymatic stability ✓ Require expensive protein phosphatases ✓ Require laboratory equipment for the signal analysis: microplate reader, potentiostat. ✓ Needs interlaboratory validation and standard protocols.
Immunology-based methods	✓ No ethical considera-tion ✓ High sensibility	✓ Require laboratory equipment for the signal analysis: microplate reader, potentiostat, luminometer, QCM, SPR. ✓ Needs interlaboratory validation and standard protocols.

Due to its many advantages, numerous aptamer-based biosensors have been developed for the detection of targets, mainly proteins [97–99]. The thrombin aptamer is the most commonly exploited [100]. In the case of small targets, like toxins, optimization of the working conditions is usually laborious [101]. In 2008, our group selected a DNA aptamer specific for ochratoxin A which is one of the most abundant food-contaminating mycotoxin [102]. Until now, no OA aptamer has been described. One objective of our group was to select an aptamer for OA. Nowadays, the development of an electrochemical aptasensor for OA detection is in progress and the aptamer is being patented by the laboratory IMAGES.

6. Conclusion

This paper presents the current analysis techniques that have been applied to the detection of OA in contaminated shellfish. Several methods are used but none of them is ideal (Table 5). Mouse bioassays are simple but they suffer from low sensitivity,

lack of reliability and ethical implications. In vitro cytotoxicity assays, based on morphological changes in cells after exposure to toxins, have been developed to provide a substitute for the mouse bioassay. However, they are time-consuming and the results show high variability and low specificity. Chromatographic techniques, such as high-performance liquid chromatography (HPLC) coupled with fluorescence detection, have been widely used for the detection of seafood toxins. Nevertheless, these methods are laborious and time-consuming and require expensive equipment and skilled personnel. Liquid chromatography–mass spectrometry (LC–MS) and liquid chromatography–tandem mass spectrometry (LC–MS/MS) are gaining increasing success due to the efficient toxin separation, high selectivity, high sensitivity, wide working range, accurate quantification, ease-of-use and rapidity. Liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) has been validated and approved by the European Regulation as the new official control method for lipophilic toxins in shellfish [26]. Even if the European Community considered the enzymatic approach as a possible alternative to the mouse bioassay, this strategy suffers from poor enzymatic stability. However, enzyme immobilization contributes in overcoming this problem. Immunoassays seem to be also a promising tool for routine detection of okadaic acid due to the high sample throughput and relatively low costs [25]. Moreover, no skilled personal is required.

The ELISA tests and the PP2A inhibition assays are the first step towards the development of immunosensors andenzyme-based biosensors, which are rapid, robust, specific and sensitive analytical methods for the detection of phycotoxins. Several configurations that depend on the immobilization technique and the transducer type can be envisaged for the development of biosensors. Nanotechnology is playing an important role in the development of efficient biosensors. Different types of nanomaterials (e.g., nanoparticles and nanotubes) with different properties can be used. Despite their potential, they have not yet been exploited for the development of biosensors for the detection of OA. However, they offer exciting new opportunities to improve the performance of biosensors for the analysis of contaminated shellfish.

In the last decade, aptamers have been used as new molecular recognition elements to develop assays and biosensors. However, unique properties of aptamers have not yet been exploited for the detection of marine toxins. We believe that this recognition element could be used in the near future for the development of efficient aptasensors allowing the detection of OA.

Even if some commercial kits are available for the analysis of DSP toxins, there is still a challenge to develop improved and more reliable devices allowing the analysis of contaminated shellfish.

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