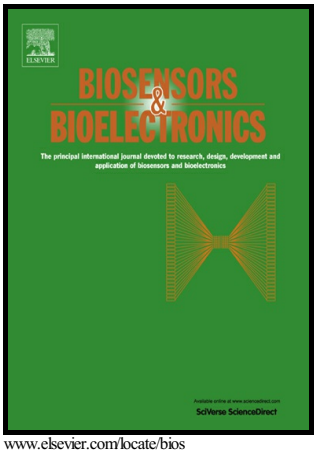

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Enzyme-linked, aptamer-based, competitive biolayer interferometry biosensor for palytoxin

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Abstract:

In this study, we coupled biolayer interferometry (BLI) with competitive binding assay through an enzyme-linked aptamer and developed a real-time, ultra-sensitive, rapid quantitative method for detection of the marine biotoxin palytoxin. Horseradish

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peroxidase-labeled aptamers were used as biorecognition receptors to competitively bind with palytoxin, which was immobilized on the biosensor surface. The palytoxin:horseradish peroxidase-aptamer complex was then submerged in a 3,3'-diaminobenzidine solution, which resulted in formation of a precipitated polymeric product directly on the biosensor surface and a large change in the optical thickness of the biosensor layer. This change could obviously shift the interference pattern and generate a response profile on the BLI biosensor. The biosensor showed a broad linear range for palytoxin (200 to 700 pg/mL) with a low detection limit (0.04 pg/mL). Moreover, the biosensor was applied to the detection of palytoxin in spiked extracts and showed a high degree of selectivity for palytoxin, good reproducibility, and stability. This enzyme-linked, aptamer-based, competitive BLI biosensor offers a promising method for rapid and sensitive detection of palytoxin and other analytes.

Keywords: Aptamer, SELEX, Biolayer interferometry, Biosensor, Palytoxin

1. Introduction

Marine biotoxins are highly active metabolites that are present in marine organisms, and are typically very toxic (Gallardo-Rodriguez et al. 2012). Palytoxin (PTX), which was first isolated from soft corals in 1971, is one of the most potent non-protein natural toxins known to date (Fig. S1) (Moore and Scheuer 1971). The estimated LD_{50} after intraperitoneal injection of PTX is 25 ng/kg in rabbits and 50 ng/kg in mice

(Patocka et al. 2015; Volpe et al. 2014). It can accumulate at very high levels in shellfish, sea urchins, and crabs through the aquatic food chain, and consumption of PTX-contaminated seafood by humans causes poisoning characterized by dizziness, weakness, muscle pain, breathing difficulties, heart failure, and even death (Louzao et al. 2008). The onset of symptoms is very rapid, and death can occur within a few minutes. Most incidents of PTX poisoning have been associated with consumption of seafood contaminated with PTX. Skin and respiratory problems can also occur after direct contact with aerosolized water during dinoflagellate blooms (Deeds and Schwartz 2010; Tubaro et al. 2011). PTX outbreaks are usually associated with Indo-Pacific waters, but have been observed worldwide, including along the Mediterranean and Atlantic Europe coastlines (Aligizaki et al. 2011; Ciminiello et al. 2013; Durando et al. 2007). Shellfish collected in the north Aegean Sea showed contamination with PTX during *Ostreopsis* blooms between 2004 and 2006 (Aligizaki et al. 2008). Because of the presence of PTX, France has prohibited sea urchin collection from April to November as a precautionary measure (Lemée et al. 2012). Although PTX is highly toxic to humans and animals and is widely distributed in the ocean, there is currently no recognized official method for the determination of this toxin (Riobo and Franco 2011). The European Food Safety Authority has recommended a maximum regulatory limit of 30 mg/kg for shellfish meat, and encouraged the development of a regulatory monitoring regime using sensitive, specific, rapid, and accurate analytical methods (EFSA. 2009).

The mouse bioassay is commonly used for detection of marine biotoxins, including PTX (CRLMB 2009; Riobo et al. 2008). However, this method has inherent ethical and logistical problems, poor sensitivity, and undefined cross-reactivity (EFSA. 2009). Some other analytical methods have been developed for the identification and detection of PTX, including liquid chromatography coupled to a fluorometer, ultraviolet-visible spectrophotometer, or mass spectrometer (Ciminiello et al. 2011; Kerbrat et al. 2011; Riobó et al. 2006). However, because these analytical methods are time consuming and require sophisticated instrumentation, they are not suitable for on-site monitoring. Also, the current limits of quantitation for these methods appear to be insufficient to meet the regulatory requirement for PTX (30 mg/kg) in shellfish (Ciminiello et al. 2011). Therefore, alternative methods that are sensitive and can rapidly detect PTX in seafood and water are required. Biosensors are ideal alternatives to conventional analytical methods for PTX detection. Some optical immunosensors have been developed for PTX detection based on surface plasmon resonance, electrochemistry, and chemiluminescence, and these show high sensitivity and specificity (Alfonso et al. 2014; Zamolo et al. 2012). However, a major drawback is that antibodies, which are expensive, have limited stability, and are difficult to produce in vivo, are used as biorecognition receptors in these methods.

Aptamers are functional single-stranded DNA or RNA oligonucleotides, which can be selected for targeted applications from random combinatorial libraries using the Systematic Evolution of Ligands by Exponential Enrichment (SELEX) technique. As

stable and inexpensive recognition receptors, aptamers can provide comparable results to and even replace antibodies (Jayasena 1999). The first aptamer was identified in 1990 (Ellington and Szostak 1990; Tuerk and Gold 1990), and since then, many aptamers with high affinity and specificity for a wide range of target analytes have been isolated. This has led to the development of various robust biosensing technologies in recent years (Ferapontova et al. 2008; Tan et al. 2013).

Biolayer interferometry (BLI) is a real-time optical analytical technique for measuring interactions between biomolecule (Concepcion et al. 2009). This method uses fiber-optic biosensors to monitor changes in the optical thickness of the sensor layer that occur with biological binding events. In this system, analytes that interact with ligands immobilized on a sensor surface form a monomolecular layer, which causes a proportional shift in the interference spectrum of the reflected light (Kumaraswamy and Tobias 2015). Interactions are measured in real-time, and can be used for monitoring binding specificity, rates of association and dissociation, or changes in concentration, with precision and accuracy. The application of aptamers as recognition receptors in conjunction with BLI technology can enable highly sensitive (limit of detection 50 pg/mL) and rapid (5–20 min) detection of analytes, such as the marine neurotoxin gonyautoxin 1/4 (Gao et al. 2016a; Gao et al. 2016b).

In the present study, to accurately measure PTX at lower concentrations, an enzyme-linked aptamer-based signal enhancement step was introduced. Horseradish peroxidase-labeled aptamers (HRP-aptamer) were used as biorecognition receptors to

competitively bind with PTX, which was immobilized on the biosensor surface. The PTX:HRP-aptamer complex was then submerged in a 3,3'-diaminobenzidine (DAB) solution, which resulted in formation of a precipitated polymeric product directly on the biosensor surface and a large change in the optical thickness of the biosensor layer. This change resulted in wavelength interference, and amplified the biosensor response. Indeed, the coupling of BLI with an enzyme-linked aptamer-competitive binding assay (ELACBA, termed BLI-ELACBA) could amplify the biosensor signal during the detection step to enable real-time, ultra-sensitive, and rapid detection of PTX in seafood and water samples. This BLI-ELACBA offers a promising alternative to traditional analytical methods for detection of the marine biotoxin PTX.

2. Materials and methods

2.1. Materials and Reagents

All nucleic acid sequences were custom synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). Okadaic acid, microcystin-LR, saxitoxin, and brevetoxin were purchased from Taiwan Algal Science, Inc. (Taiwan). Dynabeads® M-270, carboxylic acid, and a Qubit® ssDNA Assay Kit were purchased from Thermo Fisher Scientific (Waltham, MA). 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), 2-(*N*-Morpholino)ethanesulfonic acid, *N*-hydroxysuccinimide (NHS), urea (ultra-pure grade), and other chemicals were purchased from Sigma-Aldrich China (Shanghai, China). Dr. GenTLE® Precipitation Carrier and 20 bp DNA Ladder (Dye Plus) were

obtained from TaKaRa Bio, Inc. (Dalian, China). GoTaqHot® Start Colorless Master Mix was purchased from Promega Corporation (Shanghai, China). Streptavidin (SA) and Amine Reactive 2nd Generation (AR2G) biosensors were obtained from ForteBio (Menlo Park, CA). The coupling buffer was 2-(*N*-morpholino)ethanesulfonic acid (pH 5.0). Binding buffer (20 mM Tris-HCl, 100 mM NaCl, 2 mM $MgCl_2$, and 5 mM KCl, pH 7.5) was purchased from Tiandz (Beijing, China). The elution buffer was nuclease-free water. All solutions were prepared using Milli-Q water (Millipore, Billerica, MA).

2.2. Coupling of PTX to Magnetic Beads

An aliquot (400 µL) of the Dynabeads®M-270 suspension (30 mg/mL) was washed several times with coupling buffer. Then, 25 mg of EDC and 25 mg of NHS were dissolved in 600 µL of coupling buffer and mixed with the 400 µL of washed beads. The mixture was incubated for 0.5 h with end-over-end rotation at room temperature. After the reaction, the supernatant was separated from the beads using a magnetic frame, and the activated beads were equilibrated with 400 µL of coupling buffer. PTX (10 µg) was dissolved in 800 µL of coupling buffer and mixed with 200 µL of the activated beads. The mixture was rotated for 0.5 h at room temperature. After the reaction, the beads were washed with coupling buffer to remove excess unreacted toxin. Ethanolamine (40 µL) was diluted in 1600 µL of coupling buffer. Then, 800 µL of the diluted ethanolamine solution was added to 200 µL of the PTX beads to block the unreacted carboxyl on the beads. The remaining 800 µL of the ethanolamine solution was added to 200 µL of the

activated beads to prepare negative beads. After another 0.5 h of rotation at room temperature, the beads were washed thoroughly with binding buffer. To confirm that PTX was successfully immobilized on the beads, the unreacted toxin was quantified in the recovered supernatant. Ultraviolet absorption spectra were recorded from 190 to 400 nm, and the maximum absorption of PTX was at 264 nm. A calibration curve of the absorption against the PTX concentration was constructed (Fig. S6), with each sample analyzed in triplicate. The coupling efficiency of PTX was calculated to be 92.79% by interpolation. Finally, PTX beads and negative beads were stored in binding buffer at 4 °C until use.

2.3. In Vitro Selection of the DNA Aptamer

Magnetic bead-SELEX (MB-SELEX) includes positive- and counter-selection processes (Fig. 1A, Table S1). A single-stranded DNA (ssDNA) library (5′-ACCGACCGTGCTGG ACTCA-N_{42}-ACTATGAGCGAGCCTGGCG-3′) was dissolved in binding buffer, heated to 95 °C for 10 min, cooled in an ice bath for 5 min, and then kept at room temperature for 5 min to fold into stable oligonucleotide conformations. Ten microliters of PTX beads was washed several times with binding buffer, and then added to the prepared library in 200 μL of binding buffer in a centrifuge tube. The mixture was incubated with rotation at room temperature. Then, the beads were washed several times with binding buffer until no DNA was detected by fluorometry (Qubit® 2.0, Thermo Fisher Scientific). The ssDNA bound to PTX immobilized on the beads was eluted using 100 μL of elution

buffer with heating at 95 °C for 15 min. The eluted ssDNA was recovered and quantified. To improve the screening efficiency, a counter-SELEX strategy was introduced. The ssDNA library was first incubated with the negative beads, and unbound ssDNA was collected and quantified in the supernatant and subjected to the same heating and cooling treatment as before. Subsequently, the prepared library was incubated with the washed PTX beads. Furthermore, counter-targets that acted as free competitors (okadaic acid, STX, microcystin-LR, brevetoxin) were added to the positive incubation system to improve the specificity of screening. The selected DNA pools were amplified by the polymerase chain reaction (PCR) in 40 parallel 50-μL reactions, each containing Go Taq® Hot Start DNA Polymerase and Colorless Go Taq® Reaction Buffer, 2 mM $MgCl_2$, 200 μM dNTP, and 0.5 μM of forward and reverse primers. The forward primer was 5′-ACCGACCGTGCTGGACTCA-3′, the reverse primer was 5′-CGCCAGGCTCGCTC ATAGT-3′, and the modified reverse primer was 5′-poly (dA20)-Spacer18-CGCCAGGC TCGCTCATAGT-3′. The PCR conditions were as follows: 94 °C for 1 min, followed by 25 cycles of 95 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s, and a final extension step of 2 min at 72 °C. The relevant ssDNA was separated from the double-stranded PCR product by 12% urea denaturing polyacrylamide gel electrophoresis (PAGE), and the gel band was eluted by boiling. The eluted ssDNA was purified by ethanol precipitation and dried at room temperature for 30 min. Finally, the dried ssDNA powders were dissolved in binding buffer and used for the next selection round.

2.4. Cloning and Sequencing of Selected DNA

After 10 selection rounds, the enriched ssDNA pool was amplified with unmodified primers. The purified PCR products were then cloned and sequenced by Sangon Biotech Co., Ltd.

2.5. Determination of Kinetic Parameters by BLI

BLI is a real-time optical analytical technique for measuring the interactions between biomolecules, and involves analysis of interference patterns of white light created by two different surface reflections (Concepcion et al. 2009). In this case, the surface reflections are from a layer of immobilized ligands on the biosensor surface, and an internal reference layer (Fig. 2B). Binding of analytes to the biosensor surface causes a shift in the interference pattern. This wavelength shift ($\Delta\lambda$) directly reflects the change in the optical thickness of the sensor layer, and is reported as a change in wavelength as a function of time. Only analytes binding to or dissociating from the biosensor surface can shift the interference pattern and generate a response profile in the Octet system used in this study (OctetRED96, ForteBio). Therefore, the affinity binding of aptamers was determined by BLI. The potential aptamers were immobilized on the biosensor surface using SA–biotin coupling between the modified biotin on the aptamers and SA on the biosensor. The assay procedure included five steps (Fig. S4): (1) baseline (2 min), (2) loading (3 min), (3) washing (2 min), (4) association (3 min), and (5) dissociation (3 min). All steps were performed at 25 °C with shaking at 1500 rpm in a 96-well plate containing

200 µL of solution in each well. The binding buffer (200 µL) was used as the baseline solution (A), loading solution (B), washing solution (C), association solution (D), and dissociation solution (E). In the Octet system, a signal was simultaneously acquired from the control surface and subtracted from the data obtained from the reaction surface for normalization to eliminate the effects of nonspecific binding and buffer-induced interferometry spectrum shifts. This was performed using the Octet Data Analysis Software (CFR Part 11 Version 6.x). The affinity parameter K_d was then obtained. A 1:1 binding mode with mass transfer fitting was used to obtain the kinetic data.

2.6. Immobilization of PTX on the AR2G biosensor

PTX was immobilized on the AR2G biosensor surface by coupling the terminal amine groups on the toxin with the carboxylic groups on the biosensor surface using an EDC/NHS method. Briefly, 25 mg of NHS and 25 mg of EDC were dissolved in 1 mL of coupling buffer, and 200 µL of the solution was immediately added onto the AR2G biosensor surface and incubated for 0.5 h to activate the carboxylic acid groups. Then, PTX (10 µg) was dissolved in 1 mL of coupling buffer, and 200 µL of this solution was incubated with the activated biosensor for 0.5 h to enable coupling of the PTX. Any remaining unreacted carboxylic groups on the sensor were blocked using 0.2 M ethanolamine. Finally, the PTX-modified biosensor was washed with binding buffer and stored in binding buffer at 4 °C until required for further use.

2.7. Extraction of Marine Biotoxins from Shellfish and Water

Scallops and clams had been harvested in the Zhoushan Archipelago (Zhejiang, China). Mussels were purchased from a local supermarket (Shanghai, China). Shellfish extracts were prepared following the AOAC 2005.06 double extraction acid procedure (Lawrence et al. 2004). Briefly, samples (5 ± 0.1 g) of shucked shellfish meat were weighed, and 3 mL of 1% (volume fraction) acetic acid in water was mixed with the meat in a centrifuge tube. The mixtures were shaken for 90 s on a vortex mixer, boiled for 5 min, cooled to room temperature, and then shaken for another 90 s. Subsequently, the mixtures were centrifuged for 10 min at 4500 rpm, and the extracts were collected. The homogenate was extracted by repeating the same procedure. The collected supernatant was evaporated in a SpeedVac concentrator (Thermo Fisher Scientific), and the residue was resuspended in 200 μL of binding buffer.

Water samples (3 mL) were spiked with PTX, which was then extracted by centrifugation for 20 min at 4500 rpm, and the supernatant was collected. The extract was evaporated in a SpeedVac concentrator, and the residue was resuspended in 200 μL of binding buffer.

3. Results and discussion

3.1. Selection and Identification of the DNA Aptamers

To obtain DNA aptamers that bind to PTX with high affinity and specificity, we immobilized PTX on M-270 Dynabeads (Fig. S2). MB-SELEX involves incubation,

separation, elution, and PCR amplification, as outlined in Fig. 1A (Gao et al. 2016a). To improve the screening efficiency, negative beads were introduced after the fourth round to eliminate any ssDNA that was bound non-specifically to the beads. Furthermore, competitive counter-molecules (e.g. okadaic acid, microcystin-LR, saxitoxin, and brevetoxin-A/B) were added in the positive incubation system to improve the specificity. After 10 rounds of selection, the ssDNA recovery ratio had greatly increased and reached a plateau, which indicated that the binding sites on the target in the system had almost reached saturation (Fig. 1B). Therefore, the selection cycles were stopped, and the enriched DNA was cloned and sequenced. The identified sequences were then analyzed by multiple sequence alignment using Clustal X software, and their secondary structures were predicted using the mfold web server. Based on sequence conservation, the alternatives were divided into nine families (Fig. S3). Then, sequences with the highest homology or minimum free energy from each group were chosen for further binding studies with PTX (Fig. S4). Eight aptamers showed high affinity for PTX, ranging from 8.43×10^{-10} to 1.03×10^{-6} M, while four sequences showed no binding (Fig. S5). The aptamer with the highest affinity for PTX (PTX-13) was used for further characterization. The binding interactions of PTX with the aptamer were studied at different concentrations (75 nM (top), 50 nM (middle), and 25 nM (bottom), Fig. 1 C). The K_{on}(1/Ms) was 7.60×10^{5}, K_{dis}(1/s) was 6.41×10^{-4}, and K_d (M) was 8.43×10^{-10}. These results revealed that the PTX-13 aptamer has fast association and slow dissociation with PTX. A random sequence was used as a negative control and did not exhibit binding.

Furthermore, other marine biotoxins structurally unrelated to PTX (e.g. okadaic acid, microcystin-LR, saxitoxin, brevetoxin-A/B) showed no change in the response. These results indicated that the PTX-13 aptamer bound to PTX with high affinity and specificity.

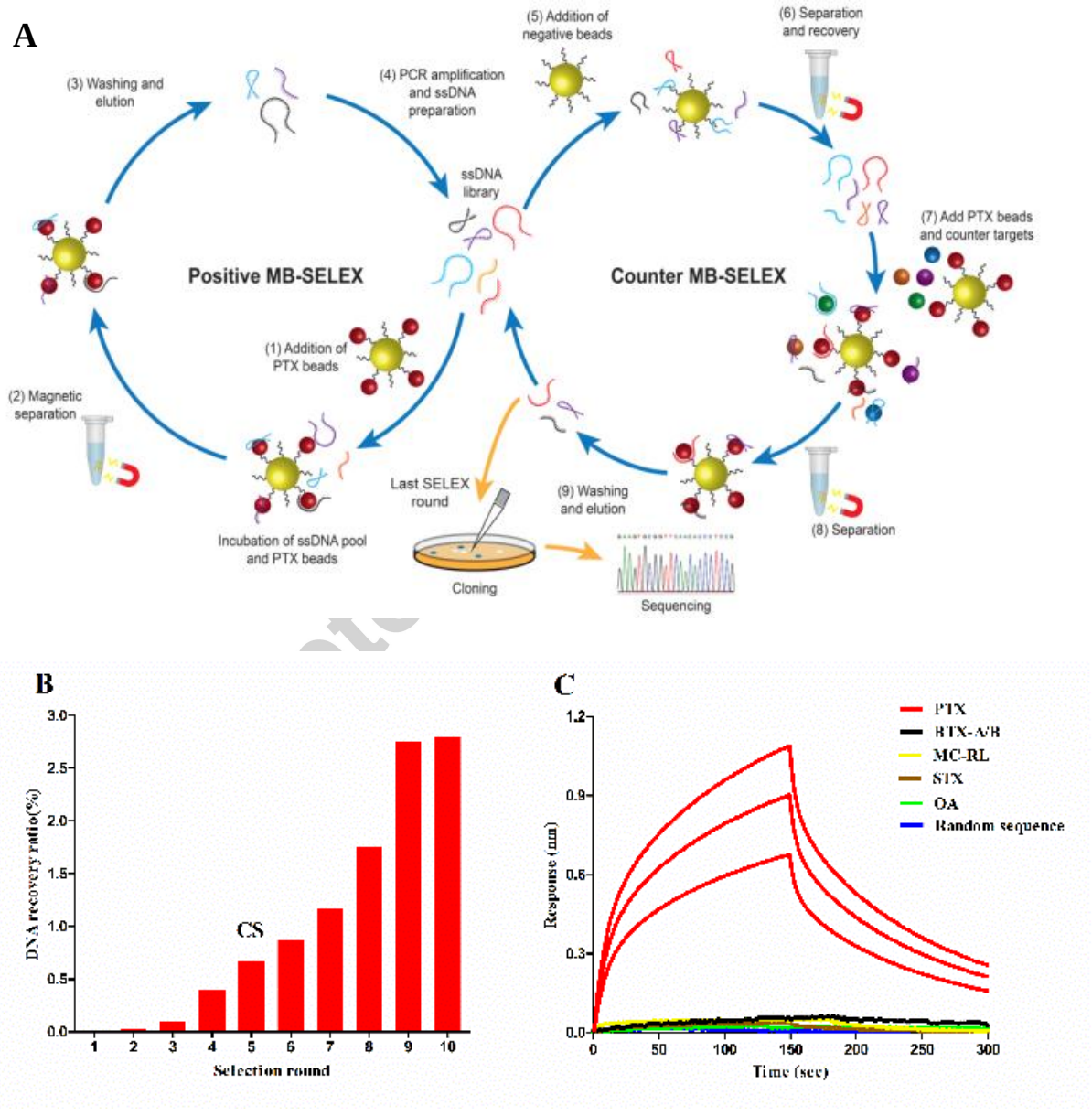

Fig. 1. (A) Schematic of the MB-SELEX protocol. (B) Enrichment of PTX-ssDNA during MB-SELEX. (C) Characterization of affinity and specificity of the PTX-13

aptamer for PTX. The red lines represent the interaction curves of the aptamer with PTX at different concentrations (75 nM (top), 50 nM (middle), and 25 nM (bottom)). The blue line represents the interaction curve of a random sequence with PTX. The black, yellow, gold, and green lines represent the interaction curves of the PTX-13 aptamer with BTX-A/B, MC-LR, STX, and OA, respectively.

3.2. Enzyme linked, aptamer-based competitive BLI biosensor

To enable rapid and sensitive detection of PTX, the PTX-13 aptamer was then used to fabricate a real-time, optical, competitive biosensor based on BLI detection. In the first step of the assay (Fig. 2A), the PTX-labeled AR2G biosensor was submerged for 1 min in the binding buffer. The sensor was then incubated with samples free of PTX in a fixed amount of the HRP-aptamer for 5 min, followed by a quick 1-min wash step. Finally, the PTX:HRP-aptamer complex on the biosensor was submerged for another 3 min in a 3,3'-diaminobenzidine (DAB) substrate solution, resulting in formation of a precipitated polymeric product directly on the biosensor surface. This caused a large change in the optical thickness and mass density of the biosensor layer, and induced wavelength interference ($\Delta\lambda$, Fig. 2B) and amplified the biosensor response (Fig. 2C).

The sensitivity was greatly improved using the BLI-ELACBA assay format. In this assay, the free HRP-aptamer concentration used for the competition step was a crucial factor. Theoretically, higher concentrations of the aptamer will lead to decreased sensitivity and lower concentrations will lead to a reduced signal. To optimize the binding

conditions, HRP-aptamer concentrations ranging from 0 to 500 pM were studied. The BLI response increased with increasing free HRP-aptamer concentrations up to 200 pM (Fig. 2D). Therefore, 200 pM was chosen as the optimal HRP-aptamer concentration. Furthermore, the ability of the biosensor to selectively capture the free HRP-aptamer from the equilibrated detection system without disturbing the PTX:HRP-aptamer complex is also crucial. An important parameter in quantifying the free PTX is the contact time between the capture surface and the equilibrated detection solution. Generally, a longer incubation time allows for the capture of more of the free HRP-aptamer for a stronger signal than that obtained with a shorter incubation time. However, as more of the free HRP-aptamer is captured from the solution over time, the PTX:HRP-aptamer complex may dissociate, disturbing the balance and altering the measured response. To evaluate this effect, incubation times from 2.5 to 60 min were studied. The complex remained stable between 2.5 and 10 min (Fig. 2E), but a 7-fold decrease in apparent affinity was observed between 10 and 60 min. Therefore, to balance the demands of stability and signal strength, an incubation time of 5 min was selected for the competitive capture stage.

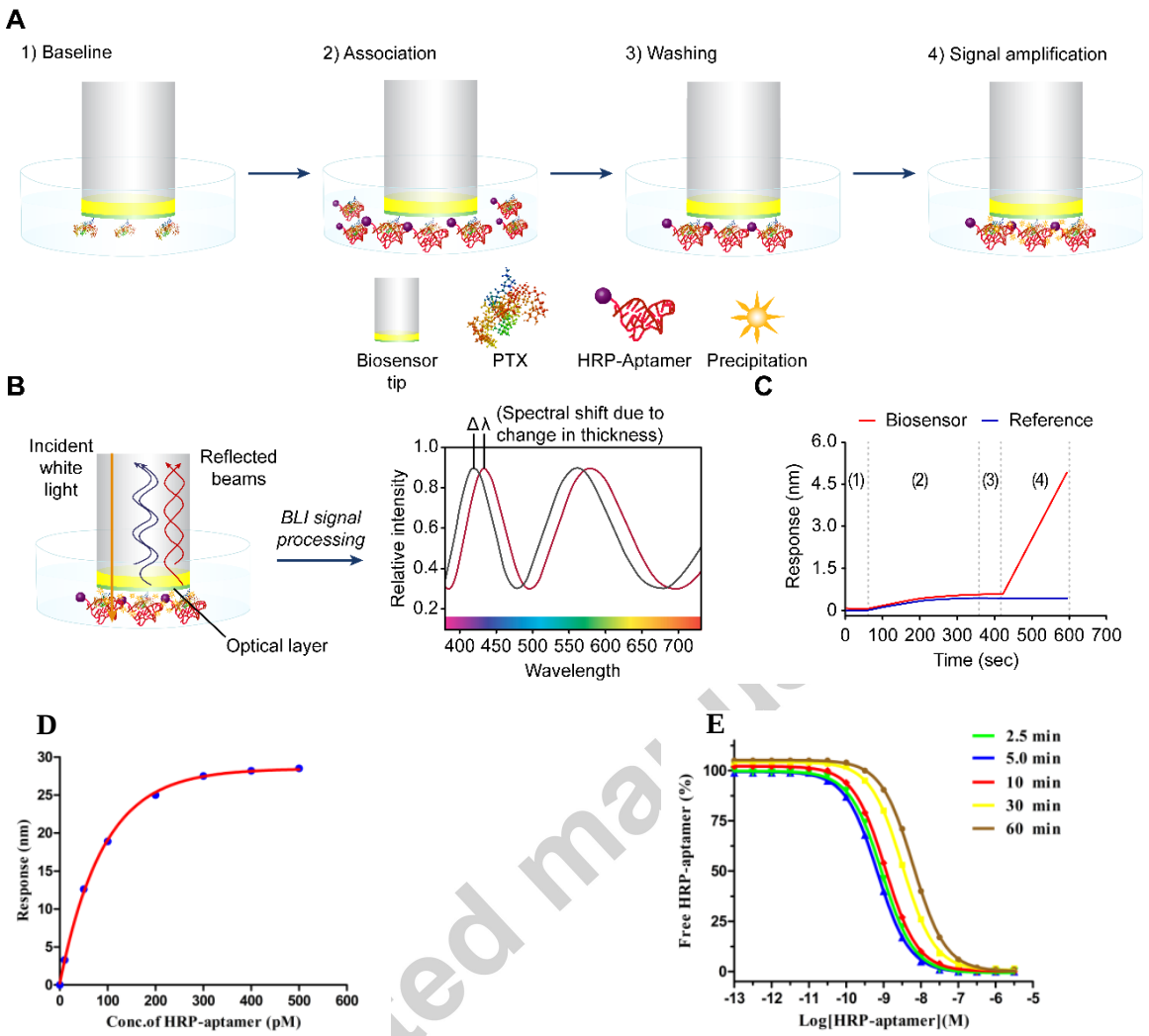

Fig. 2. (A) Schematic of the working principles of the biosensor with the competitive assay. (B) Schematic of the BLI-based detection system. (C) Schematic of the BLI-based detection steps: (1) baseline (1 min), (2) capture of free HRP-aptamer (5 min), (3) washing (1 min), and (4) signal amplification (3 min). (D) Calibration curve of the biosensor response with changes in the HRP-aptamer concentration (0 to 600 pM) obtained in a non-competitive system. (E) The effect of the contact time between the capture surface and the equilibrated detection solution, monitored by measuring the free HRP–aptamer using the BLI-enzyme-linked aptamer-competitive binding assay.

To examine the detection of PTX using the BLI-ELACBA, time-dependent changes in response to PTX at different concentrations (0 to 1000 pg/mL) were investigated under the optimal experimental conditions. As the concentration of PTX increased, the BLI response decreased (Fig. 3A). A calibration curve of the responses against the reciprocal of the PTX concentration was obtained (Fig. 3B), with each sample analyzed in triplicate. This was fitted to a sigmoidal logistic four-parameter equation:

$$y = (R_{max} - R_{min}) / [1 + (x/EC_{50}) \wedge b] + R_{min} \quad (1)$$

where R_{max} and R_{min} are fitting parameters that correspond to the maximum and minimum response, respectively; b is the additional fitting parameter that corresponds to the slope of the curve; and *EC50* is the reciprocal of PTX concentration leading to 50% of the maximum response. The regression equation was y = (25.05427 – 0.10812) / [1 + (x/ 0.00180) ^ –1.50701] + 0.10812, with a correlation coefficient R^2 of 0.999. Moreover, the biosensor exhibited a linear detection range from 200 to 700 pg/mL of PTX, with good agreement (R^2=0.992). The limit of detection (LOD, 0.04 pg/mL) was calculated as the average plus three times the standard deviation of the background signal. This LOD is comparable to the LODs reported for biosensors for small molecules and previously reported detection methods for PTX (Alfonso et al. 2012; Alfonso et al. 2014; Fraga et al. 2016; Zamolo et al. 2012). Furthermore, compared with earlier work, the present work uses advanced BLI technology and complicated sensor designs to enable fast, highly sensitive, and real-time detection in crude samples.

3.3. Analysis of PTX in real shellfish and water *samples*

To assess the feasibility of detecting PTX in real samples, spiked extracts were analyzed using the developed BLI-ELACBA. Free PTX was spiked in shellfish and seawater samples at three levels with the concentrations of 50, 200, and 800 pg/mL. As shown in Table 1, good recovery (100.27%–108.24%) was obtained, which indicates there is little interference from the shellfish and water matrices. The coefficient of variation (2.27%–6.76%) was acceptable, and the biosensor is promising for practical use in shellfish and water samples. Furthermore, cross-reactivity experiments with extracts spiked with 500 pg/mL of okadaic acid, brevetoxin-A/B, saxitoxin, and microcystin-LR showed the response was close to the maximum signal (Fig. 3C). This indicates nonspecific interference of these toxins in the BLI-ELACBA does not occur. Moreover, for a mixed extract containing PTX (0.5 ng/mL) and other toxins (50 ng/mL okadaic acid, brevetoxin-A/B, saxitoxin or microcystin-LR), the response (8.90) was comparable to the case when only PTX was present (response = 9.15). These results demonstrate that PTX can be detected with high selectivity even in toxin mixtures, and no cross reactivity with other marine toxins occurred.

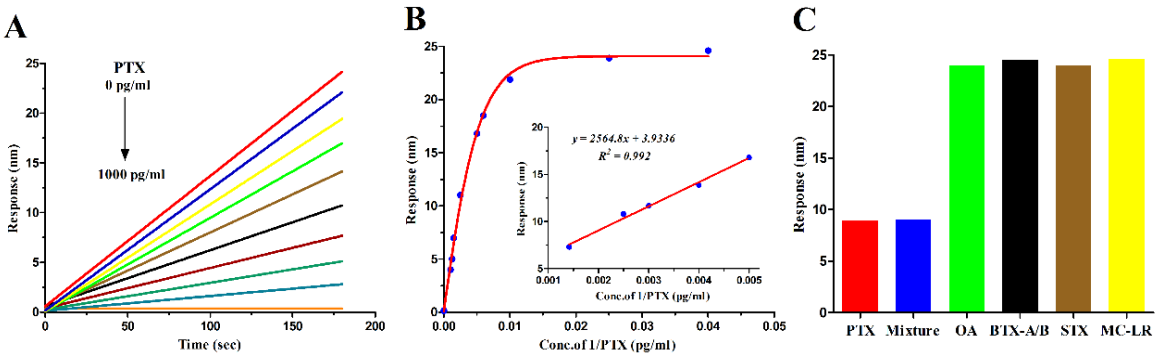

Fig. 3. (A) Response plot for the biosensor after addition of PTX at different concentrations (0–1000 pg/mL). (B) The calibration curve for PTX, plot of response vs. the reciprocal of PTX concentration. (C) Cross-reactivity of the biosensor with OA, BTX-A/B, STX, MC-LR (each at 500 pg/mL), and a mixture of all the toxins (PTX at 500 pg/mL and the other toxins at 50 ng/mL each).

Table 1. Recoveries for samples spiked with PTX at three concentrations and analyzed by BLI-ELACBA (n = 3).

samples	Spiked (pg/mL)	Theoretical response	Average response ±SD	Recovery (%)	CV (%)
mussels	50	23.697	24.044±0.834	101.46	3.46
—	200	17.137	18.021±0.409	105.16	2.27
—	800	5.354	5.697±0.367	106.41	6.44
scallops	50	23.697	24.217±0.860	102.19	3.55
—	200	17.137	18.343±0.494	107.04	2.69
—	800	5.354	5.795±0.392	108.24	6.76
clams	50	23.697	24.117±0.817	101.77	3.01
—	200	17.137	18.260±0.472	106.55	2.75
—	800	5.354	5.748±0.371	107.36	6.45
Water	50	23.697	23.761±0.716	100.27	3.01
—	200	17.137	17.591±0.483	102.65	2.75
—	800	5.354	5.624±0.380	105.04	6.75

4. Conclusion

In summary, this work shows the first time DNA aptamers have been identified for and

applied to biosensing of the marine toxin PTX. The developed BLI-ELACBA allowed for real-time, ultra-sensitive (LOD = 0.04 pg/mL), rapid (10 min) detection of PTX. The biosensor showed good selectivity for PTX against other marine biotoxins that are structurally unrelated to PTX, such as okadaic acid, microcystin-LR, saxitoxin, and brevetoxin-A/B. Moreover, the biosensor showed good reproducibility and stability in the analysis of spiked samples. We believe that this BLI-ELACBA offers a promising alternative to traditional analytical methods for detection of the marine biotoxin PTX.

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Highlights

- Thiswork is the first report of successful selection and identification of DNA aptamers that bind with high affinity and specificity to palytoxin.
- We report for the first time the use of an enzyme-linked aptamer with acompetitivebiolayer interferometry(BLI)biosensor.
- The real-time optical biosensorshowed a broadlinear range for the concentrations of palytoxin from 200 to 700 pg/mL with a low detectionlimit of 0.04 pg/mL.
- The biosensor was applied for the detection of palytoxin inspiked extracts showing a high degree of selectivity for palytoxin, and a good reproducibility and stability.

- The novel enzyme-linked, aptamer-based, competitive BLIbiosensor offers a promising detection method for palytoxin and other analytes in a rapid and sensitive manner.