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DNA aptamer selection and detection of marine biotoxin 20 Methyl Spirolide G

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ABSTRACT

This study reports the selection of DNA aptamer for the detection of 20 Methyl Spirolide G (SPXG). After 10 rounds of selection, the enriched pool of aptamers specific to SPXG was cloned, sequenced and clustered into seven families based on similarity. Three sequences SPX1, SPX2 and SPX7, each belonging to different clades were further evaluated for their binding affinity. Surface plasmon resonance studies determined the highest affinity K_D of $0.0345x10^{-8}$ M for aptamer SPX7. A label-free microscale thermophoresis-based aptasensing using SPX7 with highest affinity, indicated a linear detection range from 1.9 to 125000 pg/mL (LOD = 0.39 pg/mL; LOQ = 1.17 pg/mL). Spiking studies in simulated contaminated samples of mussel and scallop indicated recoveries in the range of 86 to 108%. Results of this study indicate the successful development of an aptamer for detection of SPXG at picogram levels. It also opens up avenues to develop other sensing platforms for detection of SPXG using the reported aptamer.

1. Introduction

Cyclic imines are an emerging group of marine biotoxins that contaminate seafood. 20 Methyl spirolide G (SPX G), a cyclic imine is a neurotoxin produced by dinoflagellate *Alexandrium ostenfeldii / A. peruvianum*. Spirolides were first identified in toxic digestive gland extracts of mussels and scallops found in the Atlantic coast of Nova Scotia, Canada. Sixteen different types of spirolides have been identified and characterized in contaminated shellfish and phytoplankton extracts from coasts of Europe, North and South America (Farabegoli, Blanco, Rodríguez, Vieites, & Cabado 2018). Accumulation of toxin in marine foods occurs as a result of ingestion of spirolide-producing dinoflagellates by the aquatic invertebrates. SPX G has been isolated from various species of shellfish, especially mussels and scallops (Guéret & Brimble, 2010). The toxin has been reported to bind neuronal nicotinic acetylcholine receptors and block skeletal muscles in mice models (Couesnon et al., 2016). Toxicity studies of SPX G, show an intraperitoneal LD_{50} value of 8 μg/kg body weight in mice (Munday et al., 2012). At present, there are no regulatory levels set by European Union (EU) or any other regulatory authorities for SPX G or other spirolide toxins in sea foods because of lack of conclusive data regarding their human poisoning (Rambla-Alegre et al., 2018). However, their toxicity and potential risk to consumers have raised growing concerns among public and policy makers to address food safety issues related to these biotoxins.

The present methods used for the detection of spirolides are classical analytical techniques such as high performance liquid chromatography, mass spectrometry and liquid chromatography – tandem mass spectrometry (Otero, Alfonso, Alfonso, Rodríguez, Vieytes, & Botana, 2011; Aasen et al., 2005; Gerssen, Mulder, McElhinney, & Boer, 2009). Mouse bioassay is the only official method accepted by EU for the determination of marine toxins including SPX G (European Commission, 2011). Intraperitoneal injection of shellfish extracts to mice in mouse bioassay has led to sacrifice of large number of laboratory animals leading to ethical concerns. Fluorescence polarization and solid-phase receptor-based assays are also reported for spirolide detection. Fluorescence polarization method works on the principle of quantitative spirolide binding to the fluorescently labelled nicotinic acetylcholine receptor

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resulting in the change of fluorescence polarization signal (Otero et al., 2011). Solid-phase receptor-based assays use the competition of spirolide with biotin labelled α-bungarotoxin for binding to nicotinic acetylcholine receptor and the immobilization of the former complex on streptavidin coated surface (Rodríguez et al., 2011). Although, these methods are accurate and reliable, they suffer from disadvantages such as long processing time, complex sample pretreatment and preparation steps, photobleaching, need for specially trained personnel and use of antibodies which restricts their wide scale application.

Aptamers are short oligonucleotide or peptide sequences generated *in vitro* against target molecules. They are screened, designed and evolved by an *in vitro* selection process known as systematic evolution of ligands by exponential enrichment (SELEX). Aptamers fold into specific three dimensional structures and bind to their ligands by complementary shape interactions and can incorporate small molecules into their folded structure or integrate into the structure of larger molecules (Hermann & Patel, 2000). The idea behind aptamer as recognition element comes from their ability to fold into three dimensional structures which facilitates their interaction with target molecules. Aptamer today are used in a wide range of applications, which comprise *in situ* bio-imaging, drug delivery and therapy, disease diagnosis, hazard detection, food monitoring, etc. (Wang et al., 2017; Jahangiri–Dehaghani, Zare, & Shekari, 2020). Relative to antibodies, aptamers have several advantages, including stability, low cost, and ease of synthesis (Jayasena, 1999). Aptamer-based biosensing platforms can be developed for both qualitative and quantitative measurements.

In this study, we report for the first time, the generation and selection of an aptamer for marine biotoxin SPX G. The selected aptamer showed high binding affinity to SPX G with dissociation constant (K_D) in the nanomolar range. Microscale thermophoresis (MST) coupled aptamer-based detection of the target, indicated ultrasensitive detection with a broad linear range (from picogram to microgram level) and no significant interference of the matrix. The aptamer also was able to detect the toxin in real spiked samples with good recoveries. Apart from biosensing applications, the MST aptamer-based method can also be extremely useful in toxicological studies and to collect data on the occurrence of SPX G in marine products.

2. Materials and methods

2.1. Chemicals and reagents

All the analytical grade chemicals were purchased from SRL (Mumbai, India). 20 methyl spirolide G (SPX G) was supplied by CIFGA laboratories, Spain. Polymerase chain reaction (PCR) reagents, streptavidin immobilized on agarose CL-4B and okadaic acid was purchased from Sigma-Aldrich (Bangalore, India). Aflatoxin B_1 (AFB_1) was purchased from Himedia Laboratories (Mumbai, India). All HPLC grade solvents were purchased from Merck (Bangalore, India). Immobilization buffer, regeneration buffer and streptavidin immobilized SA chip for SPR studies were provided by GE Healthcare Life Sciences (Bangalore, India). For the SELEX cycles, based on previous literature reports (Silverman, 2009; Paniel et al., 2017), a ssDNA library with a central randomized region of 49 bases flanked by primer binding sites on either sides was selected and was synthesized and purchased from Sigma-Aldrich, Bangalore, India.

2.2. In vitro selection of aptamers against SPX G

2.2.1. Coupling of SPX G to streptavidin – Agarose column

The coupling was performed by biotinylating SPX G via 1-Ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDC) mediated ester linkage-based on a method given by Wang et al. and Tsakos et al. (Wang, Aleiwi, Wang, & Kurosu, 2012; Tsakos, Schaffert, Clement, Villadsen, & Poulsen, 2015). The reaction was carried out in dichloromethane (DCM). To 1 ml of DCM, 0.16 μmoles of biotin was added, followed by 0.19 μmoles of EDC, and 26 μL of N, *N*-Diisopropylethylamine (DIPEA). The mixture was magnetically stirred for 1 h at room temperature. To the above mixture, 0.155 μmoles of SPX G was added and magnetically stirred at room temperature for 2 h or until reaction was complete. The mixture was diluted with ultrapure water and the water layer was separated, the DCM layer was dried over anhydrous sodium sulfate, and concentrated. The resultant syrup was dissolved in methanol (1 ml) and the product (biotynalated SPX G) was added to streptavidin coated agarose column. A 10X molar excess of biotinylated target was added to streptavidin – agarose column to ensure that the binding sites on the column were filled by target.

2.2.2. Selection of SPX G aptamer

In vitro selection involves construction of a large pool of random sequence of nucleic acid, followed by repeated cycles of enrichment for species with desired affinity and sensitivity. The SELEX process was initiated with a DNA library of~ 10^{15} different DNA sequences of 90 bp length, with 49 bp of random sequence flanked by two constant regions of 20 bp and 21 bp. The enriched aptamer pool was obtained at the end of the SELEX cycles by affinity chromatography on streptavidin – agarose column. To start with, in order to eliminate DNA sequences from the library that may have binding affinity to the agarose matrix itself, a pre-column (or negative column) of 0.5 ml was prepared without the toxin conjugate. A 100 μg of DNA library diluted in binding buffer (1 M NaCl, 5 mM $MgCl_2$, 25 mM HEPES, pH 7.4) was loaded on to the pre-column and incubated for 2 h at room temperature with an end-over-end rotation. The column was then washed with 10–12 column volume (CV) of wash buffer (1 M NaCl, 5 mM $MgCl_2$, 25 mM HEPES, 0.01% tween 20, pH 7.4) to remove unbound DNA. This non-specific unbound DNA from the pre-column was introduced to the SPX G immobilized column (2 ml CV) for further rounds of SELEX.

For each selection cycle, 8–10 μg of DNA was loaded followed by incubation for 2 h at room temperature with end-over-end rotation. The column was then washed with 10–12 CV of wash buffer to remove the unspecific and unbound DNA. The toxin-affinity DNA was eluted with 3 CV of elution buffer (1 M NaCl, 5 mM $MgCl_2$, 25 mM HEPES, 0.75 M $MgCl_2$, pH 7.4). The eluted DNA was concentrated by freeze drying and the excess salt was removed using dialysis against ultrapure water. The DNA sequences were then amplified by PCR and again subjected to the next SELEX cycle.

The PCR was carried out with 50 μL reactions, each comprising of 1.25 U of Taq polymerase, 1X reaction buffer, 2 mM $MgCl_2$, 200 μM dNTP, 10 pmoles of forward and reverse primer. The PCR amplification conditions were as follows: 94 °C for 5 min followed by 30 cycles of 94 °C for 45 s, 56 °C for 30 s and 57 °C for 45 s. After the final cycle, the reaction was held at 72 °C for 5 min before cooling at 4 °C. Electrophoresis was carried out using 3% agarose gel to characterize the PCR amplification product. The amplified DNA (dsDNA) was further purified to ssDNA by passing it through a streptavidin affinity column. This resulted in the binding of biotinylated antisense DNA strand to the column and the sense ssDNA was eluted and further subjected to the next cycle of SELEX. The confirmation of the successful elution of ssDNA was confirmed by Nanodrop 2000 spectrophotometer quantification system. These eluted ssDNA were further amplified with PCR as well as confirmed and characterized using 3% agarose gel. This aptamer selection process was repeated for a total of 10 rounds.

2.2.3. Cloning and sequencing of selected aptamer

The product from the final round of SELEX carrying the enriched aptamer pool was cloned and sequenced by Chromous Biotech (Bangalore, India). The obtained aptamer sequences were subjected for multiple sequence alignments using CLUSTAL W software (Thompson, Higgins, & Gibson, 1994). Prediction of the ssDNA secondary structure was performed by a free energy minimization algorithm available in the Mfold web server (Zuker, 2003).

2.3. Surface plasmon resonance studies

Surface plasmon resonance (SPR)-based dissociation constant (K_D) analysis was performed using Biacore T200 biosensor system (GE Healthcare Life Sciences, Bangalore, India). All the analyte and ligand stock solutions were diluted in HBS-EP + buffer (0.1 M HEPES, 1.5 M NaCl, 0.03 M EDTA and 0.05% v/v surfactant P20) prior to flowing them on the sensor surface. The data were evaluated using Biacore T200 Evaluation Software Version 3.0 and data were fit to two state binding.

Stock solution of aptamers, dissolved in TE buffer (pH 7.4) were further diluted (0.2 μM) in HBS-EP + buffer for immobilization to the SA chip using the streptavidin – biotin interaction. The aptamers were injected for 8–10 mins with a flow rate of 30 μL/min for immobilization. A total of 1065 RU of aptamer was immobilized and flow cell 1 was kept as blank (without aptamer) for using as a reference.

For K_D analysis, stock SPX G (2.36 mg/mL) dissolved in methanol was further diluted in HBS-EP + buffer. The same buffer was used as running buffer. A series of SPX G solutions at different concentrations (0, 0.7, 1.4, 2.8, 5.6, 11.2 μg/mL) were injected. Association time of 60 *sec*, dissociation time of 600 *sec* and flow rate of 30 μL/min was maintained constant throughout the kinetics experiment. Regeneration was carried out with 10 mM glycine (pH 2.5) for 30 *sec*.

2.4. Microscale thermophoresis studies

SPX G aptasensor, cross-reactivity and real sample analysis was performed using MST on a NanoTemper Monolith NT Automated with label-free mode (NanoTemper Technologies GmbH, Munich, Germany). Samples were prepared in the MST buffer (10 mM Tris HCl pH 7.4; 100 mM NaCl; 10 mM $MgCl_2$; 0.05% Tween-20) and loaded into standard/premium treated capillaries. Measurements were performed at 25 °C using 40% MST power with laser off/on times of 5 s and 20 s, respectively. All the experiments were repeated three times for each measurement. In order to test the technical reproducibility, the same capillaries can be scanned several times (technical repeats). Data analysis was performed using the NanoTemper® MO Affinity analysis software Version 2.3. For aptasensor experiment, concentrations of 500 nM of SPX 7 aptamer and 0.24 pg/mL to 0.5 μg/mL of SPX G was used. Recovery studies were carried out using 500 nM of SPX 7 aptamer and 0.2, 25 and 50 ppb of SPX G spiked in mussel samples. Cross-reactivity studies were done using 50 nM and 200 nM of SPX 7 aptamer, 5 μM of okadaic acid and 6.25 μM aflatoxin B_1 (AFB_1).

2.5. Shellfish sample preparation

Mussel and scallop samples were prepared according to Otero et al. 2011 where known concentrations of SPX G toxin was spiked to 10 g of mussel/scallop homogenate and kept at room temperature (27 °C) for 1 h. To the above spiked sample, 30 ml of methanol was added, vortexed thoroughly and then centrifuged at 3000 rpm for 10 min at 4 °C. This extraction step with methanol was repeated twice after which the supernatants were pooled together and concentrated. The concentrated pellet was dissolved in 60 ml ultrapure water followed by partitioning twice with an equal volume of dichloromethane. The organic layer was evaporated, dissolved in assay buffer and filtered through 0.45 μm syringe filters. The filtered extracts were then used for further experiments.

2.6. Inter- and intra-assay validation and statistical evaluation

Method validation was carried out through inter- and intra-assay precision test. Intra-assay precision is a measure of the variance in sample points within an assay, meaning sample replicates which are analyzed within the same run. Inter-assay precision is a measure of the variation of results obtained from repeated experiments. The results of intra- and inter-assay precision were expressed as standard deviation (SD) of the values during the assays. All experiments were performed with a sample size of n = 6 and SD was calculated.

All other experiments in the study were carried out in triplicates and SD was calculated. During MST aptasensing assay, paired-*t* test (performed using Jamovi) was carried out to evaluate the significance of the data points for the assay.

3. Results and discussion

Aptamers have proven to be one of the best suitable ligands for the detection of small molecules. Aptamers as bio-recognition elements have various advantages over antibodies, especially for development of biosensors. Aptamers can be synthesized *in vitro* without requiring the need for experimental animals. This reduces the cost, time and batch-to-batch variation during production. Aptamers can be developed against small molecules and toxins which is difficult in case of antibodies. They are also amenable to chemical modifications and therefore can be labelled with linkers, functional groups and reporter molecules for easy detection. Aptamers are also highly stable in terms of temperature and ionic strength compared to antibodies (Toh, Citartan, Gopinath, & Tang, 2015). These features of aptamers have enabled them to replace antibodies in various biosensor detection platforms. Recently, there has been an upsurge in overcoming some of the limitations in the application of antibodies and the advent to look for alternative recognition elements which could offer the same sensitivity and selectivity along with good stability. In this direction, aptamers have emerged as a lucrative alternative in the biosensing field because of the numerous advantages they offer in comparison to these molecules (Ngundi, Kulagina, Anderson, & Taitt, 2006). Aptamers for marine toxins like okadaic acid, saxitoxin, gonyautoxin, brevetoxin and palytoxin (Eissa, Siaj, & Zourob, 2015; Handy et al., 2013; Gao et al., 2016; Gao, Zheng, & Wu, 2017a; Gao, Zheng, & Wu, 2017b) have been previously reported. To the best of our knowledge, there are no reports on aptamers or aptamer-based biosensors for spirolide detection. In this study, we report the screening, selection and identification of SPX G aptamers showing high affinity for SPX G.

3.1. SPX G aptamer selection

The general protocol of SELEX involves the immobilization of the target and then its exposure to a library of variable oligonucleotides. Immobilization of SPX G to streptavidin – agarose column was carried out by biotinylating the toxin via EDC mediated ester linkage. EDC can be used to facilitate the formation of ester bonds between carboxyl group of biotin and hydroxyl group of SPX G. This biotinylated product was then added to the column for final immobilization. The conjugation of the toxin to the column was confirmed by an initial spectroscopic analysis where the absorbance at 220 nm was analyzed for all the reactants. The reactants included SPX G toxin, EDC, biotin, unconjugated mixture of these reactants and conjugated mixture (flow through). As evident from Fig. S1, the peak obtained at 220 nm in toxin and unconjugated mixture was replaced with a plateau in the conjugated mixture sample confirming conjugation of the toxin to the streptavidin – agarose column. Further, FTIR was carried out for confirmation of the conjugation (Fig. S2). In the IR spectra of conjugate mixture, the peak corresponding to the hydroxyl stretching frequency at 3433 cm^{-1} is absent as compared to the same for biotin, indicating the involvement of the hydroxyl group in the formation of the conjugate.

A DNA library consisting of 10^{14} –10^{15} nucleotide sequences was screened through a SPX G immobilized column by SELEX process. After 10 rounds of selection, when there was significant increase in the amount of eluted DNA and decrease in the amount of unbound DNA (Fig. S3), the selection cycles were stopped and the eluted DNA pool from the last cycle was cloned and sequenced.

Table 1
Aptamer sequences selected for binding affinity measurements.

Aptamer	Random sequence (5′ to 3′)
SPX 1	GGCGGTGTGGGTACCACGAGGTTTGGACGCGCGTAGCACCCCATTCAGC
SPX 2	GAGGCTGGCCGGATCGGCTCTAACCGAAGTCGCGGGCACTCACCGTGGG
SPX 7	CACGACGAGCGATAGGTTGTGGACATTGACAGGACCGAACACGCGCCCC

3.2. Cloning, sequencing and structural analysis of selected aptamers

Thirty randomly selected clones were sequenced and the identified sequences were analyzed by multiple sequence alignments using CLUSTAL W software. The alignments grouped these sequences into seven families based on their similarities (Fig. S4). Three out of seven aptamer sequences, one belonging from each clade were further selected for affinity analysis through SPR. These aptamers were designated as SPX 1, SPX 2 and SPX 7 and their sequences have been listed in Table 1.

Secondary structure for most of the aptamers are defined by stems, loops, hairpins, bulges and quadruplexes. Aptamers bind to their targets through Van der Waals forces, hydrogen bonds, electrostatic interactions, stacking of aromatic rings or a combination of these effects (Hermann, & Patel, 2000; Jayasena, 1999). The highest affinity aptamer (SPX 7) was subjected to secondary structure prediction by Mfold web server. This was done to get insights into the probable target binding motifs of the aptamer. The prediction was carried out at 27 °C with 1 M NaCl, 5 mM $MgCl_2$, whereas other parameters of the software were set to default, leading to a broad variety of secondary structures. The secondary structure analysis of the SPX 7 aptamer showed a typical stem-loop structure with the lowest Gibbs free energy value of −3.66 kcal/mol (Fig. 1).

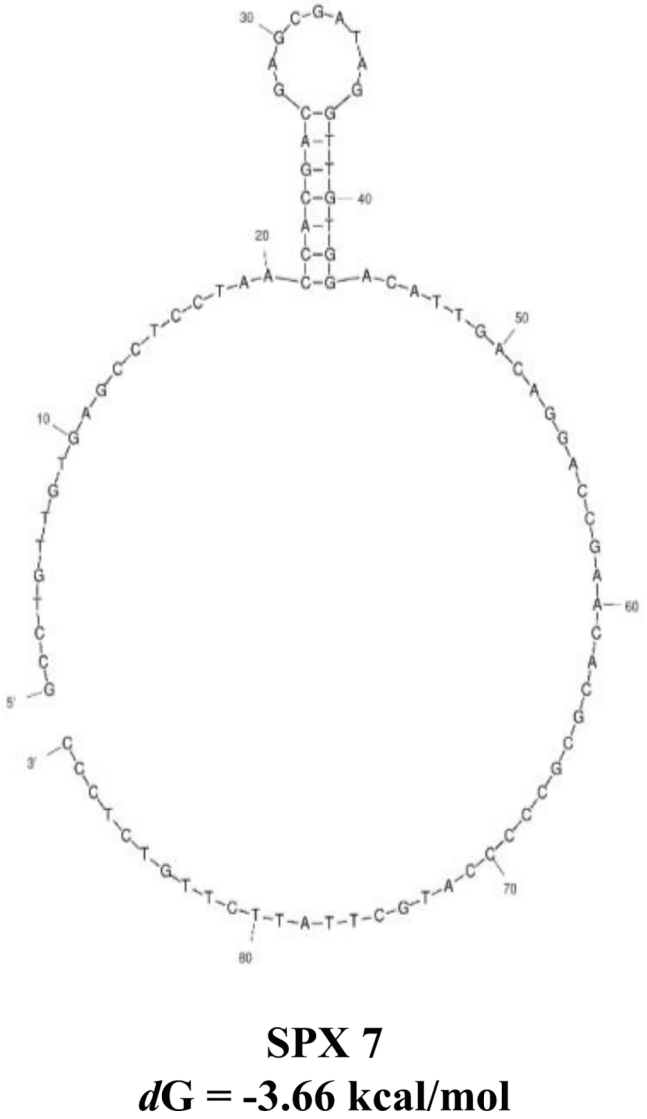

Fig. 1. Predicted secondary structure of the aptamer sequences with their lowest Gibbs free energy values using Mfold software.

3.3. Affinity measurement using SPR

The interaction kinetics between SPX G and the three selected aptamers was studied using SPR (Biacore T200). For kinetic profiling, standard solutions of SPX G at different concentrations (1, 2, 4, 8, 16 μM) were tested and their sensograms were fitted to two state binding model. Table 2 lists the binding kinetic values of the three aptamers with SPX G.

The results obtained after kinetic screening showed that the colored experimental curves matched well with the black fitted curves. SPX 1 showed the lowest affinity for SPX G with a K_D value of 5920×10^{-8} M (Fig. 2A) compared to SPX 2 (3.45×10^{-8} M; Fig. 2B) and SPX 7 (0.0345×10^{-8} M; Fig. 2C). Among the aptamers, SPX 7 also had a faster on rate and slow off rate for the binding of the aptamer to toxin as indicated by the k_{a1} and k_{d1} rate constants (Table 2). The structural changes or conformational changes as evidenced by K_{a2} and K_{d2} also indicate significant faster complex formation and slower dissociation of SPX7 toxin complex when compared to the other aptamers. This on rate and off rate evaluation and comparison is also helpful in calculating the dosing for other toxicological studies.

The half-life calculations for the aptamers based on the equation; $t_{1/2} = 0.69/k_{off}$, showed SPX 7 had a higher half-life for the binding and complex formation with the toxin. k_{off} in the equation denotes the first-order dissociation rate constant of the ligand-analyte binding. The k_{off} value directly relates the lifetime of the aptamer (ligand) and SPX G (analyte), where a higher k_{off} indicates shorter lifetime and vice versa (Corzo, 2006). Based on these observations, SPX7 was further used for aptasensor studies using Microscale thermophoresis (MST).

Eissa and group (Eissa, Ng, Siaj, Tavares, & Zourob, 2013) also performed conventional SELEX to generate aptamers against marine toxin okadaic acid (OA). The highest affinity OA aptamer reported by this group, had a K_D value of 77 nM. In the present study, among the aptamers selected, the aptamer with highest affinity had a dissociation constant of 0.3 nM for SPX G using the conventional affinity chromatography SELEX protocol. The difference in affinity of aptamers towards their analyte is governed by a number of factors including library selection, stringency of SELEX cycles, molecular size of analyte, target immobilization strategies etc.

Table 2
Interaction kinetics of SPX G toxin with aptamers, fit to two state binding model.

Aptamer	k_{a1} (1/Ms)	k_{d1}(1/s)	K_{a2} (1/s)	$K_{d2} \times 10^{-5}$ (1/s)	$K_D \times 10^{-8}$ (M)
SPX 1	1925 ± 96	2.097 ± 0.105	0.0028 ± 0.0001	15.84 ± 0.79	5920 ± 296
SPX 2	2667 ± 133	0.289 ± 0.014	0.0040 ± 0.0002	0.127 ± 0.006	3.45 ± 0.17
SPX 7	3905 ± 195	0.261 ± 0.013	0.0051 ± 0.0002	0.002 ± 0.0001	0.0345 ± 0.0017

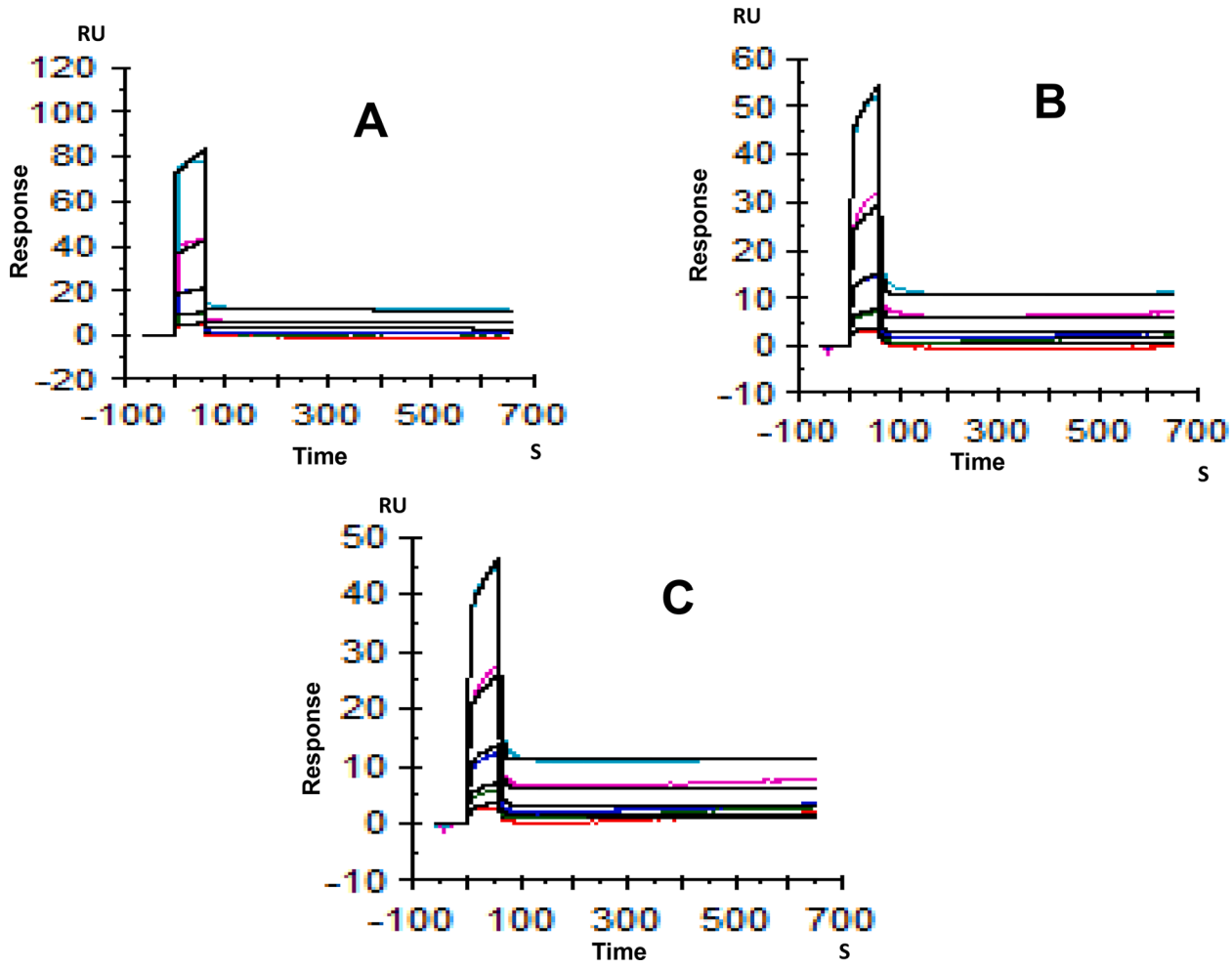

Fig. 2. SPR sensogram for dissociation constant (K_D) analysis of (A) SPX 1, (B) SPX 2 and (C) SPX 7 using SPR.

3.4. Label-free MST-based aptasensing of SPX G

MST is a biophysical technique used for the quantification of biomolecular interactions. It is based on the principle of thermophoresis, where the movement of molecules is directed by a temperature gradient, which depends on even slight change in charge, size and conformation. It can be a very useful technique for detection of analytes at ultrasensitive levels using biomolecular ligands. In the present study, the aptasensing assay was carried out at different concentrations of toxin prepared in MST buffer and recording the responses as relative fluorescence units. All the responses were blank deducted and the sensing experiments were performed at 25 °C using 40% MST power with laser off/on times of 5 s and 20 s, respectively. Fig. 3 shows the MST depletion curve and calibration curve for sensing of SPX G by the selected aptamer (SPX 7). The aptasensing assay exhibited a linear detection range from 1.9 pg/mL – 0.125 μg/mL with R^2 value of 0.9876. The limit of detection

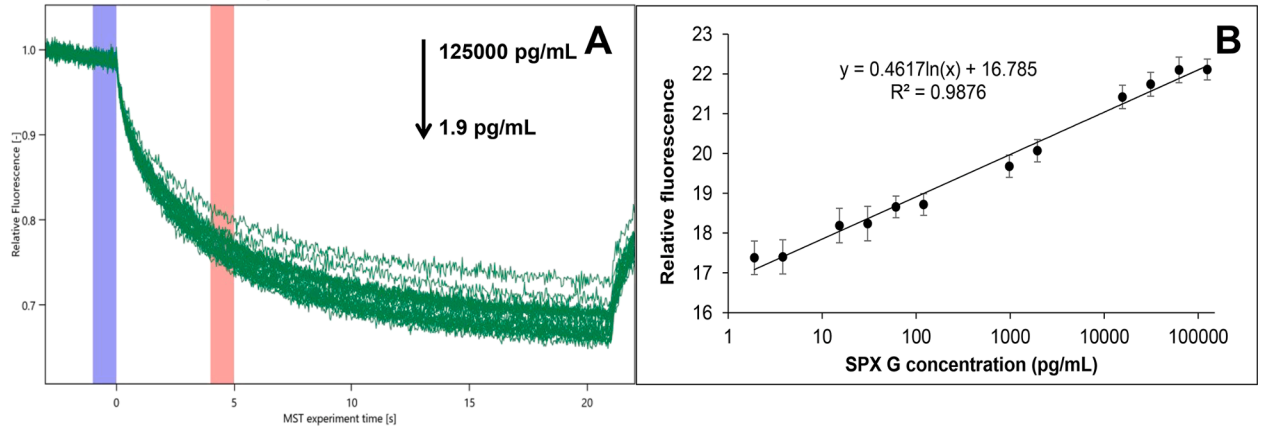

Fig. 3. Label-free MST aptasensor; binding of aptamer (SPX 7) to different concentrations of SPX G toxin (A) MST depletion curve (B) Standard binding curve of (SPX solutions were prepared in MST buffer (10 mM Tris-HCl)).

(LOD) and limit of quantification (LOQ) of the MST coupled aptasensing assay was calculated to be 0.39 pg/mL and 1.17 pg/mL, respectively (calculated using S/N ratio of 3) (Shrivastava & Gupta, 2011). The signal response was found to be greater at higher concentrations of the toxin (>15.63 ng/mL) and gradually decreased with decrease in concentration. Paired-*t* test showed that all results obtained are statistically significant ($p < 0.001$).

Aptamers against spirolides or spirolide group of toxins have not been reported earlier. Eissa et al. (Eissa, Siaj, & Zourob, 2015) developed aptamers against brevetoxin-2 using divinyl sulfone activated bead SELEX, where the highest affinity aptamer had a K_D value of 92 nM. This aptamer was then used to develop an electrochemical competitive aptasensor with a LOD of 106 pg/mL. Gu and group (Gu et al., 2018) developed a fluorescence aptasensor for the detection of marine biotoxins namely saxitoxin, tetrodotoxin and domoic acid with a LOD of 0.39, 1.21 and 0.45 ng/mL, respectively. Our study reports the development of high affinity aptamer (0.3 nM) and a highly sensitive MST aptasensing technique (LOD = 0.39 pg/mL; LOQ = 1.17 pg/mL) for SPX G detection.

In comparison to their biological counterparts, aptamers can be *in vitro* synthesized and suitably tagged with a wide range of markers for sensitive and specific detection of targets. They offer several advantages in biosensor development. Antibodies in immunosensor-based methods suffer from batch-to-batch variations which may alter the sensitivity of the developed sensor. Enzyme-based sensors suffers from stability issues because even slight modification in physiological conditions may lead to instability of enzymes.

3.5. Analytical performance in real samples

In order to evaluate the performance of the aptasensor in real samples and to study possible matrix interference for the assay, known concentrations of SPX G were spiked to mussel (0.2, 25, 50 ppb) and scallop (1.5, 3, 6 ppb) samples and recovery studies were carried out. Spirolides were first identified in digestive gland extracts of mussels and scallops and have been identified as toxins of concern to human health (Gao et al., 2017a). Table S1 represents the % recovery of SPX G from mussel and scallops using the label-free MST technique. Appreciable recovery of SPX G from mussel (86 to 108% at 0.2, 25 and 50 ppb levels) and scallop (86.87 to 107.26% at 1.5, 3 and 6 ppb levels), indicated no significant matrix interference and suitability of the assay for detection of the toxin at ultrasensitive ppb levels.

The inter- and intra-assay precision of the MST-based aptasensing method was determined at different SPX G concentrations in the range of 1.9 to 125000 pg/mL. The intra-assay precision was assessed by analyzing six replicates of each sample in a single run, and inter-assay precision was assessed by analyzing the same by repeated analysis of samples of SPX G (Table S2). The standard deviation of six assays of each standard was satisfactory, with however less precision at lower analyte (SPX G) concentrations.

Spirolide group of toxins have mostly been detected through methods like liquid chromatography-tandem mass spectrometry (Aasen, Hardstaff, Aune, & Quilliam, 2006) and fluorescence polarization (Otero et al., 2011). As mentioned earlier, no aptamer or antibody-based reports on SPX G detection are available. The previous reports on detection of spirolide toxins namely 13- desmethyl spirolide C and 13, 19-didesmethyl spirolide C are based on nicotinic acetyl choline bioreceptor and these studies report LOD in the range of 4.2–20.35 ng/mL in samples of mussel and scallop (Table 3). Much lower LOD and LOQ values (in picogram levels) were obtained for SPX G detection in the present work. A comparison with immuno-assays, enzyme or aptamer-based biosensors for other closely related biotoxins also indicate the present aptamer-based biosensor to be superior in sensitivity, with good recoveries in real samples (Table 3).

3.6. Cross-reactivity studies

Specificity of the SPX 7 aptamer was studied with okadaic acid (OA), belonging to the same family of spirolides. OA is a heat-stable, lipophilic phycotoxin, produced by *Dinophysis* dinoflagellate. Similar to SPX G, this polyether toxin mostly accumulates in marine sponges and shellfishes like mussels and scallops. OA is found to coexist with spirolides in contaminated shellfishes and hence was selected for the cross-reactivity study (Otero et al., 2011; Chand, 2009). The thermophoresis of 50 nM SPX 7 aptamer with 5 μM okadaic acid was measured in this study. As it is shown in Fig. S5A, binding between SPX 7 aptamer and OA was not observed, which confirms the specificity of aptamer towards the marine toxin. Even at concentration as high as 5 μM, OA does not interfere with SPX G. Cross-reactivity was also tested with a different group of toxin namely mycotoxin AFB_1. Fig. S5B shows the thermophoretic depletion of 200 nM SPX 7 aptamer with 6.25 μM AFB_1, where no binding was observed between the aptamer and the mycotoxin.

4. Conclusion

To summarize, highly specific DNA aptamers that bind to SPX G were selected by conventional SELEX. After sequence analysis of the DNA pool, three different aptamers were further studied for their binding

Table 3
Reported biosensors for the detection of major marine biotoxins.

Marine toxin	Bioelement	Sensor format	LOD	Real sample analysis	Recovery %	Reference
13 desmethyl spirolide C	Nicotinic acetylcholine receptor	Solid-phase bioreceptor-based competitive assay	11.08 ng/mL	Cockle	68	Rodríguez et al., 2011
			4.2 ng/mL	Scallop	80	Rodríguez, Vilariño, Molgó, Aráoz, & Botana, 2013
		Fluorescence polarization	8.66 ng/mL	Mussel	87	Vilarino, Fonfría, Molgó, Aráoz, & Botana, 2009
13, 19 didesmethyl spirolide C	Nicotinic acetylcholine receptor	Fluorescence polarization	17.31 ng/mL	Mussel	88	Otero et al., 2011
			20.35 ng/mL	Mussel	78	Fonfría et al., 2010
Saxitoxin	Aptamer	Square wave voltammetry	275.35 pg/mL	Seawater	94.4–111	Qi et al., 2020
		Biolayer interferometry	0.5 ng/mL	Shellfish, ribbon fish and water	101–107	Gao et al., 2017a
Brevetoxin-2	Aptamer	Electrochemical impedance spectroscopy	106 pg/mL	Shellfish	102–110	Eissa, Siaj, & Zourob, 2015
Tetrodotoxin	Antibody	Planar waveguide	2.5 μg/L	Puffer fish	85–115	Reverté et al., 2017
20 Methyl Spirolide G	Aptamer	Microscale thermophoresis	0.39 pg/mL	Mussel and scallop	86–108	Present study

affinities to the toxin. SPX 7 aptamer with K_D in the nanomolar range had a better half-life, faster on-rate and slow off-rate for binding to the toxin and was therefore selected for further studies. A label-free MST coupled aptasensing using SPX7 aptamer showed a broad detection range and good performance in real samples. Results thus indicate the suitability of the aptasensing technique for detection of SPX G in contaminated samples. The study also opens the possibility of application of the developed aptamer in developing other qualitative and quantitative biosensing formats. The MST aptamer-based method can also be extremely useful in toxicological studies and to develop data on their occurrence in marine products.

CRediT authorship contribution statement

Monali Mukherjee: Investigation, Formal analysis, Methodology, Writing - original draft. **Srinivas Sistla:** Investigation, Data curation, Writing - review & editing. **Shivakumar R. Veerabhadraiah:** Investigation, Formal analysis, Methodology. **B.K. Bettadaiah:** Validation, Writing - review & editing. **M.S. Thakur:** Conceptualization, Writing - review & editing. **Praveena Bhatt:** Supervision, Resources, Project administration, Validation, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2021.130332.

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