1 A saxitoxin-binding aptamer with higher affinity and inhibitory activity **2 optimized by rational site-directed mutagenesis and truncation**

3 Zheng X, Hu B, Gao SX, Liu DJ, Sun MJ, Wang LH^{*,1}

4 ¹Department of Biochemistry and Molecular Biology, College of Basic Medical Sciences, Second
5 Military Medical University, No. 800, Xiangyin Rd., Shanghai 200433, People's Republic of
6 China.

7 *Address correspondence to the author at No. 800, Xiangyin Rd., Shanghai 200433, People's
8 Republic of China; Tel: 86-21-81870967; Fax: 86-21-65334344; E-mail: wsh928@hotmail.com.

9 **Abstract:** Saxitoxin (STX), a member of the family of paralytic shellfish poisoning toxins, poses
10 toxicological and ecotoxicological risks. To develop an analytical recognition element for STX, a
11 DNA aptamer (APT^{STX1}) was previously discovered via an iterative process known as Systematic
12 Evolution of Ligands by Exponential Enrichment (SELEX) by Handy et al. Our study focused on
13 generating an improved aptamer based on APT^{STX1} through rational site-directed mutation and
14 truncation. In this study, we generated the aptamer, M-30f, with a 30-fold higher affinity for STX
15 compared with APT^{STX1}. The K_d value for M-30f was 133 nM, which was calculated by Bio-Layer
16 Interferometry. After optimization, we detected and compared the interaction of STX with
17 aptamers (APT^{STX1} or M-30f) through several techniques (ELISA, cell bioassay, and mouse
18 bioassay). Both aptamers' STX-binding ability was demonstrated in all three methods. Moreover,
19 M-30f performs better than its parent sequence with higher suppressive activity against STX. As a
20 molecular recognition element, M-30f has good prospects for practical application.

21 **Keywords:** Aptamer, saxitoxin, mutagenesis, truncation, inhibitory activity, G-quadruplex.

22 1. INTRODUCTION

23 Saxitoxin (STX), with the molecular formula C₁₀H₁₇N₇O₄ (Molecular Weight = 299), is one of the
24 most potent natural neurotoxins known; it is highly polar due to the presence of two guanidinium
25 groups (Figure 1, Wiese et al., 2010). Along with dozens of analogues, STX makes up a family of

1 neurotoxins, which are classified as paralytic shellfish toxins (PSTs), and may cause paralytic shellfish
2 poisoning (PSP; Etheridge, 2010). As reported, STX can be synthesized by multiple species of
3 cyanobacteria and dinoflagellates (Perreault et al., 2011). In vivo accumulation of STX by
4 filter-feeding bivalves and fish, and the subsequent transfer through the food chain, can result in human
5 illnesses or even death (Cusick and Saylor, 2013). STX is toxic mainly because of its effect on the
6 nervous system, where it blocks voltage-gated sodium channel in excitable cells (Tian et al., 2014). Its
7 LD50 in mice is 8–10 $\mu\text{g kg}^{-1}$ i.p., 3.4 $\mu\text{g kg}^{-1}$ i.v. and 260 $\mu\text{g kg}^{-1}$ by oral administration (Falconer, 2008).
8 These data demonstrates the importance of monitoring programs for STX. In many countries, the
9 regulatory limit for PSP toxins in shellfish has been established as 800 μg of STX equivalents/kg of
10 shellfish meat or 4 mouse unit (MU) of PSP toxins/g of shellfish meat(Kawatsu et al., 2014).

11 The longstanding regulatory method for validating biological threats is the internationally
12 accredited AOAC biological method (Mouse bioassay 959.08), with only the United Kingdom using
13 the relatively newly accredited AOAC Official HPLC Method 2005.06 as an alternative (Campbell et
14 al., 2009). However, a rapid, high throughput screening assay for effective monitoring is still needed,
15 which could also reduce animal usage. The most important part of a screening assay is its recognition
16 element, e.g., the antibodies and receptors that are usually adopted (Handy et al., 2013). However,
17 many challenges remain, e.g., the limited availability of antibodies and the use of animals. Handy et al.
18 (2013) reported the discovery of a DNA aptamer named APT^{STX1} that targets STX, which could serve
19 as an alternative analytical recognition element in diagnostic assays for STX (Figure 2; Handy et al.,
20 2013).

21 Aptamers are short strands of DNA or RNA that can fold into unique three-dimensional structures
22 and bind with high affinities and specificities to targets such as ions, proteins, low molecular weight

1 metabolites, sugar moieties, lipids, and even whole cells. They are typically screened out from a
2 random library comprising 10^{12} – 10^{15} oligonucleotide sequences that are synthesized in vitro by a
3 SELEX process (Systematic Evolution of Ligands by Exponential Enrichment) (Radom et al., 2013 ;
4 Shigdar et al., 2013). Because of their numerous advantages compared to antibodies, e.g., stability and
5 non-immunogenicity, aptamers are promising for detection and clinical applications (Wang et al., 2011).
6 To date, several aptamers have entered clinical trials as antagonists, agonists or targeting molecules in
7 drug delivery; the most successful one, pegaptanib, has been approved by the FDA for the treatment of
8 vascular ocular disease (Radom et al., 2013). There has also been considerable progress towards
9 applications of aptamers for laboratory techniques and medical diagnostics (Radom et al., 2013).

10 However, primary aptamers obtained by SELEX are not suitable for direct clinical or laboratory
11 application. Optimization is needed to improve and regulate their functions (Wang et al., 2011). In
12 recent years, a number of attempts have been made to optimize present aptamers through several
13 common strategies, e.g., truncation, chemical modification, LNA replacement, mutation and 3' or
14 5' capping (Wang et al., 2011; Yang et al., 2011; Nonaka et al., 2013; Bullock et al., 2000). In this study,
15 we adopted rational site-directed mutagenesis and truncation to optimize APT^{STX1}. First, we introduced
16 mutations to improve its conformational stability and strengthen its interaction with STX. Although a
17 number of aptamers have been identified by SELEX, it sometimes fails to identify aptamers that bind
18 with their target with high affinity, because of amplification bias of PCR and reduced library diversity
19 caused by experimental manipulation (Nonaka et al., 2013). Different groups have engineered mutated
20 aptamers that bind to targets with improved affinity compared to the wild type (Nonaka et al., 2013;
21 Bullock et al., 2000). Similarly, by introducing site-directed mutations on the basis of secondary
22 structure prediction, we attempted to get an improved underlying aptamer with higher affinity. It is

1 known that G-quadruplexes are nucleic acids structures, characterized by unique highly ordered
2 architecture and high stability (Ruttikay-Nedecky et al., 2013). Some sequences remain folded under
3 physiological conditions and at temperatures above 90 °C (Ruttikay-Nedecky et al., 2013). Therefore,
4 we presume that if an aptamer can form a G-quadruplex after slight mutation, its affinity could be
5 improved based on its strengthened structural stability. We designed experiments to verify our
6 hypothesis. As expected, we obtained a mutated quadruplex-forming aptamer with higher affinity to
7 STX compared to the parent atamer. Subsequently, we adopted truncation to reduce the cost of the
8 molecule and find the key binding structure. Generally, not all nucleotides of a certain aptamer are
9 necessary for direct interaction with the target or for folding into the structure that facilitates target
10 binding. Moreover, longer sequences result in lower yield and higher cost of synthesis (Shangguan et
11 al., 2007). Thus, truncation is necessary during the optimization process. At the same time, it aids in
12 exploring the relationship between the aptamer structure and function and in characterizing the target
13 binding motif.

14 After optimization of aptamer APT^{STX1}, we investigated the interaction of the pre- and
15 post-optimized aptamers with STX through different methods: ELISA, a cell bioassay and mouse
16 bioassay. By these methods, STX was detected as a control following standard procedures. After the
17 introduction of aptamers in these detection systems, differences could be observed compared to
18 controls, which verified aptamers' ability of binding with STX. Besides, we compared the
19 performances of different aptamers in these assays. Through these three methods, we obtained an
20 overall understanding of their STX-binding ability and function.

1

2 **Fig. 1. Structure of STX.**

3 **Fig. 2. Structure of APT^{STX1} generated by the mfold web server**

4 **2. Experimental**

5 **2.1. Materials and Reagents.**

6 All synthetic oligonucleotides were synthesized by Sangon Biotech (Shanghai,China). STX

7 diacetate was purchased from Taiwan Algal Science Inc (Taiwan). Ultrafiltration tubes used were

8 Amicon Ultra-0.5 mL and Ultra-4 mL centrifugal desalting filters with a 3 KD molecular cutoff,

9 obtained from EMD Millipore (Alberta, Canada). Ouabain(10 mM, aqueous) and Veratridine(1 mM,

10 in 0.01 M HCl) were purchased from Sigma-Aldrich Co. LLC(mainland, China). Nuclease free water

11 (IDT) was used in the preparation of all reagents used for experiments. PBS-T used was 10 mM

12 phosphate buffer, 2.7 mM KCl, 140 mM NaCl, 0.05% Tween-20, pH 7.4 (Sigma, St. Louis, MO). All

13 other chemical reagents used were analytical grade.

2.2. Evaluation of STX-aptamers binding using Bio-Layer Interferometry sensors

STX-aptamers binding was evaluated using Bio-Layer Interferometry sensors in an OctetRED 96 system (ForteBio, Shanghai). The principle and analysis procedures used herein were as detailed in Concepcion et al. (2009). Briefly, aptamer immobilization was performed using the streptavidin-biotin coupling method. SSA(enhanced streptavidin) sensors (ForteBio) were prepared by the procedure of equilibration (1 min), biotinylated aptamer coupling(1 μ M, 5 min), dissociation(5 min), and equilibration (2 min); One reference channel was also prepared via equilibration. The running buffer used for immobilization and sample analysis was PBS-T at room temperature. Lyophilized aptamers were reconstituted in PBS-T to a concentration of 100 μ M, then diluted in PBS-T to 10 μ M and 1 μ M, heated at 95°C for 5 min, and cooled on ice for 10 min prior to use. All aptamers used for experiments herein were required to undergo the heating and cooling procedure to promote their refolding. STX at different concentrations (5 μ M, 10 μ M and 20 μ M) was analyzed for association time over 5min and dissociation time over 5 min, along with a blank sample containing only running buffer for reference. In addition, GTX2 and GTX3 (gonyautoxin, 20 μ M) were used to evaluate the interaction of aptamers with similar paralytic shellfish toxins. The response data from the control surface were subtracted from the responses obtained from the reaction surface using the Octet Data Analysis Software CFR Part 11 Version 6.x; the affinity parameter K_d was similarly obtained. A 1:1 binding mode with mass transfer fitting was used to obtain the kinetic data.

2.3. Optimization of APT^{STX1}

During the optimization process, single-point mutations were first introduced into the sequence of the STX-binding aptamer (APT^{STX1}). A set of 22 oligonucleotide single-point mutants were obtained with mutation sites at both sides of guanine bases, since sets of guanine bases are necessary for G-quadruplex structures. The sequences of all mutants produced are listed in Table S-1, Supporting Information. Prediction by QGRS Mapper revealed that 9 sequences (M-6, M-8, M-12, M-13, M-15, M-20, M-22, M-30 and M-32) among the 22 mutants could form G-quadruplex structures. To verify our hypothesis that aptamers with G-quadruplex structures could exhibit improved target affinities, the 9 sequences and the parent sequence were synthesized and their STX-binding abilities were evaluated by Bio-Layer Interferometry. Similarly, multi-point mutagenesis was also attempted. 6 quadruplex-forming multi-point mutant sequences were synthesized and their K_d values calculated. Ultimately, M-30 was found to have the strongest STX affinity. To remove redundant nucleotides on

1 M-30, we conducted truncation analysis according to predictions from the mfold web server. 6
2 truncated sequences derived from M-30 (M-30a, M-30b, M-30c, M-30d, M-30e; Figure 4), with bases
3 at either ends truncated or one of four stem-loop structures removed, were synthesized and their Kd
4 values were calculated by Bio-Layer Interferometry. In combination with QGRS Mapper prediction
5 results, we were able to determine the core functional sequence of M-30 without affecting its affinity
6 (M-30f).

7 2.4. ELISA

8 ELISA kits for the analysis of STX were purchased from Abraxis LLC (Warminster, U.S.A).The
9 kits employ a direct competitive ELISA, coated with a second antibody (sheep anti-rabbit). **STX in the**
10 **sample and a STX-enzyme-conjugate compete for the binding sites of rabbit anti-STX antibodies in**
11 **solution. The STX antibodies are then bound by the second antibody immobilized on the plate. After a**
12 **washing step and addition of the substrate solution, a color signal is produced. The intensity of the blue**
13 **color is inversely proportional to the concentration of the STX present in the sample. The color reaction**
14 **is terminated after a specified time and the absorbance at 450nm was measured using a Thermo**
15 **Scientific Multiskan FC Microplate Photometer. Each sample, control or standard solution was**
16 **analyzed in duplicate.**

17 In order to study the interaction of STX with M-30f or APT^{STX1}, we detected STX (0.8 ng/ml) as a
18 control and the mixture of STX and an aptamer as a sample after incubation for 30 min at room
19 temperature (0.8 ng/ml STX; 10⁻⁶, 10⁻⁷ and 10⁻⁸mol/L aptamers). Then, we conducted statistic analysis
20 of the results to find out whether the introduction of aptamers could lead to changes in absorbance at
21 450nm. However, no significant differences were observed among aptamer groups and control. Since
22 STX also exists in enzyme conjugate (enzyme-STX), aptamers might bind with not only free STX but
23 also enzyme-STX, leading to negative results above. To avoid the interference from enzyme-STX, we
24 detected free STX remaining after incubation with an aptamer and ultrafiltration (0.5mL, 3KD,
25 14,000rpm, 30 min). Aptamers and aptamer-STX complexes were retained in the concentrate by
26 ultrafiltration. The free STX in the mixture of aptamer and STX was tested following the same
27 procedure as detailed above. The efficiency of ultrafiltration was verified by denaturing polyacrylamide
28 gel electrophoresis to confirm that aptamers and the aptamer-STX complex were not filtrated. Besides,
29 to demonstrate that STX alone can be completely filtrated, we also designed ELISA experiments. Pre-
30 and post-filtrated STX were both detected as samples and compared.

1 2.5. Cell bioassay

2 Neuro-2a cells (mouse neuroblastoma cells, ATCC CCL-131) were grown in RPMI 1640 complete
3 medium (Hyclone, South America) supplemented with 10% fetal bovine serum, 2 mM glutamine, 1
4 mM sodium pyruvate, 50 µg/ml streptomycin, and 50 units/ml penicillin. The culture was maintained at
5 37°C in a humidified CO₂ (5%) atmosphere (Humpage et al., 2007). For the bioassay, 96-well
6 microplates were seeded with 200 µL complete medium RPMI 1640 containing 10⁵ cells per well and
7 then incubated for 24 h. Culture wells were then filled with 10 µl each of sample, 10 mM ouabain, and 1
8 mM veratridine. Mixture of STX (3.2, 0.8, or 0.2 ng/well) and aptamer (10⁻⁷ mol/L, M-30f or APT^{STX1})
9 after incubation was subjected to replicates of 3 wells as samples, with STX (3.2, 0.8, or 0.2 ng/well)
10 alone as controls. 6 wells were processed as untreated controls (without additions of sample, ouabain,
11 and veratridine) or as ouabain/veratridine controls (without addition of sample). After incubation for 24
12 h, the medium was wicked off with sterilized paper-towels. The wells were then given 200 µL fresh
13 medium and 20 µL CCK-8, and incubated for another 45–60 min. Finally, the absorbance was measured
14 with a Thermo Scientific Multiskan FC Microplate Photometer at a wavelength of 450 nm (Manger et
15 al., 1995).

16 2.6. Mouse bioassay

17 A mouse bioassay was performed according to the official method of the Association of Analytical
18 Communities (AOAC) (2005). We aimed to adopt this standard method to study and compare the
19 interaction of STX with aptamers pre- and post- optimization. Briefly, the assay for PSP toxins is
20 performed as follows: Five or more female ICR mice weighing 19.6±0.4 g (approximately 4 weeks old)
21 were intravenously (i.v.) injected with 300 µL of sample, and the survival ratio was observed. Mice
22 were first randomly assigned to one of five treatment groups: mixture of STX and APT^{STX1} [4.95 mg/kg]
23 after incubation; mixture of STX and M-30f [4.95 mg/kg] after incubation; aptamer (APT^{STX1} or M-30f)
24 alone, and STX alone as a control. The concentration of STX suitable for use as the control was first
25 researched prior to performing the bioassays. However, in the complicated environment in vivo,

1 aptamers didn't protect mice from death. With the aid of ultrafiltration, mice were randomly assigned
2 to one of following treatment groups : ultrafiltration separated STX after incubation with APT^{STX1} [4.95
3 mg/kg]; ultrafiltration separated STX after incubation with M-30f [4.95 mg/kg], ultrafiltration
4 separated STX alone as a control. By ultrafiltration, in vivo interference to the interaction of aptamers
5 with STX was avoided.

6 2.7. Statistics

7 GraphPad Prism (GraphPad Software, San Diego, CA, USA) was used for all statistical tests.
8 Statistical significance was accepted at $p \leq 0.05$.

9 3. RESULTS

10 3.1. Improvement of aptamer STX-binding ability through rational site-directed mutagenesis and 11 truncation

12 Aptamers with high STX-binding ability are required for use as recognition elements in
13 STX-detection systems. The affinity of a STX-binding aptamer (APT^{STX1}) that was identified in a
14 previous study¹² was re-evaluated by Bio-Layer Interferometry. Bio-Layer Interferometry is a label-free
15 technology. Once the molecule bound to the sensor surface fluctuates in number, the spectrometer
16 captures the real-time phase shift of the interference spectrum, which directly reflects the thickness of
17 the bio-layer. To date and to our knowledge, no reported studies have measured the interaction of
18 aptamers with small molecules using Bio-Layer Interferometry. As shown in Figure 3, increasing
19 concentrations of STX elicited corresponding increases in the sensor response. As a negative control,
20 running buffer was exposed to the reference channel, and no change in response was observed (data not
21 shown); as such, non-specific binding of STX to the sensor was ruled out. By global fitting of the
22 responses of three concentrations of STX over the oligonucleotide surface of constant density, we
23 obtained the association-dissociation curves and the K_d values for the interactions. The curves revealed
24 that the association and dissociation rates were both slow; therefore, aptamers needed to be incubated
25 with STX for 30 min in our study to obtain a sufficient binding reaction. With a K_d value of 3.84 μM

1 for the STX-APT^{STX1} interaction, it was evident that the binding ability of APT^{STX1} required
 2 improvement in order to construct a sensitive STX-detection system. Moreover, studies to determine
 3 specificity of the APT^{STX1} sensor surface for STX versus its congeners (GTX2 and GTX3), that often
 4 co-occur in regions where STX may be present, followed the same assay procedures. Binding response
 5 was not observed, thus indicating that APT^{STX1} didn't cross-react with GTX2 or GTX3(data not shown)
 6 and further supporting that the selected aptamer, APT^{STX1}, was specific to STX.

7
 8 Fig.3. Bio-Layer Interferometry association and dissociation curves. Sensors were immobilized with
 9 oligonucleotides (M-30f or APT^{STX1}). Different phase shifts to the interference spectrum were induced
 10 by different concentrations of STX (20 μM, 10 μM and 5 μM)

11

1 Table 1 List of quadruplex-forming sequences and their kinetic parameters.

Names	K _d (μM)	K _{on} ((1/Ms)	K _{dis} (1/s)
APT ^{STX1}	3.84	4.87×10 ³	1.87×10 ⁻²
M-6	4.27	3.47×10 ³	1.48×10 ⁻²
M-8	3.50	3.04×10 ³	1.06×10 ⁻²
M-12	51.0	3.29×10 ²	1.68×10 ⁻²
M-13	3.28	4.63×10 ³	1.52×10 ⁻²
M-15	39.3	4.97×10 ²	1.95×10 ⁻²
M-20	29.1	4.13×10 ²	1.2×10 ⁻²
M-22	4.17	1.27×10 ³	5.28×10 ⁻³
M-30	0.128	1.20×10 ⁴	1.54×10 ⁻³
M-32	24.0	8.76×10 ²	2.1×10 ⁻²

2

3 In the optimization process for aptamer APT^{STX1}, we first introduced individual point mutations.
4 In the 22 resultant single-point mutants, nine sequences (M-6, M-8, M-12, M-13, M-15, M-20, M-22,
5 M-30 and M-32) were predicted to form G-quadruplex structures based on QGRS Mapper. By
6 Bio-Layer Interferometry, the association and disassociation parameters of each oligonucleotide were
7 determined and are shown in **Table 1**. The results signify that M-6, M-8, M-13 and M-22 have similar
8 affinities for STX as APT^{STX1}, whereas M-12, M-15, M-20 and M-32 have reduced affinities for STX.
9 However, one mutant shows stronger binding to STX: M-30 (5'-GGTATTGAGGGTCGCAT
10 CCCGTGGAAACAGTTTCATTGGGCGCACTCCGCTTTCTGTAGATGGCTCTAACTCTCCTCT-
11 3'). The K_d value for M-30 was 128 nM, indicating that it had a 30-fold stronger binding ability than
12 APT^{STX1}. The dissociation rate constant (K_{dis}) for M-30 was 1.54×10⁻³(s⁻¹), whereas the K_{dis} for
13 APT^{STX1} was 1.87×10⁻² (s⁻¹). The association rate constant (K_{on}) for M-30 was 1.20×10⁴(M⁻¹·s⁻¹),
14 whereas that for APT^{STX1} was 4.87×10³ (M⁻¹·s⁻¹). Therefore, the improvement in STX-binding ability
15 was mainly accomplished by increase of the association rate constant and reduction of the dissociation
16 rate constant. Prediction by the mfold web server revealed that M-6, M-8, M-13, M-22, which
17 maintained similar affinities for STX, had the same secondary structures from APT^{STX1}, whereas M-12,
18 M-15, M-20 and M-32, which had reduced affinity, formed different stem-loop structure as APT^{STX1}.
19 From these results, we conclude that both the G-quadruplex and stem-loop structure in M-30 are

1 involved in its interaction with STX.

2 We next attempted multi-point mutagenesis on the basis of the results detailed above. Six
3 additional mutants were synthesized and evaluated by Bio-Layer Interferometry. Results revealed that
4 the affinity increased five folds at most with multi-point mutants (Table S-2, Supporting Information),
5 and all were lower than that of M-30. We speculated that aptamer M-30, with its single point mutation
6 at site 30, showed the strongest STX-binding ability because of its particular size and the positioning of
7 the mutation for G-quadruplex formation. Therefore, we chose M-30 as the aptamer for further
8 optimization.

9 Not every nucleotide may be necessary for the STX-binding ability of M-30. We therefore
10 conducted a truncation analysis to reduce the cost for synthesis and find the minimal functional core
11 structure of M-30. Since stem-loop structures were involved in the interaction of aptamers with STX,
12 five truncated sequences of M-30 (M-30a, M-30b, M-30c, M-30d, M-30e) were synthesized according
13 to prediction from the mfold web server and their K_d values were determined (Table 2). Sequences
14 M-30a, M-30d and M-30e, with bases at both ends, the third stem-loop and the fourth stem-loop
15 structures separately removed, were found to have K_d values very close to that of the full-length M-30.
16 However, the removal of the first or the second stem-loop from M-30 (M-30b, M-30c) resulted in
17 dramatically reduced binding affinity, suggesting that the two stem-loop structures were critical for
18 target recognition. According to the information generated by QGRS Mapper on the composition and
19 distribution of putative quadruplex-forming sequences in M-30, we found that the core nucleotides for
20 a G-quadruplex were located just in the first and second stem-loop structures. Based on these results
21 and our inference, sequence M-30f was synthesized with the third and fourth stem-loop structures
22 removed and redundant nucleotides at both ends truncated. As expected, its affinity was similar as that
23 of the full-length M-30. Thus, we took M-30f as the final optimized STX-binding aptamer. Moreover,
24 no response was observed when GTX 2 and GTX3 (20μM) were used as targets, which indicated that
25 M-30f did not cross-react with GTX2 or GTX3(data not shown) and maintained its specificity for STX.

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2 Fig. 4. Secondary structure of truncated aptamers.

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4 Table 2 Kinetic parameters of truncated aptamers.

Names	K _d (μM)	K _{on} ((1/Ms)	K _{dis} (1/s)
M-30a	0.131	1.53×10 ⁴	2.00×10 ⁻³
M-30b	13.8	1.62×10 ³	2.24×10 ⁻²
M-30c	10.4	2.74×10 ³	2.85×10 ⁻²
M-30d	0.137	2.15×10 ⁴	2.95×10 ⁻³
M-30e	0.143	1.37×10 ⁴	1.96×10 ⁻³
M-30f	0.133	1.40×10 ⁴	1.86×10 ⁻³

5

3.2. M-30f interacts with STX better than APT^{STX1} (ELISA)

In order to evaluate the interaction of STX with aptamers (M-30f and APT^{STX1}), we first adopted the ELISA method. With STX filtrated as a control, the mixture of STX and an aptamer after incubation was added as a sample. However, there is no significant statistic difference among aptamer groups and control. After some analysis, we speculated that the negative results were caused by aptamers' ability of binding with both free STX and enzyme-STX. To avoid the potential interference from enzyme-STX, STX separated by ultrafiltration (0.5mL, 3KD, 14,000rpm, 30 min) after incubation with aptamers (M-30f or APT^{STX1}) of different concentrations was subjected to the ELISA kit. The addition of aptamers, both M-30f and APT^{STX1}, resulted in a decrease in absorbance at 450nm, which can be attributed to a reduction in the amount of free STX in the assay. In addition, at the concentrations of 10⁻⁷ and 10⁻⁶M, absorbance decrease at 450nm caused by M-30f was more than that caused by APT^{STX1}. Upon comparison between the M-30f and APT^{STX1} groups, statistically significant differences could be observed (p<0.05, Figure 5).

In order to verify the efficiency of ultrafiltration, STX alone was separately added to the ELISA kit, before and after ultrafiltration. Similar absorbance at 450 nm was obtained without significant difference. This demonstrates that STX could completely pass through the ultrafiltration membrane. We also verified the efficiency of ultrafiltration for DNA aptamers by denaturing polyacrylamide gel electrophoresis. APT^{STX1} before ultrafiltration exhibited a light band, while the filtrate revealed nothing, indicating that ultrafiltration could almost completely retain aptamers (≥20KD) in the concentrate (Figure S-1, Supporting Information).

Fig. 5. Comparison of two aptamers' STX-binding ability by ELISA. STX (0.8 ng/ml) alone was taken as a control. After interaction with aptamers(M-30f and APT^{STX1}) at different concentrations (10⁻⁸,10⁻⁷ and 10⁻⁶ M), remaining free STX was obtained by filtration and added to the analyte solution. Free STX was reduced by the addition of aptamers. A significant difference was observed between M-30f and APT^{STX1} groups. (*p<0.05)

3.3. Improved inhibitory activity for M-30f compared to APT^{STX1} (cell bioassay)

The cell bioassay for STX is based on its ability to block sodium channels. Veratridine and ouabain, which are known as sodium channel agonists and sodium pump inhibitors separately, reduce cell viability by 50-74% (mean, 62%). The effect of added STX is shown by the standard curve in Figure S-2. **Neither Ouabain nor veratridine alone had fatal effect on Neuro-2a cells.**

With STX as the control, mixture of STX and aptamer after incubation was subjected as a sample in cell bioassay. Then, different groups and the control were brought into comparison. As shown in Figure 6, addition of M-30f or APT^{STX1} along with STX resulted in decreased cell viability, compared to control. What's more, significant difference (p<0.05) was observed between M-30f and APT^{STX1} groups. From these results, we can conclude that **both aptamers could interact with STX, and** the suppression of sodium channel activity by STX was also inhibited by M-30f and APT^{STX1}, with the M-30f performing better.

Fig. 6. Comparison of two aptamers' STX-binding ability in cell bioassay. STX alone (0.2, 0.8 and 3.2 ng/well) was used as the controls. After interaction with aptamers(M-30f and APT^{STX1}, 10⁻⁷ M), aptamer-STX mixtures were added to the analyte solution. Cell survival was reduced by the addition of the mixtures. A significant difference was observed between M-30f and APT^{STX1} groups. (*p < 0.05)

3.4. M-30f and APT^{STX1} can both interact with STX (mouse bioassay)

In order to evaluate the interaction of STX with M-30f or APT^{STX1} in vivo, we adopted the mouse bioassay method. First, we determined a suitable concentration of STX to use as a control. The number of survivors decreased as the dose of toxin was raised (Table S-3). A lethal effect of STX was observed in five out of six mice when given 300μL of STX (11.25μg/kg, i.v); this concentration was subsequently used in the following study. Then, 4.95 mg/kg aptamers alone or mixed with STX was administered to mice. Aptamers alone were not lethal to mice; however, in the STX mixture groups, aptamers did not protect mice from death. To study the effect of aptamers on the amount of free STX, we filtrated the mixture before injection. STX of the same concentration pre- or post ultrafiltration was injected into mice to verify the efficiency of ultrafiltration and no difference was observed, which was consistent with the results obtained above in ELISA. STX preprocessed by incubation with aptamers(M-30f or APT^{STX1}) and ultrafiltration didn't cause death to mice, which indicated that a portion of the STX was maintained in the concentrate in the form of complexes with the aptamers (Table 3). Although a difference between M-30f and APT^{STX1} at the animal level was not observed, both M-30f and APT^{STX1} showed high affinity towards STX and resulted in the direct reduction of the STX dose administered to mice.

Table 3. STX toxicity after interaction with aptamers and filtration. STX and aptamers were used at concentrations of 11.25 µg/kg and 4.95 mg/kg, respectively.

saxitoxin	M-30f	APT ^{STX1}	Alive/tested
+	+	-	6/6
+	-	+	6/6
+	-	-	1/6
-	+	-	6/6
-	-	+	6/6

4. DISCUSSION

In this study, we obtained a significantly improved STX-binding aptamer (M-30f) with higher affinity (30-fold) and inhibitory activity than the parent aptamer identified by SELEX (APT^{STX1}). It is well known that the secondary structure of oligonucleotides affects the amplification efficiency of PCR. Therefore, oligonucleotides with higher order structures may not be easily amplified in SELEX, which reduces the efficiency of obtaining aptamers with high target-binding ability through this technique. Thus, post-SELEX optimization is necessary to find potential sequences with desired properties. As demonstrated in this study, site-directed mutagenesis is a promising strategy for continued aptamer optimization.

The relation between structure and function was also researched in our study. To reduce the cost of synthesis and remove unnecessary nucleotides, the length of aptamer M-30f was truncated to only 34 nucleotides. We find that the core target-binding structure is formed by the first and second stem-loops, which is consistent with the assumption by Handy et al. (2013) that the primer-binding section is involved in the binding reaction. Additionally, these two stem-loop structures can form a G-quadruplex, as predicted by QGRS Mapper. It is assumed that STX-binding aptamers can form a pocket for binding of small marine toxin; the formation of a G-quadruplex might tighten the interaction of the pocket with STX, which results in a slower dissociation rate and higher affinity as observed.

1 Finally, using a range of techniques (ELISA, cell bioassay and mouse bioassay), we compared the
2 STX-binding abilities of M-30f and APT^{STX1}. All the results demonstrate that both M-30f and APT^{STX1}
3 can bind STX, though M-30f shows increased binding compared to APT^{STX1} in the ELISA and cell
4 bioassays. Moreover, both aptamers showed STX inhibitory activity in the cell bioassay, whereas the
5 same phenomenon was not observed in mice. This difference is probably due to the aptamers'
6 inadequate nuclease stability in serum, a topic for further research in the future.

7 As a recognition molecule for STX, M-30f has great potential for practical application. For
8 monitoring programs, a compact Bio-Layer Interferometry sensor can be researched and developed,
9 allowing for real-time and high-throughput detection, with M-30f as a molecular recognition element
10 for STX. With high affinity and good selectivity, M-30f may be able to outcompete antibodies in STX
11 diagnostics. It is anticipated that the use of M-30f in STX detection methods could reduce or eliminate
12 animal-based models, as well as the numerous challenges associated with antibody-based methods.
13 Furthermore, M-30f can also be adopted to filter STX from ocean water to protect human from
14 paralytic shellfish poisoning. For clinical applications, M-30f still needs to be further optimized to
15 improve and regulate its functions and in vivo behaviors such as stability, bio-distributions, blood
16 clearances, etc. This may be done through more diverse optimization methods, e.g., phosphorothioate
17 internucleotide linkages, 2'-O-CH₃ nucleotide modification, 5'-modified pyrimidines and LNA
18 replacement. Such modifications may be applied to STX-binding aptamers as the aim of future
19 research.

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