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New Analytical Methods

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Magnetic separation-based multiple SELEX for effectively selecting aptamers against saxitoxin, domoic acid, and tetrodotoxin

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ABSTRACT: In this study, a novel magnetic separation based multiple systematic evolution of ligands by exponential enrichment (SELEX) was applied to select aptamers simultaneously against three kinds of marine biotoxins, including domoic acid (DA), saxitoxin (STX), and tetrodotoxin (TTX). Magnetic reduced graphene oxide (MRGO) was prepared to adsorb unbound ssDNAs and simplify the separation step. In the multiple SELEX, after initial twelve rounds of selection against mixed targets and the subsequent four respective rounds of selection against each single target, the three resulting ssDNA pools were cloned, sequenced and analysed. Several aptamer candidates were selected and subjected to the binding affinity and specificity test. Finally, DA-06 (K_d = 62.07 ± 19.97 nM), TTX-07 (K_d = 44.12 ± 15.38 nM) and STX-41 (K_d = 61.44 ± 23.18 nM) showed high affinity and good specificity for DA, TTX, and STX, respectively. They were also applied to detect and quantify DA, TTX, and STX successfully. The other two multi-target aptamers, DA-01 and TTX-27, were also obtained, which can bind with either DA or TTX. These aptamers provide alternative recognition molecules to antibodies for biosensor applications.

Keywords: Marine biotoxins, Aptamer, Systematic evolution of ligands by exponential enrichment, Magnetic reduced graphene oxide

Introduction

Recently, food poisoning due to the consumption of toxin-contaminated aquatic products has occurred more frequently [1]. There is a wide variety of marine biotoxins, which can be classified into two categories, lipophilic and hydrophilic toxins, according to their different molecular polarity. Saxitoxin (STX), domoic acid (DA), and tetrodotoxin (TTX) are three major hydrophilic marine biotoxins [2]. STX and TTX are both powerful sodium channel blockers [3, 4]. They can selectively bind to voltage-gated sodium ion channel, prevent transmission of sodium ions, and then inhibit conduction of neuropotentials, which in turn induce neural paralysis, dyspnea, and even death. DA is an excitatory neurotoxin with a high affinity for the propanoic acid receptors, kainate subclasses of glutamate receptors, and N-methyl-D-aspartate receptors in the central nervous system [5]. Activation of these receptors leads to over-accumulation of both calcium ions and sodium ions in the neurons, production of reactive oxygen species, and consequent damage to the nervous system. The typical symptoms are loss of memory, disorientation, seizures, and coma. Additionally, these toxins also have negative influences on the aquatic products industry and the environment [6]. Thus, it is essential to detect the marine biotoxins in aquatic products, and aquatic environments, rapidly and accurately.

Mouse bioassay (MBA) and chromatography-based chemical analytical techniques are the most widely used official reference methods for marine biotoxin detection in many countries [7-9]. However, MBA suffers from its being time-consuming, of poor repeatability, and various associated ethical issues [10, 11].

Chemical analytical techniques require expensive equipment, skilled operators, and complex sample pre-treatments [6, 12]. Therefore, they are not suitable for the increasingly important in-field fast detection. Immunoassay is a rapid detection technology, which is based on the specific recognition and binding between antibodies and antigens coupled with colorimetry, fluorescence, chemiluminiscence, electrochemistry, quartz crystal microbalance (QCM), surface plasmon resonance (SPR), and other signal transduction techniques [13]. However, the preparation of the antibody is laborious and difficult, especially for such marine biotoxins. The low molecular weight toxins may be too small to stimulate the immune system to produce antibody responses, thus they even have toxicity to experimental animals or cells. The long-term storage of antibodies requires more stringent conditions. Furthermore, cross-reactivity is another unavoidable limitation thereof [14].

Aptamer is an ssDNA or RNA molecule which is selected from a synthetic oligonucleotide library to bind to its target [15]. Compared with antibodies, aptamers possess some distinct advantages: *in vitro* screening of aptamers avoids ethical problems associated with animal experiments arising in antibody preparation. This further excludes the negative influences of immunogenicity and toxicity, and extends the range of targets to include metal ions [16], small molecules [17], biomacromolecules [18, 19], and even cells [20, 21]. The obtained aptamer sequence can be rapidly produced by chemical synthesis with high purity, at low cost, and with minor batch-to-batch variations[22, 23]. They can also be easily modified to enhance their chemical and thermal stability for long-term storage, and are labelled with various signal molecules

to facilitate detection [24-26]. The antibody-based immunosensors have already been made commercially available, such as enzyme-linked immunosorbent assay (ELISA) kits and immunochromatographic strips [27, 28]. Due to the numerous advantages of aptamers, they have been promising alternative molecular recognition elements to antibodies in analytics, medical diagnosis, and food safety inspection [29-31]. So it is of great significance to screen aptamers with high affinity and specificity.

The *in vitro* aptamer selection technology, named systematic evolution of ligands by exponential enrichment (SELEX), was first established in 1990 by both the Gold research group and the Szostak research group [32, 33]. Since then, a variety of SELEX variants have been developed against different types of targets [34]. The immobilised SELEX has been used as the conventional method for low molecular weight marine biotoxins, which fixes target molecules on magnetic beads or agarose beads to separate affinity oligonucleotides from non-affinity ones [35, 36]. Through use of this beads-based method, aptamers against microcystins (MC), STX, okadaic acid (OA), TTX, and some other marine biotoxins have been obtained [37-40]; however, the steric hindrance of the immobilisation process, the non-specific adsorption of the beads, and the possible conformational change of the immobilised target all have negative influences on selection efficiency and aptamer properties [41]. In 2012, the Gu group from Korea University developed a novel graphene oxide assisted immobilisation-free SELEX (GO-SELEX) to screen a DNA aptamer against Nampt protein [42]. This method, without fixing targets on a solid matrix, can avoid the various disadvantages of the immobilised SELEX. Additionally, the aptamer selected against the free target

with native conformation may be more suitable for detection in real samples. Later in 2014, the same group further developed an extended multiple GO-SELEX and obtained aptamers against three small molecular pesticides [43]. This multiple method improved screening efficiency, and the result confirmed its availability for small molecular targets.

In this study, the multiple GO-SELEX was further improved by replacing GO with magnetic reduced graphene oxide (MRGO). Involvement of the magnetic separation technology can simplify the separation step and thus improve screening efficiency. Through the use of multiple MRGO-SELEX, the aptamer against DA was obtained for the first time. Compared with the previous study, the aptamers against STX and TTX in their native conformation were selected, and the affinity and specificity thereof were evaluated. These aptamers provide alternative recognition elements for both marine biotoxin detection in aquatic products, or in the aquatic environment, and sample cleaning by affinity enrichment, separation, and removal.

Materials and methods

Reagents

All sequences used in this study are listed in **Table 1**. The ssDNA library which consisted of a central randomized region of 40 nucleotides (nt) flanked by two 20-nt PCR primer regions was synthesized by Integrated DNA Technologies (Coralville, USA). The primers used in PCR amplification were obtained from Sangon Biotech Co., Ltd. (Shanghai, China).

Saxitoxin diacetate salt (STX), Domoic acid (DA), Tetrodotoxin (TTX), Gonyautoxins-1&4 (GTX-1&4), Gonyautoxins-2&3 (GTX-2&3), Gonyautoxin-5 (GTX-5), Okadaic acid (OA) were purchased from Puhuashi Technology Development Co., Ltd (Beijing, China). Kainic acid (KA), Acrylamide/ bis-acrylamide (30% solution) were purchased from Sigma-Aldrich (IA, USA). Taq Plus DNA polymerase (5 U/μL), dNTP mixture (each 25 mM), 10×PCR buffer (containing Mg^{2+}), and other electrophoresis components were purchased from Sangon Biotech Co., Ltd. (Shanghai, China). Lambda exonuclease enzyme (5 000 U/mL) and 10×lambda exonuclease reaction buffer were purchased from New England BioLabs (Ipswich, MA). Graphene oxide (GO) was purchased from XFNANO Materials Tech Co.,Ltd (Nanjing, China). Other chemicals and reagents of analytical purity were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). All solutions were prepared with Milli-Q grade water.

Instrumentation

PCR amplification was carried out in a C1000 Touch Thermal cycler (Bio-Rad Laboratories, Inc., Hercules, USA). DNA concentration was measured by a NanoDrop-2000 Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, USA). Centrifugation was performed in an Eppendorf centrifuge 5424R (Eppendorf AG., Hamburg, Germany). Fluorescence intensity was obtained using a FL-7000 fluorescence spectrophotometer (Hitachi, Ltd., Tokyo, Japan). DNA was detected by polyacrylamide gel electrophoresis in Mini-PROTEAN® tetra cell system (Bio-Rad Laboratories, Inc., Hercules, USA). Gel was captured using Gel Doc EZ Imager

(Bio-Rad Laboratories, Inc., Hercules, USA). The prepared MRGO was characterized by JEM-2100 Transmission electron microscopy (JEOL Ltd., Tokyo, Japan), UV-1800 spectrophotometer (Shimadzu Corporation, Kyoto, Japan), Nicolet iS10 Fourier transform infrared spectrophotometer (Thermo Fisher Scientific Inc., Waltham, USA), 7400-S Series VSM (Lake Shore Cryotronics, Inc., Westerville, USA), and NanoBrook Omni (Brookhaven Instruments Corporation, New York, USA). The affinity and specificity tests were performed in a 96-well, clear bottomed, black polystyrene microplate (Corning Inc., Tewksbury, USA) and measured by Synergy H1 multi-mode microplate reader (BioTek Instruments, Inc., Highland Park, USA).

Preparation and characterisation of MRGO

MRGO was prepared by one-pot synthesis. In detail, GO was suspended in ultrapure water to a concentration of 1 mg/mL by ultrasonic dispersion. Then 300 mg of glucose and 333.3 mg of $FeCl_3 \cdot 6H_2O$ were added to 25 mL GO suspension. The mixture was adjusted to alkaline by adding 15 mL of 14 M ammonium hydroxide and then stirred vigorously for 20 min. Subsequently, the mixture was transferred into a Teflon-lined stainless-steel autoclave and reacted at 180 °C for 48 h. After the autoclave cooled, the dark brown suspension was transferred to a pair of centrifuge tubes and centrifuged at 5000 rpm for 10 min. The black precipitate was rinsed three times with ultrapure water under ultrasonic conditions, and then dried at 60 °C. Finally, the powdered MRGO was obtained by grinding the product. The synthesised MRGO was then characterised by transmission electron microscopy (TEM), ultraviolet-visble (UV-Vis) spectra, Fourier transform infrared spectroscopy (FTIR)

spectra, and its magnetisation curve. The MRGO suspension was prepared in ultrapure water to a concentration of 15 mg/mL by ultrasonic treatment, and stored at 4 °C for use.

In vitro selection of aptamers against three marine biotoxins by multiple MRGO-SELEX

Sixteen repeated rounds of SELEX, including binding, magnetic separation, PCR amplification, purification of the PCR product, preparation of ssDNA, and purification of the sub-library, were performed to obtain aptamers recognising DA, TTX, and STX with high affinity and specificity (**Fig. 1**). The first twelve rounds entailed mixed screening against three biotoxins simultaneously, and the subsequent four rounds of single screening were against DA, TTX, and STX, respectively.

Initially, the original ssDNA library or the sub-library was dissolved in binding buffer, heated at 95°C for 10 min, and immediately cooled in an ice-bath for another 10 min. Then, the ssDNA pool and target marine biotoxin were mixed in a molar ratio of 1:10 and incubated at 37 °C with mild shaking. Following the addition of MRGO (the mass ratio of MRGO/ssDNA was 300:1), 1 h of shaking at 37 °C was undertaken. The mixture was then subjected to magnetic separation to collect the supernatant containing target-bound ssDNAs as a template for amplification by PCR. A 50 μL PCR mixture was composed of 2 μL of supernatant, 1 μL of 5 mM dNTPs, 1 μL of 10 μM forward primer, 1 μL of 10 μM phosphorylated reverse primer, 0.5 μL of 5 U/μL Taq Plus DNA polymerase, 5 μL of 10× PCR buffer, and 39.5 μL of ultrapure water. The thermal cycle conditions were pre-denaturation at 95 °C for 5 min,

followed by 20 cycles of denaturation at 95 ℃ for 30 s, annealing at 60 ℃ for 30 s, extension at 72 ℃ for 30s, and a final extension at 72 ℃ for 5 min. The PCR product was identified by 8% native polyacrylamide gel electrophoresis (PAGE), purified by a PCR product purification kit (Shanghai Generay Biotech Co., Ltd), and the concentration therein determined by a NanoDrop-2000 Spectrophotometer. Thereafter, ssDNAs were prepared by digesting phosphorylated antisense strands of PCR products. A 400 μL digestion mixture consisted of 358 μL of purified PCR product, 40 μL of 10× reaction buffer, and 2 μL of Lambda exonuclease. The digestion conditions were incubation at 37 ℃ for 1 h, and reaction termination at 75 ℃ for 10 min. The digestion product was verified by 8% denatured polyacrylamide gel containing 7 M urea. Finally, the digestion product was purified and recovered by ethanol precipitation with the help of Dr. GenTLE Precipitation Carrier (Takara Biomedical Technology (Beijing) Co., Ltd), dissolved in binding buffer, and the concentration determined as a sub-library for the next round of selection.

To enhance the affinity of the selected aptamers, the screening stringency was strengthened with increasing numbers of SELEX rounds [38]. As shown in **Table S1**, the volume of the ssDNA library was reduced from 1000 to 10 pmol, and the incubation time of ssDNA pool and targets was decreased from 3 to 0.25 h.

To improve the specificity of the aptamers, several analogous or coexisting toxins, including GTX-1&4, GTX-2&3, GTX-5, KA, and OA, were used for the counter SELEX process in the seventh, ninth, and eleventh rounds. Due to the single screening undertaken in the thirteenth and fifteenth rounds, the counter-targets

involved not only the toxins described above but also another two marine biotoxins besides the target. (**Table S1**). Briefly, following heating and cooling treatment, the sub-library from the previous round was incubated with the counter-targets at 37 ℃ for 2 h. Then, MRGO was added and incubated at 37 °C for 1 h. After removing the counter-target bound ssDNAs in the supernatant by magnetic separation, the precipitation of MRGO adsorbing unbound ssDNAs was washed several times with binding buffer. Then, target biotoxins were added to the MRGO which resulted in affinity-based desorption of targets bound ssDNAs going back into solution. The ssDNAs specific to targets were recovered by the second magnetic separation step, which was followed by PCR amplification, dsDNAs purification, ssDNAs preparation, and purification as described above.

Cloning, sequencing, and analysing of selected aptamer candidates

After sixteen rounds of selection, the enriched aptamer pools against three marine biotoxins were amplified by using PCR with the unmodified primers. The purified PCR products were cloned and sequenced by Shanghai Personal Biotechnology Co., Ltd. (Shanghai, China). The homology of the obtained sequences was analysed using DNAMAN V6. The secondary structures and free energies (ΔG) of the sequences were predicted by the Mfold web-server (http://unafold.rna.albany.edu/?q=mfold) under the conditions of 37 °C, 150 mM Na^+, and 2mM Mg^{2+}. Several aptamer candidates were selected and the complete sequences with carboxyfluorescein (FAM) at the 5' end were synthesised for fluorescence assay.

Characterisation of selected aptamers by fluorescence assay

A fluorescence assay was used to assess the affinities of the selected aptamer candidates (**Fig. S3**). Initially, each FAM-labelled aptamer candidate was subjected to the same heating and cooling treatment as a screening process. Then, in a total of 300 μL reaction solution, 2 μM of target toxin was incubated with gradient concentrations of the sequence ranging from 5 to 200 nM at 37 °C in the dark for 1 h. With the same mass ratio to ssDNAs, corresponding amounts of MRGO were added to different reaction solutions, and shaken gently in dark conditions for 30 min. After magnetic separation, 200 μL of supernatant containing target-aptamer complex were transferred into a 96-well, clear bottomed, black polystyrene microplate. Negative controls were composed of different concentrations of sequence and corresponding amounts of MRGO without adding target to eliminate self-desorption of sequences from MRGO. The fluorescence intensities of the supernatants in the microplate were read by a Synergy H1 multi-mode microplate reader. GraphPad Prism 5.0 software was used to plot the binding saturation curve and calculate the dissociation constants (K_d) through non-linear fitting of the data.

The specificity was determined using a similar fluorescence test. In 300 μL reaction solution, 150 nM of treated FAM-labelled aptamer candidate and 2 μM of different toxins (DA, TTX, STX, GTX-1 & -4, GTX-2 & -3, GTX-5, KA, and OA) were mixed and incubated at 37 °C in dark conditions for 1 h, respectively. A negative control without toxin was performed simultaneously. MRGO was then added and shaken in the dark for 30 min, followed by magnetic separation, supernatant transfer,

and fluorescence intensity measurement. The specificity of each sequence was estimated by comparing the relative fluorescence ratios of different toxins. The relative fluorescence ratio is given by $\Delta F_{toxin}/\Delta F_{target}$, where ΔF_{target} and ΔF_{toxin} are the ΔF values of the target and other toxin, respectively. $\Delta F = F - F_0$, where F and F_0 are the fluorescence intensities at 520 nm with, and without, the addition of toxin, respectively. All assays were performed in triplicate.

Results and discussion

Preparation and characterisation of MRGO

In the one-pot synthesis process, glucose reduced $FeCl_3 \cdot 6H_2O$ and GO simultaneously: the reduced products Fe_3O_4 nanoparticles and reduced GO were connected by gluconic acid, forming MRGO. The TEM image in **Fig. 2(a)** shows that Fe_3O_4 nanoparticles (the black dots) had been anchored onto the surface of the reduced GO (the leaf shaped translucent layer) and were well dispersed. In **Fig. 2(b)**, the UV-Vis spectra shows that a redshift of the absorbance peak occurred from 230 nm of GO to 273 nm for the MRGO. The FTIR spectrum of MRGO in **Fig. 2(c)** shows a significant difference from that of GO. An additional transmittance band located at 582 cm^{-1} was characteristic of the stretching vibration of the Fe-O bond, confirming that Fe_3O_4 nanoparticles and reduced GO had been connected [44]. The magnetisation curve for MRGO is shown in **Fig. 2(d)**, and the saturation magnetisation was determined to be approximately 33 $emu \cdot g^{-1}$. The Zeta Potential of MRGO was – (8.20 ± 1.81) mV. These results demonstrate that MRGO has been

283 synthesised successfully.

284 It is reported that ssDNA sequences of different lengths showed different binding
285 kinetics to the GO surface [45]. Thus, it is necessary to obtain an appropriate mass ratio,
286 which ensures the complete adsorption of ssDNA on MRGO, then, different mass
287 ratios of MRGO/ssDNA ranging from 50:1 to 500:1 were used to adsorb 80-nt ssDNA:
288 a ratio of 300:1 was determined as the optimal mass ratio of MRGO/ssDNA for the
289 following experiment (**Fig. S1**).

290 If the marine biotoxins are adsorbed on MRGO, their binding with ssDNA will
291 be disturbed, and the adsorption efficiency between ssDNA and MRGO will be
292 decreased, so, it is essential to determine whether, or not, MRGO adsorbs the
293 biotoxins. The results shown in **Fig. S2** confirmed that DA, TTX, and STX are not
294 adsorbed on MRGO.

295 *Multiple selection of aptamer against three marine biotoxins assisted by MRGO*

296 It is a challenge to screen aptamers recognising low molecular marine biotoxins
297 with high affinity and specificity from a vast random library: GO is an interesting and
298 attractive material, which can adsorb single strand oligonucleotides by π-π stacking
299 interaction between the hexagonal cells of GO and the ring structures in the DNA
300 bases [42]. Relying on this property, an immobilisation-free SELEX assisted by GO has
301 been designed and applied to select aptamers against small molecular toxins [41, 46].
302 Comparing with beads-based immobilisation SELEX, GO-SELEX avoids
303 complicated connection of targets to beads, prevents the probable barrier to epitope,
304 and reduces potential non-specific interactions. The application of MRGO instead of

GO in the screening process further simplifies the separation step without using expensive instrument(s). A magnet can separate the target-bound ssDNAs remaining in solution from the unbound ssDNAs adsorbed on the MRGO.

As shown in **Table S1**, with the increasing rounds of SELEX, the stringency of the SELEX protocol was enhanced by decreasing both the amounts of ssDNA and the incubation time. These operations reduced the probability of binding between targets and ssDNAs, because only high-affinity ssDNAs can bind tightly to the target and form a stable complex, whereas low-affinity ssDNAs may detach from the target. In this way, the affinity of the aptamers was improved by gradually removing non-affinity and low-affinity ssDNAs.

The counter SELEX method is commonly used to eliminate non-specific binding. In this screening process, five counter targets were selected, including three STX analogues, GTX-1&4, GTX-2&3, GTX-5, a DA analogue, KA, and another main marine biotoxin OA, which were likely to coexist in a heterogeneous environment. The first magnetic separation step removed the counter-target-bound ssDNAs in the supernatant to enhance the specificity of the aptamers. In the second magnetic separation step, only the high-affinity ssDNAs were induced to change conformation and release from MRGO by adding targets. The affinity based desorption process also improved the affinity.

Sequencing and analysis of selected aptamer candidates

The ssDNA enriched pools from the last round were sent for cloning and sequencing. A total of 45, 46, and 45 sequences were successfully obtained targeting

DA, TTX, and STX, respectively. Polymerase jumping [47], meant that some sequences had fewer than 40 nucleotides in the randomised region, but the missing nucleotide also appeared frequently in other research [41, 48], and would not influence the selection if the resulting short sequence exhibited high affinity and specificity to target. They were then aligned using DNAMAN V6. According to a comparison of the homologies, the sequences against DA, TTX, and STX were grouped into six, eight, and seven families, respectively. Considering that the primer regions may exert an influence on the binding between target and aptamer due to their being involved in the formation of the stem-loop in the second structures, the complete sequences were analysed by Mfold, and one or two sequences were chosen from each family as aptamer candidates, according to their sequence repetitiveness, representative secondary structures, and lower predicted ΔG (**Fig. 3**, **Tables S2 to S4**). The repetitive sequences may be the enrichments of affinity aptamers, and the representative structures may possess conserved motifs to bind with targets. Meanwhile, the lower free energies generate more stable secondary structures, which contribute to the formation of stable complexes of target and aptamer.

Characterisation of selected aptamer by fluorescence assay

Graphene-like materials are effective quenchers for most of the fluorescent materials, and have often been applied as fluorescent donors in fluorescence resonance energy transfer (FRET) assays [49]. Herein, a fluorescence assay based on MRGO adsorption and quenching was used to estimate the affinity and specificity of the aptamer candidates. As shown in **Fig. S3**, adding MRGO to the mixture of

aptamer candidate and target resulted in the adsorption of unbound sequences and the quenching of their fluorescence of the FAM label. The higher the concentration of sequence used, the stronger the measured fluorescence emission. The binding saturation curves of the selected aptamers are shown in **Fig. 4**, and the K_d values of all the candidates are listed in **Table S5**. The lower K_d values represented a higher affinity. Therefore, four aptamer candidates with lower K_d values for each biotoxin (DA-01, DA-04, DA-06, DA-42, TTX-07, TTX-27, TTX-42, TTX-45, STX-14, STX-32, STX-38, and STX-41) were selected for the next specificity test. The specificity of each candidate was characterised by the relative fluorescence ratio value, which was calculated using the formula $\Delta F_{toxin}/\Delta F_{target}$ to describe the fluorescence intensity ratio of the counter-target and target toxin. The lower values of this ration for the counter-targets suggested an aptamer with higher specificity. As presented in **Fig. 5**, DA-06, TTX-07, and STX-41 exhibited lower relative fluorescence ratios in the present of counter-targets compared with their respective target, which demonstrated that they bound to their respective target more strongly than the other toxins. These results confirmed that DA-06 (K_d = 62.07 ± 19.97 nM), TTX-07 (K_d = 44.12 ± 15.38 nM), and STX-41 (K_d = 61.44 ± 23.18 nM) were suitable aptamers against DA, TTX, and STX with appropriate affinity and specificity, respectively.

Characterisation of multi-target aptamers

Fig. 5 showed that DA-01 possessed higher relative fluorescence ratios for both DA (100%) and TTX (81.61%). The similar result was found for TTX-27, which had higher relative fluorescence ratios for TTX (100%) and DA (48.22%). Thus, the

fluorescence assay described above was applied to determine the affinity of DA-01 for TTX and the affinity of TTX-27 for DA. As shown in **Fig. 4** and **Table S5**, the K_d values of DA-01 for DA and TTX were 134.70 ± 36.65 nM and 192.60 ± 77.94 nM, respectively; the K_d values of TTX-27 for TTX and DA were 90.73 ± 41.55 nM and 160.60 ± 49.28 nM, respectively: they were, therefore, multi-target recognised aptamers, which exhibited affinity for both DA and TTX.

Application of aptamers in a fluorescence assay

A fluorescence assay was performed to confirm the availability of the selected aptamers. The detailed process was described in Supplementary Material. As shown in **Fig. S4**, a linear relationship (R^2 = 0.9967) existed between the relative fluorescence intensity (ΔF) and the logarithm of DA concentration in the range of 0.5 to 50 ng·mL^{-1} for the aptamer DA-06. The limit of detection (LOD) was found, by calculation, to be 0.45 ng·mL^{-1} (by using the equation LOD = 3 SD/slope, where SD was the standard deviation of blank samples, and the slope was obtained from the standard curve). For TTX-07, the ΔF values were linearly proportional to the concentrations of TTX ranging from 5 to 150 ng·mL^{-1}, and the LOD was 1.21 ng·mL^{-1}. For STX-41, the ΔF values increased with the increasing STX concentrations from 1 to 100 ng·mL^{-1}, and the LOD was 0.39 ng·mL^{-1}. Then, clam samples spiked with different concentrations of DA, STX, and TTX were tested by use of this method. As shown in **Table 2**, the recovery rates were 84.66-104.63%, 84.59-96.13%, and 83.01-98.69% for DA, STX, and TTX, respectively; the relative standard deviations (RSD) were 8.23-13.65%, 4.99-14.50%, and 5.03-11.21%,

393 respectively. These results suggested that these selected aptamers (DA-06, TTX-07,
394 and STX-41) have the potential for use in the quantitative determination of DA, TTX,
395 and STX, respectively.

396 In summary, this study is the first report of using MRGO assisted
397 non-immobilised multiple SELEX to select aptamers recognising three low-molecular
398 weight marine biotoxins simultaneously. The TTX aptamer A3 [40] and the STX
399 aptamer APT^{STX1} [38] have already been obtained by beads-based immobilisation
400 SELEX, but the dissociation constants and specificity tests of these aptamers have not
401 been provided. More importantly, immobilization-based methods suffer from complex
402 conjugating, steric hindrance, non-specific adsorption, and possible conformational
403 change. Therefore, the non-immobilised SELEX offers significant advantages,
404 especially for screening aptamers against small molecules. Furthermore, use of a
405 multiple mode assay can improve screening efficiency. Depending on this
406 non-immobilised multiple MRGO-SELEX, DA-06 (K_d = 62.07 ± 19.97 nM), TTX-07
407 (K_d = 44.12 ± 15.38 nM), and STX-41 (K_d = 61.44 ± 23.18 nM) with appropriate
408 affinity and specificity for DA, TTX, and STX were selected. Although the K_d values
409 were in the same range with the aptamers obtained by GO-SELEX [41, 46, 50, 51], the
410 magnetic separation technology can further simplify the separation process. These
411 aptamers were then used in a fluorescence assay to detect spiked clam samples, which
412 demonstrated their potential application in the quantitative detection of marine
413 biotoxins. We also obtained two multi-target aptamers, DA-01 and TTX-27, which
414 can bind with either DA or TTX. We believe that these aptamers will provide

415 alternative molecular recognition elements for marine biotoxin detection.

416

417 **ASSOCIATED CONTENT**

418 **Supporting Information**

419 The Supporting Information is available free of charge on the ACS Publications
420 website.

421 Adsorption of ssDNA on MRGO, adsorption of toxins on MRGO, fluorescence
422 aptamer assay, pre-treatment of clam sample, schematic illustration of the
423 fluorescence assay, standard curve of the relative fluorescence intensity (ΔF) versus
424 logarithm concentration of toxins plotted by the fluorescence assay, the selection
425 conditions in each round of SELEX, list of the selected aptamer candidates, the
426 dissociation constants (K_d) of the selected aptamer candidates.

427

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Notes

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585

Figure captions

Fig. 1 Schematic illustration of the multiple MRGO-SELEX

Fig. 2 Characterisation of the synthesised MRGO. a) TEM image of MRGO; b) UV-Vis spectra for GO and MRGO; c) FTIR spectra for GO and MRGO; d) magnetisation curve of MRGO.

Fig. 3 Secondary structures and free energies of the selected aptamers predicted by the Mfold web-server.

Fig. 4 Binding saturation curves of selected aptamers. $\Delta F = F - F_0$, where F and F_0 were the fluorescence intensities at 520 nm in the presence and absence of the target, respectively. Error bars show the standard deviations of the mean with $n = 3$.

Fig. 5 Characterisation of the specificity of aptamers. The relative fluorescence ratio is given by $\Delta F_{toxin}/\Delta F_{target}$, where ΔF_{target} and ΔF_{toxin} are the ΔF values of the target, and other toxin, respectively. Error bars show the standard deviations of the mean with $n = 3$.

Tables

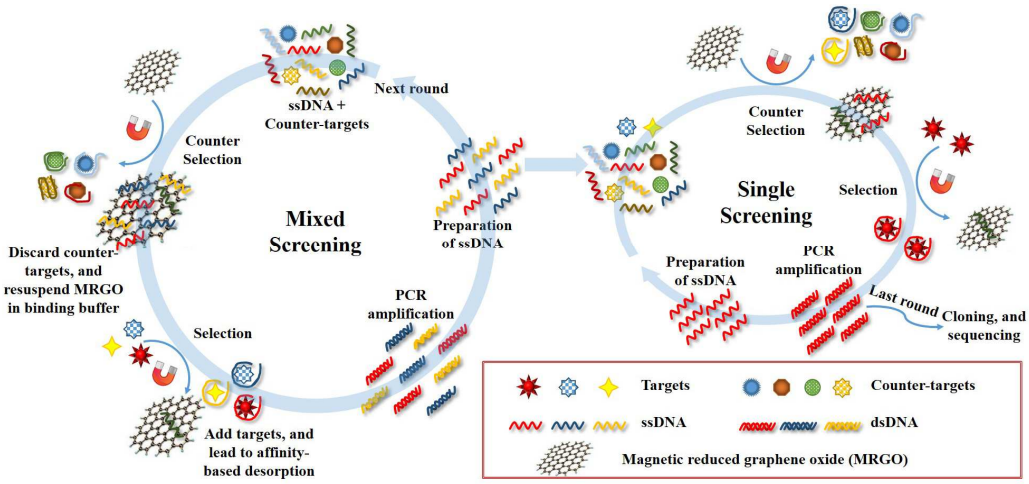
Table 1. The sequences used in this study

Name	Sequences
ssDNA library	5'- ATAGGAGTCA CGACGACCAG -N_{40}- TATGTGCGTC TACCTCTTGA -3'
Forward primer (FP)	5'- ATAGGAGTCA CGACGACCAG -3'
Reverse primer (RP)	5'- TCAAGAGGTA GACGCACATA -3'

Table 2 Recovery study of the three marine biotoxins in clam samples (n=5)

Marine biotoxins	**Spiked concentration ($ng \cdot mL^{-1}$)**	**Measured concentration ($ng \cdot mL^{-1}$, mean ± SD)**	**Recovery (%, mean)**	**RSD (%)**
DA	1	0.85 ± 0.10	84.66	12.09
	5	5.23 ± 0.43	104.63	8.23
	25	23.90 ± 3.26	95.58	13.65
STX	5	4.23 ± 0.21	84.59	4.99
	20	19.23 ± 2.07	96.13	10.77
	80	72.05 ± 10.44	90.06	14.50
TTX	10	9.15 ± 0.73	91.54	8.01
	50	41.51 ± 4.65	83.01	11.21
	100	98.69 ± 4.97	98.69	5.03

Figure graphics



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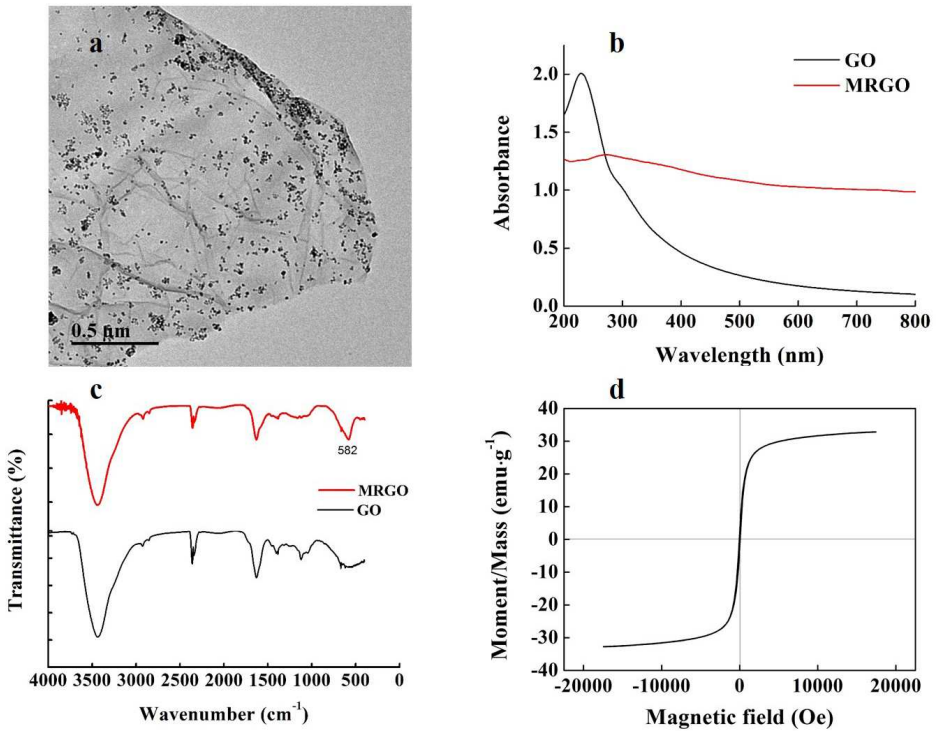

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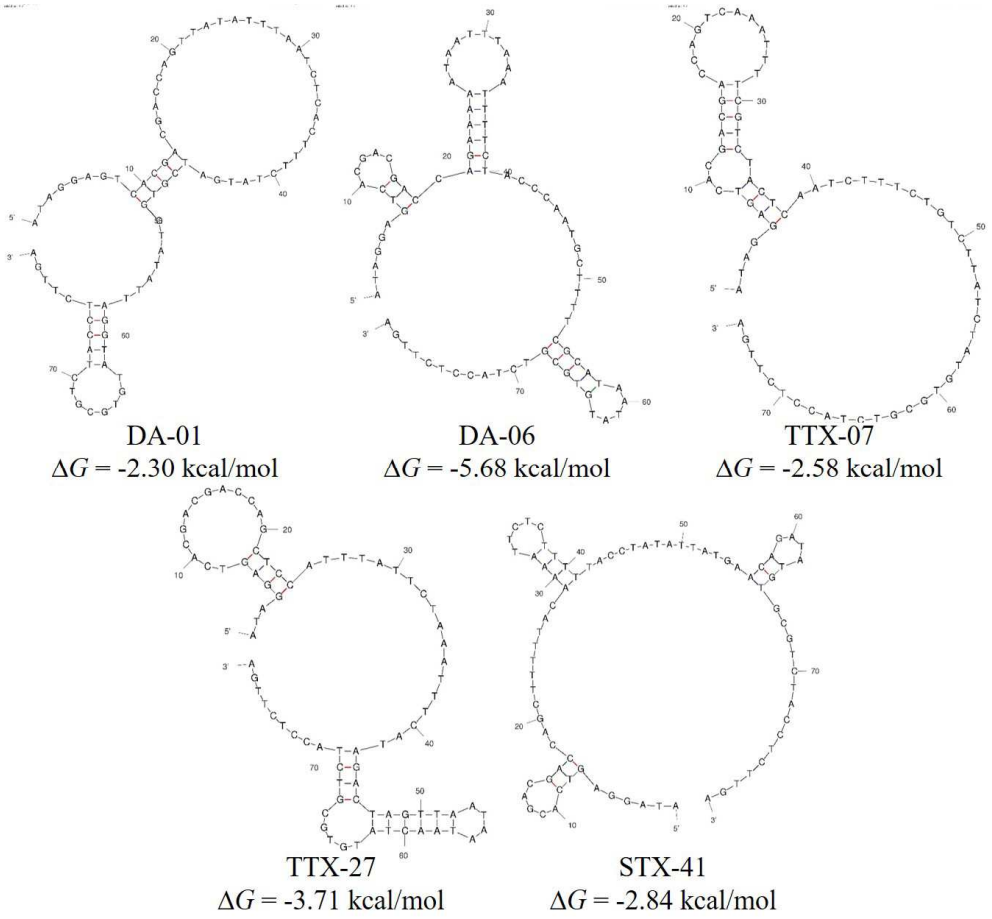

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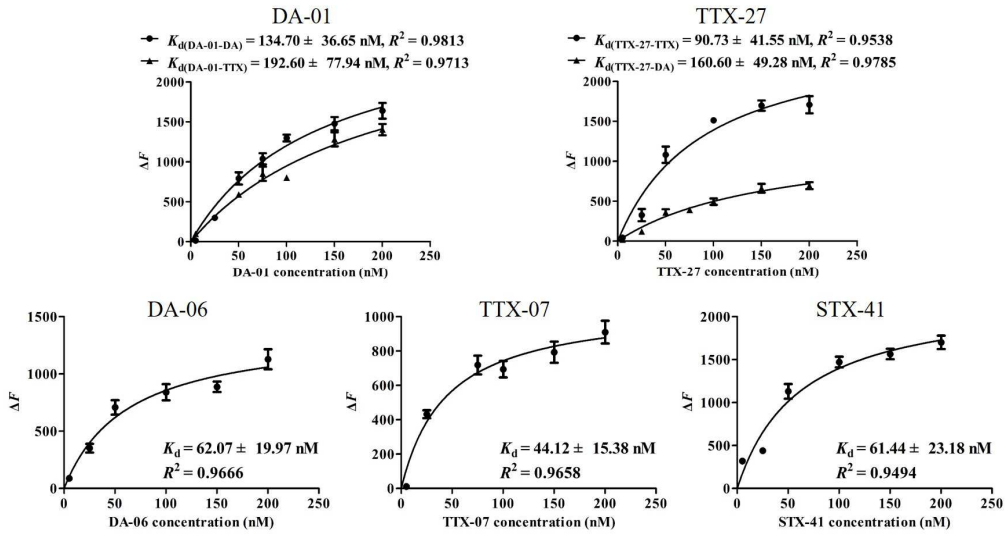

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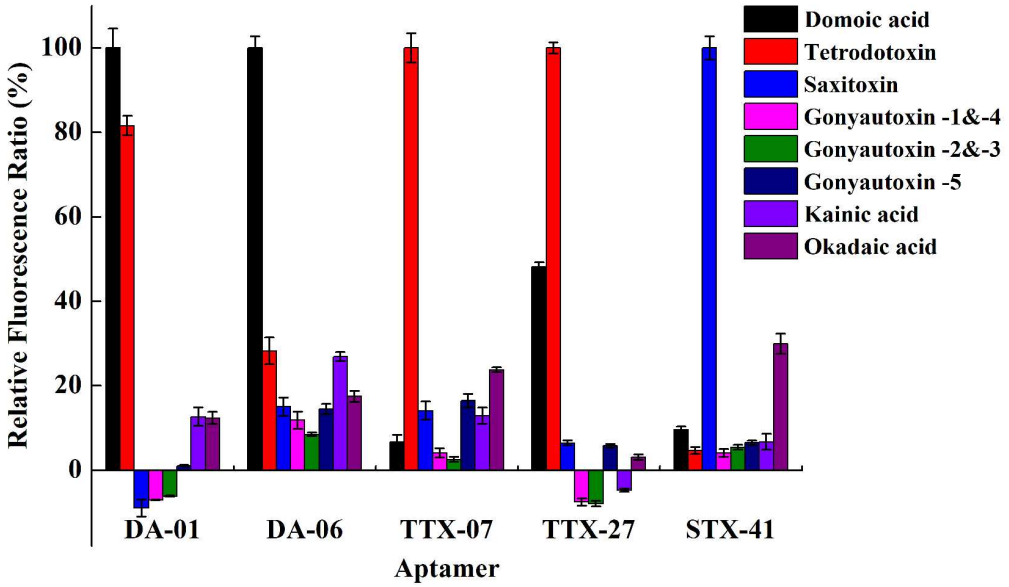

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Graphic for table of contents