

DNA aptamer for use in a fluorescent assay for the shrimp allergen tropomyosin

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Abstract Shrimp allergy is a common cause of allergenic diseases. The authors describe an aptamer-based assay for tropomyosin, one of the major allergens of shrimp. After six rounds of SELEX based on nitrocellulose membrane, several aptamers with high affinity ($K_d = 109.6$ nM) were identified that can selectively recognize tropomyosin but not other proteins over a large range of concentrations. By using a fluorescent and graphene oxide based platform, a tropomyosin assay was worked out that has a 4.2 nM detection limit and work in the 0.5 to 50 $\mu\text{g}\cdot\text{mL}^{-1}$ concentration range. The aptamers selected hold a great potential for tropomyosin detection in practical biosensor assays.

Keywords Biosensor · Allergen detection · Graphene oxide · Tetramethylbenzidine · Horse radish peroxidase · Binding assay

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Introduction

Tropomyosin is the major heat-stable shrimp allergen that was first described by [1] Hoffman et al. and later identified by [2] Santi et al. Shrimp tropomyosin is a muscle myofibrillar protein that naturally containing two subunits (38.5 and 36.6 KDa) with a ratio of 1 to 1, which can assemble into nanofibers at high ionic strength induced by ATP [3]. As an important type of seafood, shrimp has been consumed worldwide but also has become a common cause of food allergy. Several reports on adverse reactions to shellfish have been presented with the increased consumption of shellfish [4]. The immunological reactivity to tropomyosin is a good predictor of shellfish allergies [5], and extensive efforts have been made to develop valid assays for the determination of tropomyosin in food products [6–8]. Antibody-based enzyme-linked immunosorbent assays have been widely used to identify and quantify allergens in food for its specificity and sensitivity [6, 9]. Although these methods provide accurate and reliable detection of allergens in food products, they are heavily reliant on the quality of the antibodies that have drawbacks in terms of stability and preparation. Additionally, immunoassays demand laborious and time-consuming work, tedious washing steps, and expert technical skill. Therefore, rapid, simple, sensitive and specific detection methods for tropomyosin attract tremendous attention.

Aptamers are short single-stranded oligonucleotides selected through combinatorial libraries to bind specifically to small molecules [10], toxins [11], proteins [12], virus [13] and pathogens [14] with high affinity. Unlike antibodies, the remarkable target diversity, tight-binding capability, high stability, easy-of synthesis and modification of aptamers hold overwhelming advantages [15]. Furthermore, aptamers are selected in vitro by Systematic Evolution of Ligands by Exponential

Enrichment (SELEX) [16] and are small with low immunogenicity [17], which can against allergens in a reproducibly synthesized and stabilized manner. Nowadays, aptamers recognized several allergens with high affinity and specificity have been reported, including lysozyme [18], Cry j 2 [19], Asp f 1 [20], Lup an 1 [21], Ara h 1 [22] and so on. Based on these aptamers, various sensing detection methods have been developed. However, aptamers against tropomyosin have not been reported.

Up to date, fluorescent assays based on the use of aptamers and graphene oxide (GO) have already been pioneering constructed. For example, Zhao and co-workers [23] utilized assemblies of a fluorescein-labeled aptamer on reduced graphene oxide for oxytetracycline (OTC) detection. The same strategy was also applied by Duan et al. [24] for the determination of *Salmonella typhimurium*. Despite this progress, fluorescent systems with aptamers for sensing have been based on fluorescein-labeling. Moreover, fluorescent assay based on aptamers for allergen detection is still a challenge. Therefore, fluorescent aptasensors especially label-free for allergen detection are urgent and appealing.

Herein, the authors describe the selection and characterization of single-stranded DNA aptamers that recognize shrimp tropomyosin (*Penaeus Vannamei*) specifically. After six rounds of Dot-blotting-SELEX method, the enriched DNA aptamers were cloned, sequenced and identified. The affinity and specificity of the aptamers were also evaluated. In addition, one of the aptamers with high binding affinity was further implemented in measuring tropomyosin in buffer in a large range of concentrations using a robust label-free fluorescent assay based on graphene oxide and OliGreen ssDNA reagent.

Materials and methods

Materials

All oligonucleotides purified by HPLC were chemically synthesized by Shanghai Sangon Biotechnology Co., Ltd. (Shanghai, China, <http://www.sangon.com>). Shrimp tropomyosin (*Penaeus Vannamei*) was purchased from Shanghai Anyan Biotechnology Co., Ltd. (Shanghai, China, <http://anyanshengwu.foodmate.net>). Bovine Serum Albumin (BSA) and streptavidin were purchased from Sigma-Aldrich Co., Ltd. (Beijing, China, <http://www.sigmaaldrich.com>). β -conglycinin was obtained from Shanghai Bangyi Biotechnology Co., Ltd. (Shanghai, China, <http://www.bangyi-sh.com>). Lysozyme, thrombin and myosin were purchased from Beijing Huayueyang Biotechnology Co., Ltd. (Beijing, China, <http://www.huayueyang.com>). Streptavidin-HRP and TMB solution were obtained from Beyotime Biotechnology Co., Ltd. (Shanghai, China,

<http://www.beyotime.com>). Single layer graphene oxide (> 99 wt%, containing 28.77% oxygen atom) was purchased from Nanjing XFNANO Materials Technology Co., Ltd. (Nanjing, China, <http://www.xfnano.com>). All chemicals for preparing the buffers and solutions were of analytical grade and purchased from Shanghai CIVI Chemical technology Co., Ltd. (Shanghai, China, <http://www.labgogo.com>). The solutions were prepared with ultra-high purity water from a Millipore water purification system. Binding buffer (20 mM Tris-HCl, 50 mM NaCl, 5 mM KCl, 1 mM MgCl₂, pH 7.2) was used for the binding reaction between the aptamer and tropomyosin.

In vitro selection of aptamers by dot-blotting-SELEX

A single-stranded DNA library contained a central randomized sequence of 40 nucleotides flanked by invariant primer-binding sites (5'-TACTAACGGTACAAGCTA-N40-AACGTTGACCTAGAAGC) was used for the initial pool. Primer-Fw (5'-TACTAACGGTACAAGCTA-3') and primer-Rv (5'-GCTTCTAGGTCAAC-GTT-3') were used for PCR amplification. Prior to use, the library (1 μ M) dissolved in binding buffer was denatured at 95 °C for 10 min, immediately cooled on ice for 5 min, and then gradually cooled to 25 °C for 30 min to fold the structures.

Selection of aptamers recognized tropomyosin was performed using a dot-blotting- SELEX method. Typically, 1 μ g of tropomyosin and BSA was specifically spotted on a nitrocellulose membrane and blocked with 2% (w/v) BSA in PBST (phosphate buffer saline containing 0.05% Tween-20) at room temperature (RT) for 1 h. and then the folded DNA library was incubated on the membrane immobilized with tropomyosin and BSA at RT for 1 h. After three times washing with PBST, the tropomyosin immobilized spot was cut out and DNA bound to the area was extracted by phenol-chloroform and purified by ethanol precipitation. To monitor the progression of SELEX, the amount of eluted ssDNA was measured by Nanodrop device (Thermo-Scientific, USA). An ssDNA library for the following round was prepared by an asymmetric PCR protocol using HSTM Mix (Dongsheng Biotech, Guangzhou, China, <http://www.dongshengbio.com>) with a molar ratio of primer-Fw/primer-Rv = 50:1. All the PCR amplifications were performed as follows: 1 cycle at 95 °C for 5 min; 30 cycles at 94 °C for 40 s, 50 °C for 30 s, 72 °C for 30 s; followed by 1 cycle at 72 °C for 10 min. The screening was repeated six times, and the last round eluted ssDNAs were amplified by standard PCR protocol. The PCR products were purified using a PCR Extraction kit (Dongsheng Biotech, Guangzhou, China) and cloned into the pMD-19 T vectors using the TA cloning kit (Takara Biotechnology, Dalian, China, <http://www.takara.com.cn>). Colonies were subsequently selected and sequenced by BGI Technology (Shenzhen) Co., Ltd. (Shenzhen, China,

<http://www.genomics.cn>). The derived sequences were phylogenetically analyzed using MEGA 6 and predicted the second structures by RNA structure version 5.7.

Comparative binding assay

Sequenced aptamers and the initial DNA library as a control aptamer were to test for their individual binding characteristics to tropomyosin based on graphene oxide (GO) and Quanti-iT™ OliGreen ssDNA reagent (Molecular Probes, Eugene, OR, <http://www.thermofisher.com>). For each binding assay, 12 pmol of aptamer ssDNA was incubated with 30 $\mu\text{g}\cdot\text{mL}^{-1}$ of tropomyosin in binding buffer at 37 °C for 2 h. 5 μg of GO was added to the mixture, incubated at 37 °C for 1 h to remove unbound aptamers, followed by centrifugation (12,000 rpm, 5 min) to remove GO. Then the aptamer bound to tropomyosin in the supernatant was quantified using the OliGreen ssDNA reagent according to manufacturer's instructions. The fluorescence intensities were recorded on a LS-45 Fluorescence Spectrometer (Perkin-Elmer, USA) with an excitation at 480 nm and an emission at 522 nm. The experiment was performed on each sample in triplicate, and the fluorescence measurements were protected from light.

Measurement of K_d values

Saturation binding assays for K_d determination were carried out by an enzyme-linked aptamer assay (ELAA). First, tropomyosin (5 $\mu\text{g}\cdot\text{mL}^{-1}$, 100 $\mu\text{L}/\text{well}$) in carbonate buffer (50 mM, pH 9.6) was immobilized in each well of a Maxisorp 96-well microtiter plate (Nunc, NY, USA) at 4 °C overnight. The wells were washed with PBST three times and blocked by 200 μL of 2% (w/v) BSA in PBST at 37 °C for 1 h. After washing with PBST five times, various concentrations of 0, 20, 80, 120, 150, 200 and 240 nM in binding buffer (200 μL) of aptamers were added to the well and incubated at 37 °C for 1 h. After the binding reaction, unbound aptamers were removed by thoroughly washing five times with PBST accompanied by constant shaking at 500 rpm. And biotin-probes (5'-biotin-GCTTCTAGGTCAACGTT-3', 50 nM, 50 $\mu\text{L}/\text{well}$) were used for hybridizing the bound aptamers at 37 °C for 1 h. Then a streptavidin-HRP (0.05 $\mu\text{g}\cdot\text{mL}^{-1}$, 100 μL) solution was added and allowed to react at 37 °C for 1 h after washing five times with PBST. At last, washing five times after the reaction, 200 μL of TMB solution was added and incubated at RT for 30 min. The absorbance at 450 nm was measured after quenching with 50 $\mu\text{L}/\text{well}$ of 2 M H_2SO_4 using a GO Multiskan counter (Thermo-Scientific, USA).

Saturation curves of relative absorbance at 450 nm (background removal) of the bound aptamers versus the concentrations of initial incubated aptamers were obtained using OriginPro 8.0 software by nonlinear regression analysis. The

data were determined by three parallel experiments, and the K_d values were calculated by Scatchard analysis.

Label-free fluorescent aptamer-based GO sensing platform

In order to investigate our selected aptamers as tropomyosin sensing probe, a label-free fluorescent GO sensing platform was established as seen in Fig. 1. The critical amount of GO and ssDNA aptamer was previously determined. The binding assays were performed as previous described. The calibration curve of the aptamer-based detection of tropomyosin was obtained by plotting the fluorescence increment against tropomyosin concentrations. Each sample of the assay was performed three times.

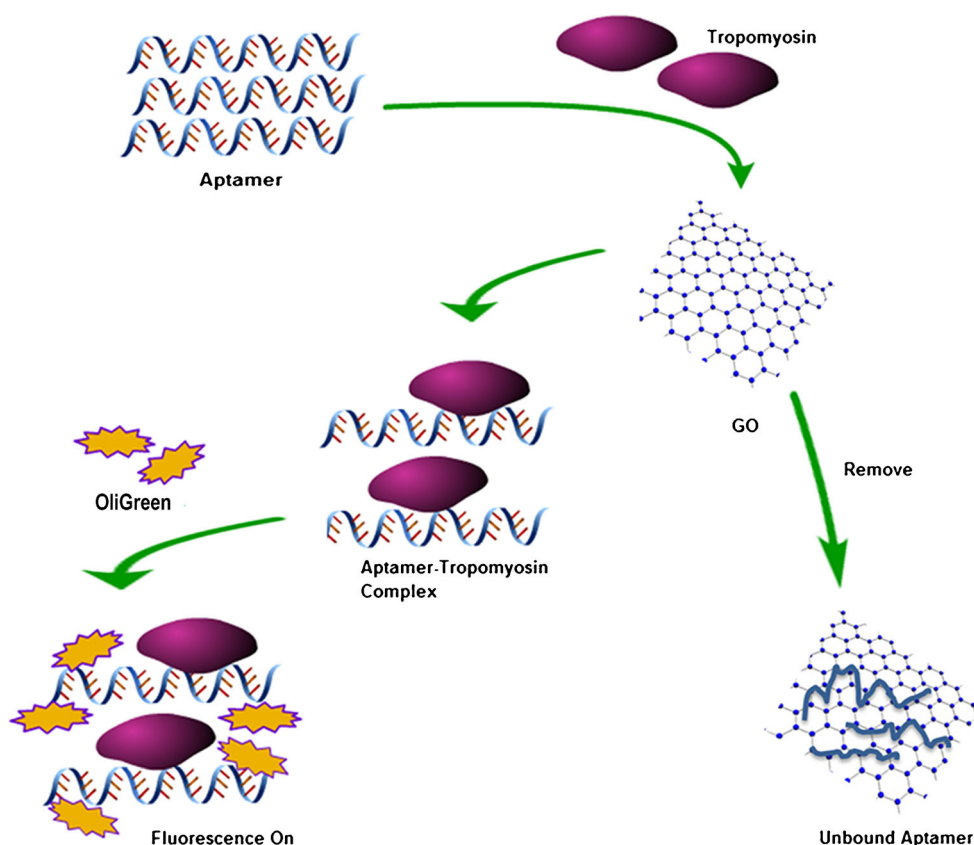
Results and discussion

Selection of aptamers against tropomyosin by SELEX

Aptamers against tropomyosin were obtained in vitro by combinatorial screening of random ssDNA library containing a 40-mer randomized region using the dot-blotting-SELEX, which is a well-known method for the selection of aptamers against proteins [25]. In this selection, tropomyosin together with BSA was spotted on the nitrocellulose membrane. Thus, DNA aptamers that bind to tropomyosin accompanied with the removal of the DNAs that bind to BSA were selected, which may result in the acceleration of the screening progress of aptamers binding to tropomyosin. As shown in Fig. S1 in supplementary materials, the recovery rate of ssDNA bound to tropomyosin increased rapidly as the rounds of SELEX progressed, and then achieved a stable level (approximately 55%) by the sixth round. These results indicate that ssDNA bound to BSA were discarded and ssDNA bound to tropomyosin were successfully enriched.

From the results of sequence analysis of the sixth round of selection ssDNAs that had cloned, 14 sequences were identified (Table S1 in supplementary materials). In order to investigate their utility for tropomyosin detection, 5 aptamers (Highlighted in red in table S1) randomly selected were screened for tropomyosin binding characteristics. Based on phylogenetic analysis, these five aptamers were grouped into two large families, I (A5, A30 and A15) and II (A1 and A32) (Fig. S2 in supplementary materials). As a result of the comparative binding assays in Fig. 2, aptamers in family I showed higher binding affinity than family II and the signals of selected aptamers were stronger than initial DNA library, indicating that aptamers against tropomyosin were successfully selected by SELEX. In addition, aptamer A5 appeared the highest affinity binding to tropomyosin followed by A15. The second

Fig. 1 Schematic of overview of tropomyosin detection via a label-free fluorescent aptamer-based GO sensing platform



structural predictions (Fig. S3 in supplementary materials) revealed that these aptamers mainly contained bulge loops and hairpin structures. Interestingly, A5 and A15 were the similar structures with two embossments unlike the others, which were speculated as the probable binding sites for recognition of the grooves of tropomyosin.

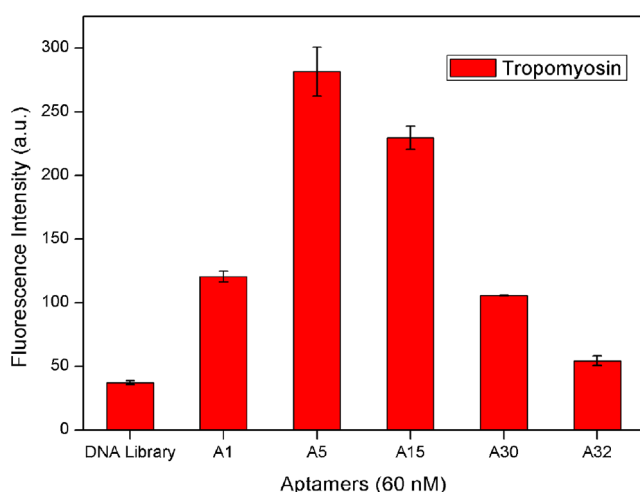


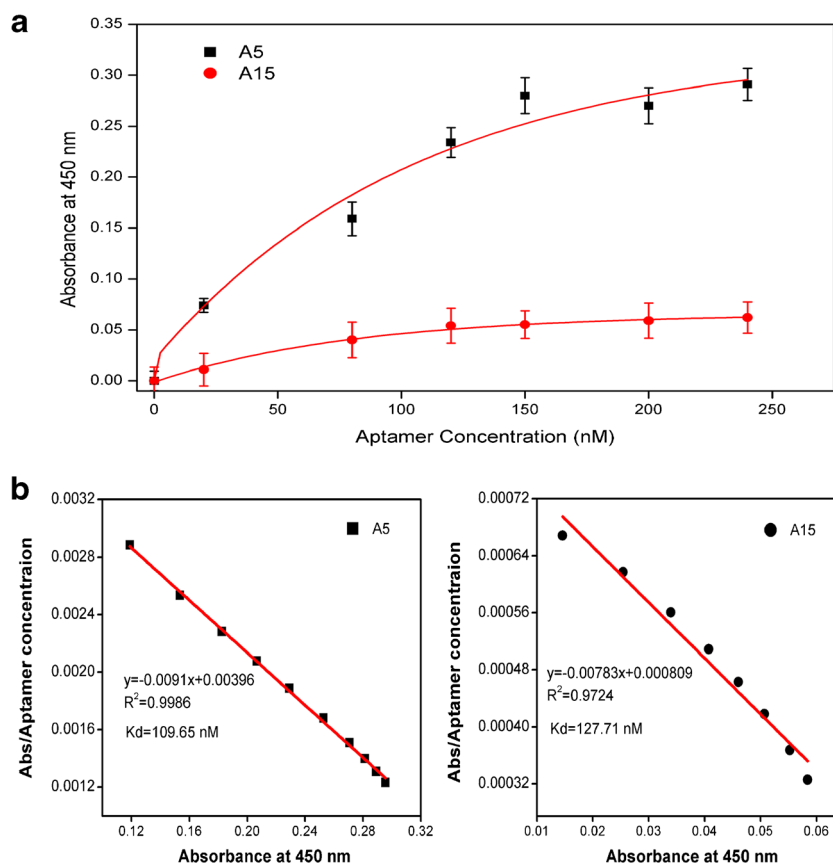
Fig. 2 Binding ability of selected aptamers against tropomyosin. The binding assays with A1, A5, A15, A30 and A32, were carried out by fluorescence quantification of aptamers bound to tropomyosin. DNA library was used as the negative control. Error bars represent standard deviation of the mean, with determination of 3 repetitions

The binding ability of aptamer A5 and A15 to tropomyosin prompts us to measure their K_d values as tropomyosin sensor. ELAA was performed to evaluate the binding affinity of aptamers for tropomyosin. To quantitative determine the aptamers bound to tropomyosin in each well of 96-well microtiter plate, biotin-probes were added. Thus, aptamers without chemical modification freely fold the structures in the ideal state and bind to targets. From the binding curves determined by nonlinear regression analysis (Fig. 3a), the two aptamers displayed relatively high affinity for tropomyosin. The K_d values of aptamers estimated by Scatchard analysis (Fig. 3b) were in the nanomolar range. And the K_d values (109.65 nM) of A5 was lower than that of A15 (127.71 nM), indicating that A5 showed higher binding affinity than A15, which is consistent with the binding assay.

Label-free sensor for tropomyosin detection based on a fluorescent GO platform

In the view of binding affinity of aptamers to tropomyosin, A5 with the highest affinity was tested for its ability as a tropomyosin sensor. GO can naturally adsorb ssDNA via noncovalent π - π stacking interaction while desorb the folded ssDNA induced by hybridization or target proteins and small molecules has been proved [26, 27]. Based on the interaction between ssDNA and GO, a mass of GO-based aptasensors

Fig. 3 Binding affinity of the aptamers (A5 and A15) to tropomyosin. **a** Binding saturation curves of A5 and A15 to tropomyosin. **b** Scatchard plot of A5 and A15 obtained from the binding curves. Binding assays with A5 and A15 were performed using the ELAA method. Error bars were standard deviation of the mean, with determination of 3 repetitions



have been successfully demonstrated for the detection of proteins [28], cells [29] and small molecules [30]. Furthermore, GO has also been applied in SELEX for screening aptamers against many targets including proteins [31] and small molecules [32]. Despite these progresses, GO-based systems with aptamer probe for sensing proteins have been mostly depended on the effective adsorption of ssDNA without

protein interference. In this study, the interaction of GO between aptamer and tropomyosin was previously conducted (Fig. S4 in supplementary materials). It can be found that GO can adsorb tropomyosin via hydrophobic effect and van der Waals force in the absence of aptamer. However, GO cannot adsorb the tropomyosin-aptamer complex, which demonstrated the availability of GO as a vehicle to remove the

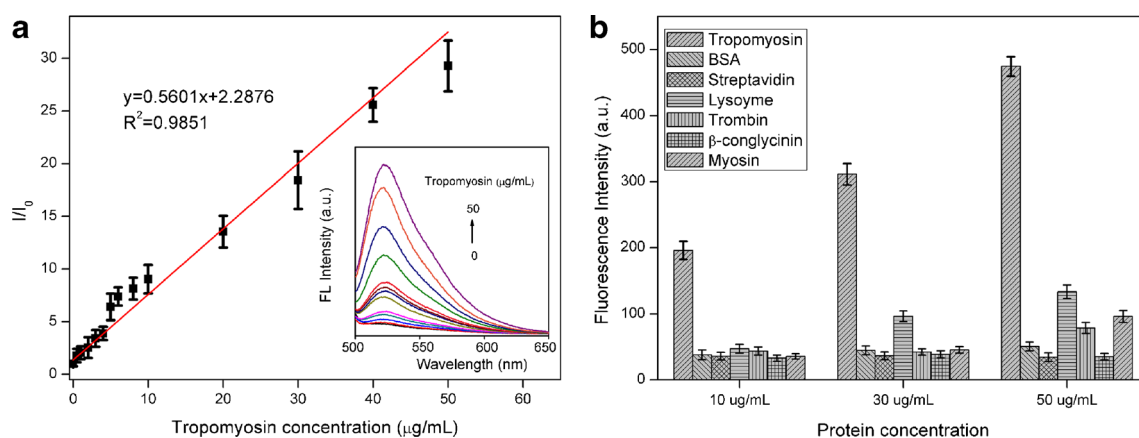


Fig. 4 Tropomyosin quantification by label-free fluorescent aptamer-based GO sensing and selectivity of the aptamer sensor. **a** Plot of relative fluorescence intensity (I/I_0) at 522 nm versus tropomyosin concentration. Inset: Fluorescence emission spectra of aptamer (A5, 80 nM) incubated with different concentrations of tropomyosin in binding buffer (pH 7.2) using the Quant-iTTM OliGreen ssDNA

reagent (a 200-fold dilution of the stock solution). I_0 denotes the fluorescence intensity without tropomyosin. **b** The fluorescence response of aptamer (A5) to BSA, streptavidin, lysozyme, thrombin, β -conglycinin, myosin and tropomyosin. Different concentrations ($10 \mu\text{g/mL}^{-1}$, $30 \mu\text{g/mL}^{-1}$ and $50 \mu\text{g/mL}^{-1}$) were tested. Error bars were standard deviation of the mean, with determination of 3 repetitions

unbounded aptamers in our detection platform. Therefore, a robust label-free fluorescent approach for tropomyosin detection was introduced by utilizing the OliGreen ssDNA reagent to quantitatively detect the aptamers bound to tropomyosin in solution with the aid of the adsorption of unfolded aptamers by GO. As illustrated in Fig. 1, aptamers freely fold the structure and bind to tropomyosin to form aptamer-tropomyosin complex while the unbound aptamer was adsorbed onto the GO surface by π - π stacking. After removal of the GO, aptamers bound to tropomyosin was detected by adding OliGreen ssDNA reagent. Here, the concentration of aptamer A5 (80 nM) was used as sensing probe and the critical amount of GO used to adsorb the total 80 nM ssDNA aptamers calculated was 5 μg (Fig. S5 in supplementary materials). Under the optimal conditions, the fluorescence intensity was rising proportionally with the increase of tropomyosin concentration (Fig. 4a). A calibration curve of fluorescence increment (I/I_0) at 522 nm as a function of tropomyosin concentration was performed over the range of 0–50 $\mu\text{g}\cdot\text{mL}^{-1}$ of tropomyosin in a good linear regression with a correlation coefficient of 0.9851 (Fig. 4a). When applying the signal to noise ratio of 3, the limit of detection for the label-free fluorescent GO sensing assay in buffer was estimated to be 0.15 $\mu\text{g}\cdot\text{mL}^{-1}$ (4.2 nM).

In order to further investigate its utility as a tropomyosin sensor, the selectivity of aptamer A5 to tropomyosin over other proteins was performed in this detection platform (Fig. 4b). The fluorescence response of A5 to BSA, streptavidin, lysozyme, thrombin, β -conglycinin, myosin and tropomyosin at various concentrations (10 $\mu\text{g}\cdot\text{mL}^{-1}$, 30 $\mu\text{g}\cdot\text{mL}^{-1}$ and 50 $\mu\text{g}\cdot\text{mL}^{-1}$) in binding buffer was measured. As it can be seen in Fig. 4b, none of the non-target proteins gave a positive response in the large range of concentrations, pointing to low cross-reactivity towards non-specific proteins and highlighting the high specificity of the aptamer to tropomyosin. It should be noted that lysozyme in high concentration cause higher fluorescence intensity than other non-target proteins due to the strong adsorption of lysozyme on GO [33]. These results obtained highlight the viability of high selectivity and sensitivity aptamer-based sensor for tropomyosin detection in food sample by constructing novel signal amplification strategy, which is of our interest and ongoing in our laboratories.

Conclusions

This study for the first time developed aptamers that recognize tropomyosin with high affinity and specificity through a six-round selection process. The optimal aptamer A5 showed the best affinity ($K_d = 109.65$ nM) and high specificity. Additionally, a robust label-free fluorescent aptamer-based GO sensing platform for tropomyosin detection in binding

buffer was performed that has a 4.2 nM detection limit and work in the 0.5 to 50 $\mu\text{g}\cdot\text{mL}^{-1}$ concentration range. These results demonstrated that a new, simple and selective probe for the sensing of shrimp allergen was established, which is of high demand in food allergen detection and clinical applications.

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Compliance with ethical standards The author(s) declare that they have no competing interest.

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