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RESEARCH ARTICLE

Fast and sensitive graphene oxide-DNAzyme-based biosensor for *Vibrio alginolyticus* detection

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Abstract

DNAzymes have been widely and effectively used for the detection of pathogenic bacteria, which pose a serious public health threat. However, the rapid and cost-effective detection of such bacteria remains a major challenge. In this study, we successfully selected *Vibrio alginolyticus*-specific DNAzymes. The activity of the candidates was assessed via fluorescence intensity and gel electrophoresis. The DNAzyme DT1 had a detection limit of 31 CFU/ml for *V. alginolyticus* and exhibited high specificity. Graphene oxide (GO) was used to develop a DNAzyme-based fluorescent sensor for the detection of *V. alginolyticus*, which significantly improved detection performance and shortened the reaction time as little as 10 s. The proposed method was then validated using crab, shrimp, fish, clam, and oyster samples. This study thus provides a new method for the rapid and sensitive detection of *V. alginolyticus*.

KEYWORDS
DNAzyme, graphene oxide, rapid detection, *Vibrio alginolyticus*

1 | INTRODUCTION

Over the past decades, aquaculture products have become a critical protein source worldwide. However, aquatic animal diseases pose a serious threat to the economy and food safety, as well as human and environmental health (Link et al., 2012). Aquatic environments constitute a breeding ground for a wide variety of microorganisms, including pathogenic bacteria (Bien Fang et al., 2011). These pathogens have been linked to aquatic animal disease and water pollution, leading to severe mortality in aquaculture ponds and causing substantial economic losses to the aquaculture industry (Xie et al., 2005). Further, contaminated aquatic products pose a severe threat to human health (Tauxe et al., 2010). Although current technologies can detect pathogenic bacteria, this method is complicated and time-consuming (Billington et al., 2014; Chen et al., 2010), which highlights the need to develop a rapid system for pathogenic bacteria detection.

Vibrio alginolyticus is a Gram-negative bacterium (Lee et al., 1996). Its optimal growth temperature is 30°C, which is common in sea water, sea products, seabed sediments, salt lakes, and coastal environments (Arunagiri & Sivakumar, 2014; Liu et al., 2001). *V. alginolyticus* is a common pathogen of marine animals and humans (Larsen et al., 1981). Several reports have indicated that this pathogen can severely affect fishes, shrimp, and bees (Yan et al., 2007; Yang et al., 2021; Yuan & Zhu, 2012). Upon infection with *V. alginolyticus*, aquatic animals develop epidermis ulcers and erythema coupled with blood vessel rupture, bleeding, and ultimately death (Gomez-Leon et al., 2005; Peng et al., 2012; Wang & Chen, 2005). *V. alginolyticus* -contaminated sea water and sea products can cause infection in humans (Chitov et al., 2009), which commonly leads to diarrhea and vomiting (Quade & Nsoesie, 2017). However, if the infection is left unattended, it can lead to severe otitis media and sepsis. Therefore, a rapid and efficient detection method is key to diagnosing and preventing infection (Chen et al., 2006). Currently, there are many

detection methods for *V. alginolyticus* including polymerase chain reaction (PCR) (Pinto et al., 2005; Wei et al., 2016; Xu et al., 2017), loop-mediated isothermal amplification (LAMP) (Cai et al., 2010; Siddique et al., 2019), immunochromatographic lateral strip flow test (Wang et al., 2013), enzyme-linked immunosorbent assay (ELISA) (Li et al., 2010), and indirect fluorescence antibody technique (IFAT) (Jin et al., 2007), among others. In traditional detection methods, identification based on morphology, physiology, and biochemistry require the skilled experience of technicians (Tait et al., 2014), and culturing bacteria is time consuming. In the method of molecular biology, extraction of bacterial genome and sequence are required for PCR, which cannot be detected on site (Xu et al., 2017; Zou et al., 2019). In immunological methods, it is sensitive, but the kits need to be preserved at low temperature (Majdinasab et al., 2018). DNAzyme is stable and easy to modify (Ma et al., 2021). It has high specificity and sensitivity with the target in the detection of pathogenic bacteria (Chang et al., 2021).

DNAzyme is a type of artificially synthesized single-stranded DNA with a variety of catalytic activities (Yu et al., 2018). Typically, single-stranded DNA of a certain length and a specific sequence acquires its catalytic function by folding into two-dimensional and three-dimensional structures and can be used in various chemical and biocatalytic reactions (Tong et al., 2019). The activity of DNAzyme includes RNA cleavage, RNA ligation, DNA cleavage, DNA phosphorylation, DNA ligation, and peroxidase activity. These structures can be obtained through the systematic evolution of ligands via exponential enrichment (SELEX) (Silverman, 2009; Sun et al., 2019). The DNAzyme featured in this study is an RNA-cleaving DNAzyme. DNAzyme was first reported in 1994 (Breaker & Joyce, 1994) by Breaker and Joyce, who used the SELEX method to obtain the first DNA sequence that could cleave RNA at a specific site from a large number of random DNA sequences (Huang et al., 2014). DNAzymes are similar to ribozymes, which are naturally occurring enzymes that exist in all life forms. Compared with RNase and protease, DNAzymes have some natural advantages. Because they are composed of DNA, they have excellent stability (Hui et al., 2010; Steele et al., 2003). Moreover, its preparation and storage costs are very low and they are easy to chemically synthesize, modify, and combine with other methods (Huo et al., 2020). These advantages have promoted its development and application in the fields of biosensing, tumor therapy, genetic diagnosis, and bioimaging (Li et al., 2018; Wang et al., 2017a). In recent years, there have been many reports on the application of DNAzyme in pathogen detection. A colorimetric paper sensor of DNAzyme was used to detect *Helicobacter pylori* (LOD: 10^4 CFU/ml) (Ali, Wolfe, et al., 2019). The fluorescence signal was used as the detection index, the RNA-cleaving fluorogenic DNAzyme was used to detect *Vibrio anguillarum* (LOD: 4×10^3 CFU/ml) (Gu et al., 2019), *K lebsiella pneumoniae* (LOD: 10^5 CFU/ml) (Ali et al., 2019). and *Aeromonas hydrophila* (LOD: 36 CFU/ml) (Ma et al., 2020). When the reaction time was extended to 72 h, the DNAzyme can detect 10 CFUs of *Legionella pneumophila* by gel-based method (Rothenbroker et al., 2020).

Nanomaterials combined with DNAzyme in the detection of pathogenic bacteria have been reported (Liu et al., 2018) The physical and chemical properties of nanomaterials can achieve high sensitivity, high efficiency, and low cost of detection. In this study, a graphene oxide (GO)-DNAzyme-based biosensor was established for the detection of *V. alginolyticus*, and a simple biosensor was designed with transparent polystyrene plates.

When the tested bacteria are added, the sensor emits a fluorescent signal that can be observed with a blue gel imager. This method is efficient, simple, convenient, and requires no specialized instruments. Our study thus developed a novel method for screening bacterial DNAzyme and established a theoretical and methodological basis for the development of other related test methods.

2 | MATERIALS AND METHODS

2.1 | Chemicals and bacterial strains

DNA sequences for in vitro selection and streptavidin-coated magnetic particles were purchased from Sangon Biotech (Shanghai, China). *Pseudomonas fluorescens* (PF), *Bacillus cereus* (BC), *Vibrio splendidus* (VS), *Vibrio cholerae* (VC), *Vibrio harveyi* (VH), *Vibrio shilonii* (VS), *Enterococcus faecalis* (EF), *Pseudomonas aeruginosa* (PA), *Aeromonas salmonicida* (AS), and *Vibrio mimicus* (VM) were obtained from the China Center of Industrial Culture Collection. *V. alginolyticus* and *Staphylococcus aureus* (SA) were provided by the Marine Resources Development Institute of Jiangsu. Tryptone and yeast extract powder were purchased from Oxoid. Agarose was purchased from Biowest. Tris (hydroxymethyl)-aminomethane (Tris), Tween-20, and metal salts were acquired from Sinopharm, all of which were of the highest purity available. N-(2-Hydroxyethyl) piperazine-N′-(2-ethane-sulfonic acid) (HEPES), 2-(N-morpholino) ethane sulfonic acid (MES), deoxynucleotide (dNTP) mix, 5× Q5 Reaction Buffer, Q5 High-Fidelity DNA Polymerase, 5×Q5 High GC Enhancer, 6× gel loading dye, and low molecular weight DNA ladder were purchased from New England Biolabs. Trehalose, Pullulan, 40% acryl/bis solution (29:1), and Gel-red (10,000×) were obtained from BBI Co., LTD. SYBR@ Green Realtime PCR Master Mix was obtained from TOYOBO LIFE SCIENCE.

2.2 | *V. alginolyticus* crude extracellular mixture (CEM-VA) preparation

According to reports, the crude extracellular mixture of bacteria can be used as targets to screen highly specific DNAZzyme (Ali, Wolfe, et al., 2019; Fan et al., 2021; Gu et al., 2019; Ma et al., 2020). First, 500 μl of *V. alginolyticus* culture was inoculated into 50 ml of LB medium and cultured at 30°C and 180 rpm for 12 h until the OD_{600} reached 0.8. The cells were then centrifuged at 13,800 *g* at 4°C for 5 min and the supernatant was stored at −20°C for subsequent experiments. The extracellular products of other bacteria

were prepared in the same way. We designed the initial library Lib-N35-pool (5′ Phosp-ATG CGA TTG CTr ACG AAC TAA CGG GGC TTA GTT-N35-GCA ATC GCA TAA CCG TAG TG 3′) using a specific forward primer (F: Biotin-ATG CGA TTG CTA CGA ACT AAC GGG GC) and reverse primer (R: CAC TAC GGT TAT GCG ATT GC) for DNAzyme screening. Before the screening, a 10% denaturing polyacrylamide gel (dPAGE) was used to purify the DNA library. The magnetic bead-SELEX method was used to screen the DNAzyme. To achieve this, 500 μl of Buffer I treated nucleic acid was combined with magnetic beads at room temperature for 30 min, after which the magnetic beads were washed with 500 μl 0.2 mM/L NaOH to remove free DNA. To form a substrate-DNA complex, 150 μl of *V. alginolyticus* crude extracellular mixture (CEM-VA) was incubated with the magnetic beads-DNA for 60 min at room temperature. After magnetic separation, the supernatant was recovered by alcohol precipitation. During the selection, *B. cereus*, *V. splendidus*, *S. aureus*, *P. aeruginosa*, and *A. salmonicida* were used as reverse selecting bacteria. The PCR method was used to amplify the DNA sequence, after which the PCR product was detected with a 2% agarose gel. Finally, the PCR products were recovered by alcohol precipitation and used as the library for the next round of screening. The PCR reaction system consisted of 1.5 μl of template DNA, 1 μl of dNTP, 2.5 μl of forward primer, 2.5 μl of reverse primer, 10 μl of 5× Q5 Reaction Buffer, 0.5 μl of Q5H high-fidelity DNA polymerase, 10 μl of 5× Q5 High GC Enhancer, and 22 μl of ddH_2O. The PCR cycle conditions were the following: 98°C preheating for 30 s, followed by 25 cycles of denaturation at 98°C for 10 s, annealing at 57.5°C for 30 s, and extension at 72°C for 30 s, and finally an extension step at 72°C for 2 min.

2.3 | Activity assay

The ninth round of PCR products was selected for high-throughput sequencing, and candidate sequences were synthesized in Shanghai. The substrate labeled with carboxyfluorescein (FAM) at the 5′ end and DNAzyme labeled with a quencher (BHQ1) at the 3′ end was complexed in Buffer B (100 mM HEPES, 300 mM NaCl, 30 mM $MgCl_2$ 0.02% Tween 20, pH 7.5) and annealed at 95°C for 5 min, then placed at room temperature to cool naturally and form a DNAzyme complex. The fluorescence method was used to detect the activity of candidate sequences in 96-well plates. Briefly, 41 μl ddH2O, 45 μl 2× Selection Buffer (2× SB), 4 μl DNAzyme complex, and 10 μl CEM-VA were added to each well. The fluorescence value was then detected within 2.5 h every 30 s in the microplate reader (excitation wavelength: 458 nm; emission wavelength: 535 nm). The control group used LB medium instead of CEM-VA. The Origin 8.5 software was then used to compare the activity of the candidate sequences.

2.4 | Optimization of detection conditions

DNAzyme activity is affected by many factors. Therefore, some parameters were optimized to improve the cleavage activity.

(a) Bacteria concentration. Bacterial culture media (i.e., CEM-VA) with different OD_{600} values ranging from 0.3 to 0.9 were evaluated to determine the optimal concentration of *V. alginolyticus*. The results were expressed in terms of fluorescence intensity. (b) pH. We also evaluated 2× SB with a pH between 4.0 and 8.5 using 100 mM MES solution (pH 4.0, 4.5, 5.0, 5.5, 6.0, and 6.5) and 100 mM HEPES solution (pH 7.0, 7.5, 8.0, and 8.5). DNAzyme reacts with CEM-VA after forming a complex in the 2× SB with different pH values, and the fluorescence intensity is detected by a microplate reader. (c) Ion species. The metal ions in the buffer were also optimized. First, Mg^{2+} was fixed and different monovalent ions were added (Na^+, K^+, $NH4^+$, Li^+, and Rb^+) to optimize the 2× SB. The concentration of monovalent ions is 300 mM. Then, Na^+ was fixed and different divalent ions were added (Mg^{2+}, Zn^{2+}, Co^{2+}, Ba^{2+}, Mn^{2+}, Ca^{2+}, Gu^{2+}, Ni^{2+}, Si^{2+}, Hg^{2+}, and Fe^{2+}). The concentration of divalent ions is 30 mM. The fluorescence method was used to detect the DNAzyme activity in different buffers. All experiments (except for the pH optimization assays) were conducted at pH 8.5.

2.5 | Specificity and sensitivity of DNAzyme

To determine the selectivity of DT1, we selected different bacterial cultures (*PF*, *BC VS*, *VC*, *VH*, *VM*, *VS*, *EF*, *SA*, *PA*, and *AS*). After centrifugation, the supernatant was used as a crude extracellular mixture. Under the optimal reaction conditions, the cleavage activity of DT1 was detected. The results were detected using fluorescence intensity and 15% dPAGE analyses.

The *V. alginolyticus* (OD600 = 0.8) culture solutions were first diluted (dilution factors: 10^1, 10^2, 10^3, 10^4, 10^5, 10^6, and 10^7) prior to the sensitivity experiment. The diluted culture solutions were then centrifuged at 13,800 *g* for 5 min, and the supernatant was used as CEM-VA to react with DNAzyme. Finally, the activity of the DNAzyme was analysed by fluorescence intensity and 15% dPAGE.

2.6 | Target determination of DNAzyme

To determine the target of the DNAzyme, the CEM-VA was pretreated with proteinase K. The CEM-VA was then filtered based on molecular weights (10, 30, 50, and 100 kDa) and then allowed to react with DT1 under optimal conditions. The results were detected via the kinetic method and gel electrophoresis.

2.7 | DNAzyme-based biosensor for *V. alginolyticus* detection

We designed a DNAzyme-based visual biosensor using the lid of a 96-well plate, which can be used to detect *V. alginolyticus*. The influence of GO, trehalose, pullulan, and dextran (MW: 20, 40, and

70 kDa) on the background fluorescence of the sensor was then evaluated to determine which of these compounds minimized the background fluorescence more effectively. GO was ultimately selected for the production of the sensor. First, different concentrations of GO (33, 40, 50, 66, 100, and 200 μg/ml) were added into 23 μl of 2× SB and 2 μl of DNAzyme mixture. The solution was then fixed on the lid of the 96-well plate to detect the intensity of the fluorescence generated when CEM-VA was added. The same procedure was repeated using different concentrations of DNAzyme (1, 2, 3, 5, 7, 10, 15, and 20 μM) to determine the optimal concentration of DNAzyme. Finally, the sensor was produced by evenly mixing 20 μl of 0.5 g/L GO, 23 μl of 2× SB, and 2.4 μl of 5 μM DNAzyme. The mixture was then added dropwise to the 96-well plate lid and placed in an oven at 60°C for drying. During detection, 20 μl of the sample (CEM-VA) was added to the sensor and the results were visualized on a blue gel imager.

The reaction time of the sensor was also optimized. The color shift of the sensor was monitored at different times (0 s, 10 s, 30 s, 1 min, 2 min, 3 min, and 5 min) after adding 20 μl of CM-VA. We also tested the storage temperature of the sensor. The sensor was first stored at 40, 50, 60, 70, and 80°C for 1 h, after which 20 μl of CEM-VA was added to observe the color change on the blue gel imager. The fully reacted solution was then transferred to a black 96-well plate to detect the fluorescence intensity, and the results were compared with those of the sensor without GO. LB was used as a blank control.

2.8 | DNAzyme-based detection

First, five types of fresh and alive aquatic animals (crab: *Portunus trituberculatus*, shrimp: *Litopenaeus vannamei*, fish: *Epinephelus* sp., clam: *Meretrix meretrix*, and oyster: *Ostrea gigas* were bought from aqua-market.) were fed for 24 h, and samples were taken to confirm that there was no *V. alginolyticus* infection by real-time PCR. Then, all the samples were cultured at 20°C for 12 h in sea water contaminated with *V. alginolyticus* (2.1×10^5 CFU/ml), after which tissue samples were collected (>10 g). The tissue samples were then ground and centrifuged at 4800 x *g* for 5 min, filtered with a 0.45 μm disposable filter, and 30 μl of the supernatant was used as CEM for DNAzyme detection.

Afterward, fish were placed in 2 L of sea water and infected with *V. alginolyticus* at a final concentration of 2.1×10^1–2.1×10^5 CFU/ml. CEM was prepared according to the above method, and TIANamp Genomic DNA Kit (Tiangen Biotech) was used to extract DNA from the sample. The *gyr*B gene of *V. alginolyticus* was detected via SYBR Green I real-time PCR (Zhou et al., 2010). The PCR program consisted of a template DNA predenaturation step at 95°C for 60 s, followed by 40 cycles of amplification (denaturation at 95°C for 15 s, annealing at 60°C for 15 s, and extension at 72°C for 45 s). The amplified products were further analysed via agarose gel electrophoresis.

3 | RESULTS AND DISCUSSION

3.1 | In vitro screening, sequencing, and analysis of DNAzyme

In this study, we used the magnetic bead-SELEX method to screen DNAzymes (Figure 1a). We used an initial library containing 35 random bases and an adenine ribonucleoside (rA) cleavage site. In this study, the extracellular product of *V. alginolyticus* (CEM-VA) was selected as the screening target and we assumed that it could form a DNAzyme with special recognition and cleavage functions when coupled with metal ions. In each round of screening, PCR was used to amplify the obtained sequence. After 9 cycles of screening, anti-screening targets were added in rounds 3, 5, and 7 to improve the specificity of the DNAzyme. The PCR products were sequenced by Sangon Biotech (Shanghai, China) and a total of 159,885 sequences were obtained (Table S1). One candidate DNAzyme sequence was then selected and synthesized, as shown in Table 1. This sequence was named "DT1" and its secondary structure was predicted as shown in Figure 1b. FAM tag substrate and DNAzymes were used to form complexes, and the fluorescence intensity was detected once they were mixed with CEM-VA. The results are shown in Figure 1c.

3.2 | Optimization of reaction conditions

To achieve higher reactivity, the concentration of *V. alginolyticus* (OD_{600}), pH, and ion species of the buffer were optimized. First, we evaluated the fluorescence intensity at different concentrations of *V. alginolyticus* (Figure 2a). The buffer was the initial 2× SB (100 mM HEPES, 300 mM NaCl, 30 mM $MaCl_2$, and pH 7.0). Add 20 μl of CEM-VA with different OD_{600} (0.3, 0.4, 0.5, 0.6, 0.7, 0.8, and 0.9) for experiment, and the intensity of fluorescence increased gradually, and it was stabilize when the OD_{600} was 0.8. Therefore, the CEM of OD 0.8 was used to conduct follow-up experiments. Similarly, different buffer pH conditions (4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, and 8.5) were also evaluated for optimization. As shown in Figure 2b, optimal DNAzyme cleavage activity was achieved when the pH was 8.5, and therefore 2× SB with a pH of 8.5 was selected for subsequent experiments. The ions in the buffer are crucial modulators of DNAzyme activity. As shown in Figure 2c, DNAzyme reactivity was optimal when Mg^{2+} and Na^+ were present. Based on the results of our experiment, when the bacterial concentration OD_{600} was 0.8, and the pH of 2× SB (100 mM HEPES, 300 mM NaCl, and 30 mM $MaCl_2$) was 8.5, the activity of D1 was the highest.

3.3 | Specificity and sensitivity analysis

Prior to conducting the specificity and sensitivity experiments, we first prepared the extracellular products of eleven kinds of

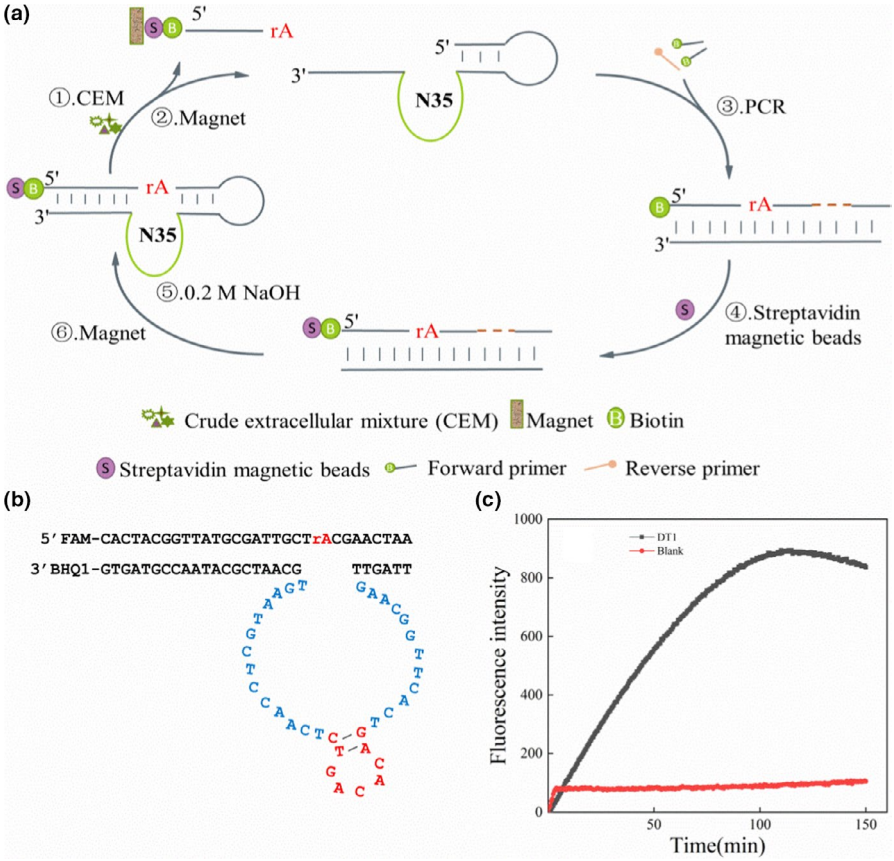

FIGURE 1 (a) CEM-VA-specific DNAzyme selection. (b) Prediction of the secondary structure of DT1. (c) Fluorescence assay to determine the activity of DT1

TABLE 1 DT1 sequence

Name	Sequence 5′–3′
>Seq1	GAACGGTTCACTGACACAGTCTCAACCTCGTAAGT
FAM-substrate	CACTACGGTTATGCGATTGCT/rA/CGAACTAACGGGGC
DT1-BHQ1	GCCCCGTTAGTTGAACGGTTCACTGACACAGTCTCAACCTCGTAAGTGCAATCGCATAACCGTAGTG

bacteria for specificity analysis. Additionally, the *V. alginolyticus* (OD_{600} = 0.8) culture solution was serially diluted for sensitivity analysis, and 2× SB was used as a control. The results were detected via fluorescence intensity, gel electrophoresis, and sensors (Figure 3a,b). The fluorescence intensity of DT1 increases significantly when the CEM-VA was added. But, the CEM of *V. splendidus*, *V. cholerae*, and *V. harveyi* could not react with DT1. And the CEM of *V. shilonii*, *V. mimicus*, *B. cereus*, and *A. salmonicida* showed a slight increase in fluorescence intensity. However, it is only 20% compared with *V. alginolyticus a* after 3 h reaction. Combined with the results of the electrophoresis and sensor in Figure 3b, DT1 was highly specific to *V. alginolyticus*.

The results of the DNAzyme sensitivity test are shown in Figure 3c. An increase in fluorescence intensity was detected when the concentration of *V. alginolyticus* reached 10^5 CFU/ml, which was consistent with the sensor results. When the concentration of *V. alginolyticus* was between 0 and 10^4 CFU/ml, the fluorescence intensity and the concentration of *V. alginolyticus* showed a good linear relationship. According to the formula 3σ/slope, where σ is the blank standard deviation and slope comes from the linear equation, the detection limits of DT1 in this study were as low as 31 CFU/ml. Therefore, the DNAzyme developed herein can be used to sensitively detect *V. alginolyticus*.

3.4 | Target determination of DNAzyme

The CEM-VA was pre-treated with proteinase K to elucidate its target, and the results are shown in Figure 4a. The fluorescence intensity of CEM-VA after treatment did not increase significantly, and there was no reaction of DNAzyme showed in the gel electrophoresis. Therefore, we speculated that the target of DT1 may be a protein. The results suggested that the extracellular protein of *V. alginolyticus* could be combined with the loop of DNA, and with the assistance of metal ions. It could induce conformational changes of DNAzyme, causing DNAzyme sequence to cleaved at

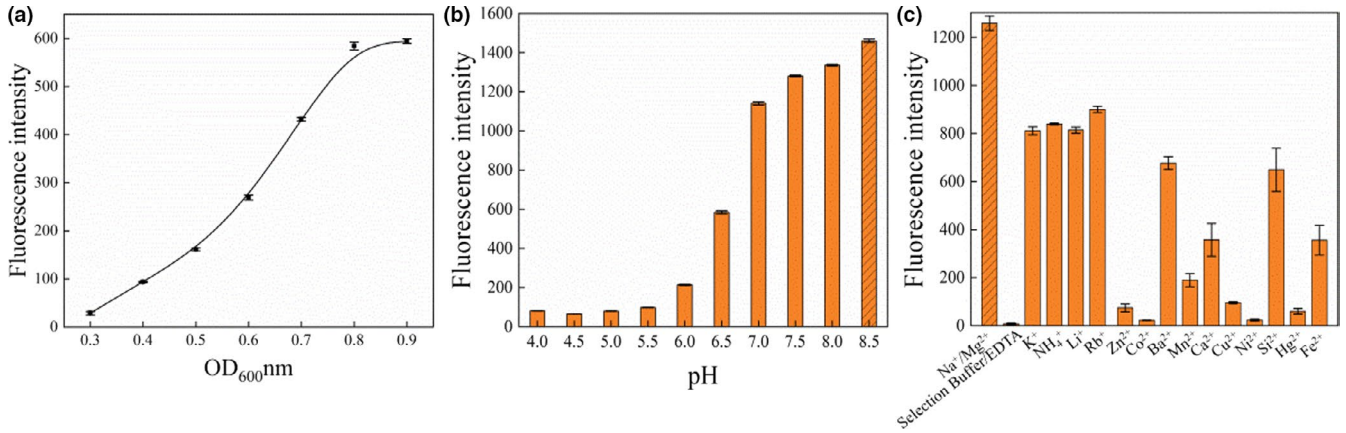

FIGURE 2 Optimization of experimental conditions. (a) Effect of different concentrations of CEM-VA on the cleavage activity of DT1 (All DNAzyme complexes were formed in 2× SB at pH 7.5). (b) Buffer pH optimization. (c) Buffer metal ion optimization

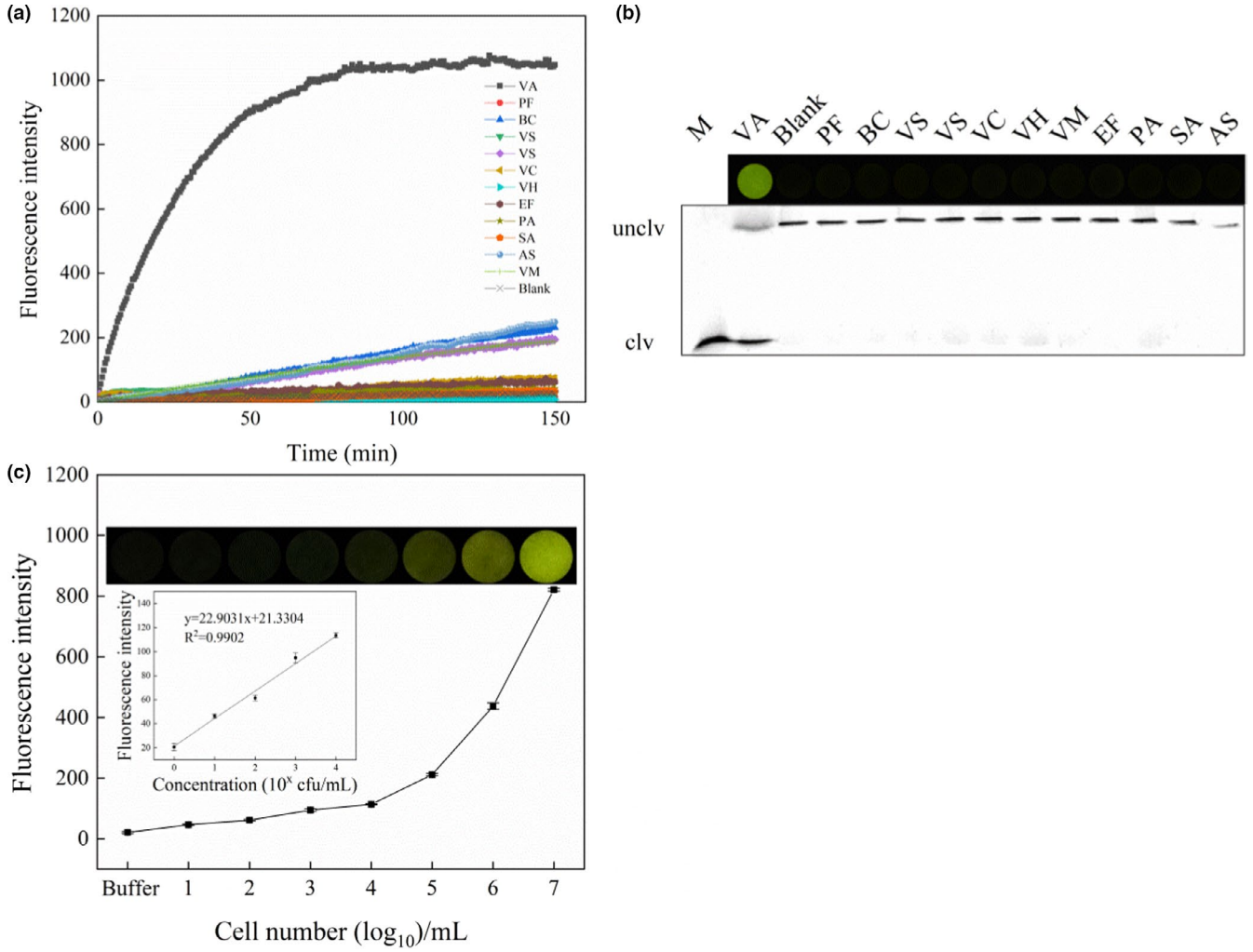

FIGURE 3 (a) DT1 specificity test. Reaction buffer (blank) and *VA*, *PF*, *BC*, *VS*, *VS*, *VC*, *VH*, *VM*, *EF*, *SA*, *PA*, and *AS*. (b) 15% dPAGE was used to evaluate the cleavage activity of DT1 under different CEMs. Unclv: 103 bp; clv: 22 bp. Inset: Biosensor-based specific detection. (c) Sensitivity test of DT1. Inset: linear relationship between fluorescence intensity and *V. alginolyticus* concentration; fluorescence images of the sensor with different bacterial concentrations

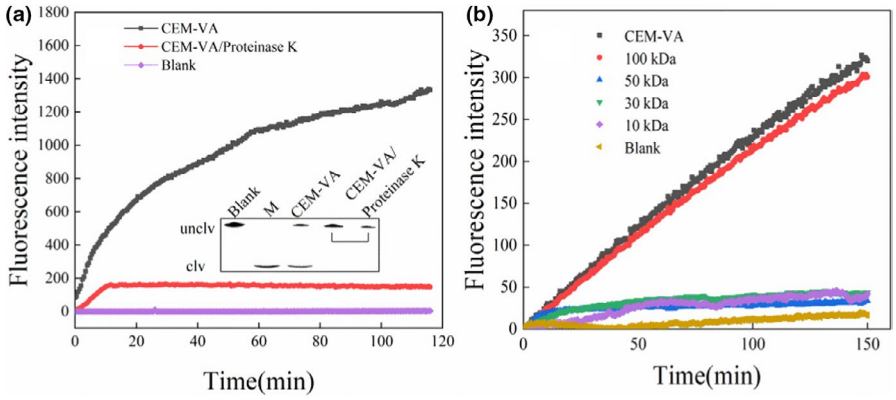

FIGURE 4 (a) Fluorescence signal of DT1 in response to proteinase K-treated CEM-VA. Inset: 15% dPAGE gel image. (b) Molecular weight prediction of the target protein

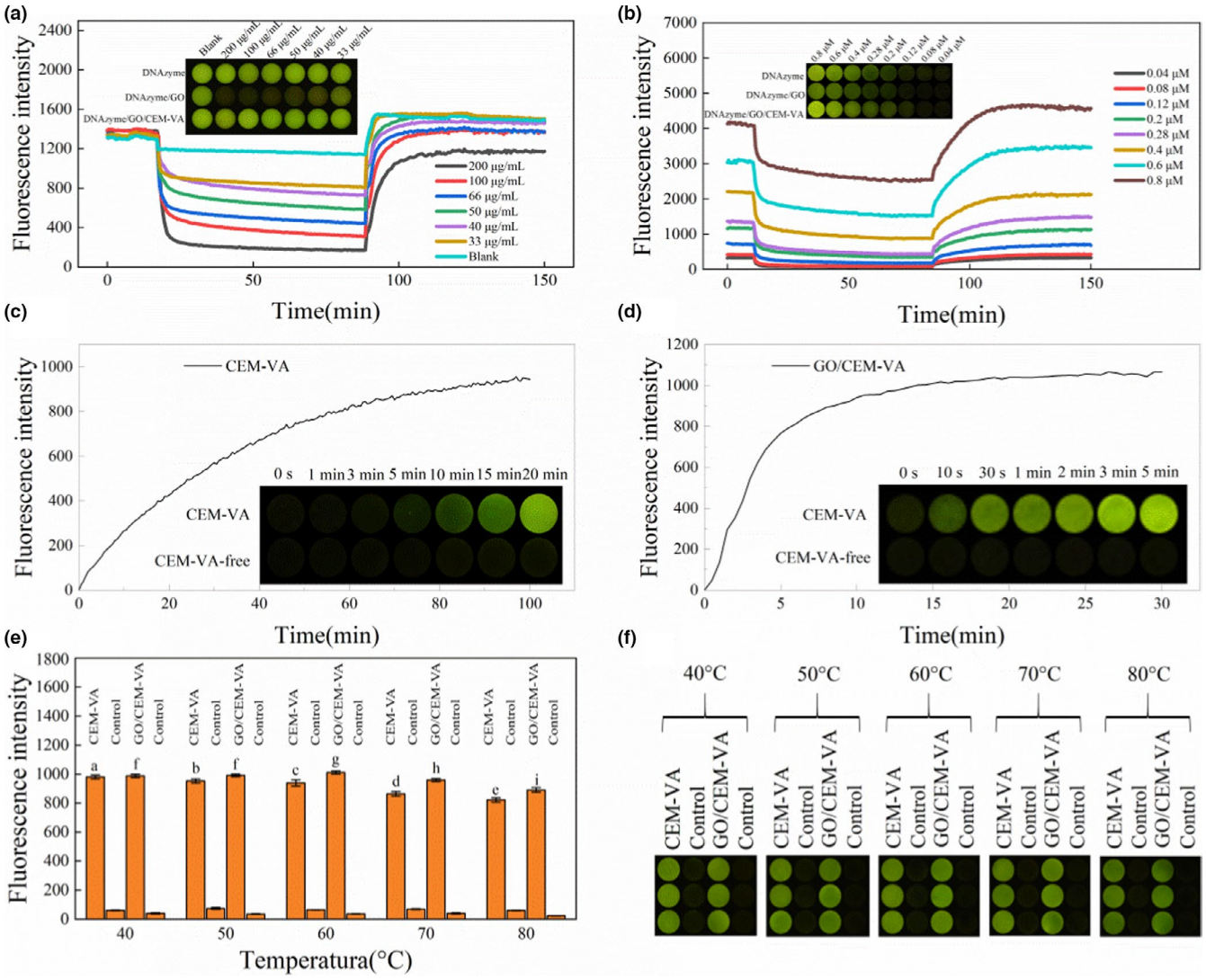

FIGURE 5 Sensor optimization. (a) GO concentration optimization. (b) DT1 concentration optimization. (c) Response time of GO-DNAzyme sensor. (d) Response time of DNAzyme sensor. (e) Fluorescence intensity of the sensor at different temperatures [a–i were considered to be statistically significant ($p < .05$), and the same letters were considered to be not significant ($p > .05$)]. (f) Photograph of the color reaction of the sensor

a specific site (Gu et al., 2019; Lee et al., 2012). Figure 4b shows that the fluorescence intensity of CEM-VA and 100 kD CEM were significantly increased, which is consistent with the gel electrophoresis results, whereas 10, 30, and 50 CEM exhibited no cutting bands. The molecular weight of the target protein was greater than 100 kDa.

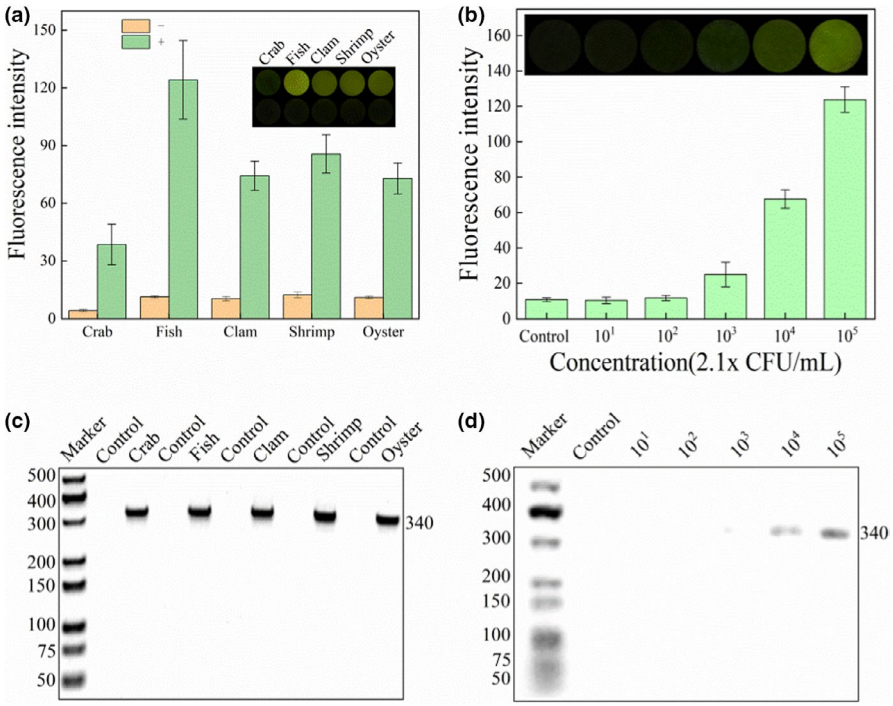

FIGURE 6 (a) Fluorescence detection of *V. alginolyticus* in crab, fish, clam, shrimp, and oyster samples. (b) Fluorescence detection of *V. alginolyticus* in fish samples at an infected concentration of 0–2.1 × 10^5 CFU/ml. Inset: Fluorescence signal of the sensor in response to the sample. (c) Agarose gel electrophoresis results for the real-time PCR detection of *V. alginolyticus* in seafood. (d) Agarose gel electrophoresis results for the real-time PCR detection of *V. alginolyticus* in fish. M: 500 bp DNA marker; Control: negative control

TABLE 2 Detection of *V. alginolyticus* in infected seafood samples

Seafood sample	Bacterial concentration (CFU/ml)	DNAzyme	Real-time PCR (Ct)
Negative control	0	–	25.62 ± 0.25
Positive control	2.1 × 10^5	+	13.70 ± 0.25
Crab	2.1 × 10^5	+	13.89 ± 0.06
Fish	2.1 × 10^5	+	12.89 ± 0.15
Shrimp	2.1 × 10^5	+	13.15 ± 0.03
Clam	2.1 × 10^5	+	13.83 ± 0.05
Oyster	2.1 × 10^5	+	13.84 ± 0.07

TABLE 3 Detection of *V. alginolyticus* in infected fish samples

Seafood sample	Bacterial concentration (CFU/ml)	DNAzyme	Real-time PCR (Ct)
Fish	Negative control	–	25.62 ± 0.25^a
	2.1 × 10^1	–	20.84 ± 0.06^b
	2.1 × 10^2	–	20.61 ± 0.34^b
	2.1 × 10^3	+	17.63 ± 0.26^c
	2.1 × 10^4	+	15.77 ± 0.13^d
	2.1 × 10^5	+	13.83 ± 0.05^e

Note: [a–e were considered to be statistically significant ($p < .05$), and the same letters were considered to be not significant ($p > .05$)]

3.5 | DNAzyme-based biosensor for *V. alginolyticus* detection

In this study, we used GO to immobilize DNAzyme and reduce the interference of background fluorescence signals. The GO and DNAzyme concentrations and the reaction time of the sensor were then optimized. The results are shown in Figure 5. When the concentration of GO was 100 μg/ml, the background fluorescence intensity contrasted sharply with the detected fluorescence intensity, as shown in Figure 5a. When the concentration of DNAzyme in the sensor was 0.28 μM and CEM-VA was present, a clear fluorescence signal could be observed after 10 s, as shown in Figure 5b–d. Compared with the sensor without GO, the response time was shortened from 5 min to 10 s, which significantly improved the detection speed. The effect of storage temperature on sensor performance was also tested, and the results are shown in Figure 5e,f. The sensors were stored at 40, 50, 60, 70, and 80 for 1 h. Our results indicated that there was almost no difference in the fluorescence intensity at 0, 50, and 60°C. The fluorescence intensity decreased slightly at storage temperatures of 70°C and 80°C but remained sufficiently high to accurately detect its target.

3.6 | DNAzyme for physical sample detection

We next sought to validate the applicability of the newly developed GO-DNAzyme by testing seafood samples (crab, fish, shrimp, clams, and oysters), after which the results were compared to those of real-time PCR detection. The results are shown in Figure 6a. Fish samples have the highest fluorescence intensity, and the sensor was the brightness, which might be caused by bacteria adsorbed on the surface of the fishes. The crab samples had the lowest fluorescence intensity and sensor brightness; the crab might not sensitive

to *V. alginolyticus*, or the crab shell performed a function of protecting bacterial infection. The sensor exhibited a strong fluorescence signal in response to the seafood samples. The sensor still emitted a clear fluorescence signal even when the concentration of *V. alginolyticus* decreased to 2.1×10^3 CFU/ml (Figure 6b). This demonstrates that the GO sensor could effectively detect *V. alginolyticus* in seafood. Additionally, the sensor results were consistent with the real-time PCR detection results, as shown in Tables 2 and 3 and Figure 6c,d. The qPCR products of the samples (crab, fish, shrimp, clams, and oysters) showed obvious target bands in the agarose gel detection (Figure 6c). When the concentration of *V. alginolyticus* reached 2.1×10^3 CFU/ml, the fish sample showed a band at 340 bp (Figure 6d).

We used GO-DNAzyme biosensor to detect *V. alginolyticus*. Peng Zuo et al. utilized an aptamer-functionalized GO nanobiosensor to detect *Lactobacillus acidophilus* with a detection limit of 11.0 CFU/ml. They also successfully extended this approach to the simultaneous detection of two infectious pathogens—*S. aureus* and *Salmonella enterica* (Zuo et al., 2013). Ziping Liu et al. designed single-stranded DNA that was complementary to *H. pylori* DNA. A method was developed for the detection of *H. pylori* in milk based on the fact that the dsDNA-QDs/GO system exhibited stronger fluorescence emission than the ssDNA-QDs/GO system. Its detection limit was 0.46 pmol/L (Liu & Su, 2017). The detection limit for *S. aureus* by Pang et al. (2013) was 0.00625 nmol/L. and the detection limit for *Vibrio parahaemolyticus* by Duan et al. (2017) was 14 CFU/ml. DNAzyme with a fluorescent group (FAM) and GO are non-covalently adsorbed to GO via π–π stacking to form a GO-DNAzyme complex (Gu et al., 2019; Liu et al., 2018; Lv et al., 2021). The proximity of the fluorophore to the GO causes the fluorescence to be quenched and the initial fluorescence intensity decreases, which is beneficial to improve the sensitivity of the sensor (Liu et al., 2014). When the target is present, the conformation of the DNAzyme changes and the DNAzyme cleaves into a long sequence of 81 bases and a short fluorescent sequence of 22 bases, and the fluorescence is released. Compared with common pathogen detection methods such as PCR, RT-PCR, ELISA, the sensitivity was significantly improved (Li et al., 2010; Xu et al., 2017; Zhou et al., 2010). The GO-DNAzyme sensor had a lower detection limit, which was reached 31 CFU/ml, while the detection limit of ELISA is 10^4–10^5 CFU/ml (Yan et al., 2001; Zhao et al., 2005). Furthermore, the detection time of the fluorescent biosensor was shorter significantly. The GO sensor could observe signals within 10 s, and the fluorescence intensity will be stable after 10 min. In contrast, the detection time of the PCR method and electrochemical method is 0.5–2 h (Liu, Hu, et al., 2014; He et al., 2019), or even longer (Pinto et al., 2012). In addition, the nano-biosensor method is usually combined with the electrochemical method, often requiring expensive instruments and unable to achieve detection in site (Nakama et al., 2021; Wang et al., 2017). The GO-DNAzyme biosensor could be carried and directly detect *V. alginolyticus* in site, without bacterial culture and laboratory processing.

4 | CONCLUSIONS

Here, we developed a DNAzyme via in vitro selection that was effective for the detection of *V. alginolyticus*. Further, a sensitive and selective biosensor based on DNAzyme was also developed for *V. alginolyticus* detection. The CEM-VA could efficiently bind to the DNAzyme, thus causing a DNAzyme structural change that resulted in a fluorescence signal. The target of DNAzyme was extracellular protein, and the detection of limit was 31 CFU/ml. Based on this principle, a *V. alginolyticus* -specific biosensor was constructed using GO. GO could adsorb DNAzyme and reduce the initial fluorescence intensity, meanwhile, the sensitivity of the sensor was increased significantly. The results of the sensitivity test showed that the sensor could detect a minimum of 2.1×10^3 CFU/ml of *V. alginolyticus*. The sensor exhibited excellent sensitivity and selectivity for *V. alginolyticus* and rendered detection results in as little as 10 s. The sensor realizes rapid detection of pathogenic bacteria without bacterial culture and quickly diagnoses the infection of *V. alginolyticus*. The method is of reference significance for the detection of different pathogens and has great prospects in the field of aquaculture, food safety, and environmental monitoring.

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CONFLICT OF INTEREST

The authors confirm that there is no conflict of interests regarding this paper.

DATA AVAILABILITY STATEMENT

The interaction data used to support the findings of this study are included within the article and the Supporting Information file. Also, all the data used to support the findings of this study are available from the corresponding author upon request.

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