

Detection of *Vibrio parahaemolyticus* based on fluorescence aptamers and graphene oxide fluorescence resonance energy transfer

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Abstract

Vibrio parahaemolyticus related food poisoning has been increasing recently. These bacteria in seawater and aquatic products can cause gastroenteritis during consumption. The outbreaks of *V. parahaemolyticus*, especially in coastal regions, need a rapid and sensitive detection method. There are several limitations to the traditional methods of identifying harmful microorganisms. Some limitations are long growing seasons, risk of cross-contamination, reliance on complicated apparatus, and expensive reagents. To address these difficulties. A rapid, accurate, and simple method for identifying *V. parahaemolyticus* becomes increasingly essential. This study aimed to develop a novel aptamer-based sensor that is rapid, susceptible, compact, and specific. One approach uses fluorescence resonance energy transfer (FRET) and graphene oxide. In this method, *V. parahaemolyticus* causes the aptamer to detach from the graphene oxide surface, which leads to fluorescence recovery and the ability to calculate bacterial concentration using fluorescence intensities. However, this method only detects large quantities of *V. parahaemolyticus* and is unresponsive to low amounts. Despite these drawbacks, the approach showed great specificity for *Vibrio parahaemolyticus* compared to other foodborne harmful microorganisms. It can identify *Vibrio parahaemolyticus* at quantities of 10⁵ CFU/mL or more, indicating potential for assuring food safety, although additional development is required. This study opened the door to developing fast, sensitive, and inexpensive technologies for identifying foodborne infections.

Keywords: Foodborne; *Vibrio parahaemolyticus*; graphene oxide; carboxyfluorescein; aptamer;

36

37 **1. Introduction**

38 Foodborne diseases present a substantial risk to human well-being, with pathogenic
39 bacteria being the main factors accountable for these infections. The large number of
40 these microorganisms in dietary sources has a significant influence on public health.
41 *Vibrio parahaemolyticus*, also referred to as *V. parahaemolyticus*, is a notable gram-
42 negative bacterium recognized for its harmful characteristics. It is frequently found in
43 seawater and aquatic products, specifically bivalve molluscan shellfish. Consuming
44 food infected with *V. parahaemolyticus* can cause the start of acute gastroenteritis,
45 which is defined by symptoms including nausea, diarrhea, stomach cramps, vomiting,
46 headache, and even death in severe cases (Jacxsens et al., 2009; Newton et al., 2012).
47 In recent times, there has been a notable increase in the occurrence of outbreaks caused
48 by *V. parahaemolyticus* worldwide, particularly in coastal regions (Dileep et al., 2003;
49 Letchumanan et al., 2014). Therefore, it is of utmost significance to develop a fast and
50 highly responsive detection technique for *V. parahaemolyticus* to ensure food safety.

51

52 Many techniques have been created to identify harmful microorganisms in food. These
53 include traditional methods like colony culture and counting (Kim et al., 2018),
54 molecular approaches using polymerase chain reaction (Ferrario et al., 2017), and
55 immunological methods like enzyme-linked immunosorbent assay (Karoonthaisiri et
56 al., 2009). However, these widely used methods have drawbacks such as lengthy
57 cultivation time, risk of cross-contamination, dependence on complex instruments and
58 expensive reagents, and the requirement for skilled professionals (Abbaspour et al.,
59 2015; Jin et al., 2017; Wang et al., 2020). As a result, there is an ongoing demand for
60 new methods that provide better speed, affordability precision or simplicity. To meet
61 the present requirements for detection, numerous new methods have been developed,
62 such as surface-enhanced raman scattering, electrochemical detection, colorimetric
63 assay, and flow cytometry technology (Han et al., 2019; P. Liu et al., 2020; R. Liu et
64 al., 2021; Shakeran et al., 2021). In particular, fluorescence detection techniques have

garnered attention from researchers due to their simplicity of operation, rapid response, and high sensitivity.

Fluorescence-based assays have gained significant popularity in detecting foodborne hazards because of their simplicity in preparation, quick reaction time, exceptional sensitivity, and precise specificity (Wu et al., 2020; Yuan et al., 2018). One of the key spectroscopic phenomena utilized in such assays is fluorescence resonance energy transfer (FRET). FRET involves a non-radiative energy transfer process between a "donor" molecule (D), acting as a fluorophore, and an adjacent "acceptor" molecule (A) (Clegg, 1992). To achieve effective FRET, the acceptor molecule must possess absorption characteristics that align with the emission wavelength of the donor molecule, and the distance between the donor and acceptor should not exceed 10 nm (Pehlivan et al., 2019).

Quenchers like graphene oxide are also used as receptors (Jung et al., 2010). Carboxyfluorescein (FAM) is among the most widely employed organic fluorescent dyes. Graphene Oxide is an important derivative of graphene obtained through oxidation with strong acid resulting in enhanced chemical stability, excellent hydrophilicity, and good biocompatibility due to the abundance of oxygen-containing groups on its surface (Lee et al., 2016; Suvarnapaet & Pechprasarn, 2017; Zheng & Wu, 2017). Currently, researchers have developed diverse biosensors utilizing graphene oxide for protein or nucleic acid detection.

In this study, we aimed to develop a fluorescence sensor for the detection of *Vibrio parahaemolyticus*, utilizing carboxyfluorescein as the signalling molecule and graphene oxide as a quenching agent. Our approach involved several critical steps: First, we assessed the fluorescence signal of aptamers modified with carboxyfluorescein at the 5' end, both before and after high-temperature treatment at 95°C, to evaluate their stability and performance. Next, we determined the optimal concentration of graphene

oxide required to achieve effective quenching of the fluorescent aptamers. Finally, we introduced *Vibrio parahaemolyticus* into the system and monitored the recovery of fluorescence, which indicated successful binding of the aptamer to the target bacteria. Throughout this process, we meticulously optimized reaction conditions to ensure accurate and reliable detection of *Vibrio parahaemolyticus*. The results demonstrate the potential of our fluorescence sensor for practical applications in monitoring food safety and environmental health.

2. Materials and methods

2.1 Materials and reagents

Graphene oxide, Alkaline peptone water medium, Thiosulfate Citrate Bile Salts Sucrose Agar Medium were bought from Luqiao Technology Co., Ltd (Beijing). *Vibrio parahaemolyticus* (CGMCC 1.1997), *Salmonella* (CGMCC 1.10603) were provided by China General Microbial Culture Collection and Management Center. *Staphylococcus aureus*, Enterotoxigenic *Escherichia coli*, *E. coli*. 0.01mol/L PH 7.4 phosphate buffer, 5'-FAM-TCTAAAAATGGGCAAAGAAACAGTGA CTC GTTGAGATACT-3' was obtained from Sangon Bioengineering. Co., Ltd. (Shanghai, China).

2.2 Processing of fluorescent aptamers

The aptamer was centrifuged at 4000 rpm for 30 s in a high-speed refrigerated centrifuge to ensure complete sedimentation and to prevent adhesion to the tube walls. Following centrifugation, PBS buffer was added to adjust the fluorescent aptamer to a final concentration of 100 μ M. The solution was then aliquoted into 10 μ L portions and stored at -20°C in brown, light-proof centrifuge tubes.

2.3 Preparation of graphene oxide solution

Graphene oxide (4 mg) was dispersed in 4 mL of PBS buffer and sonicated in an ultrasonic cleaner for 1 h to ensure uniform dispersion. The resulting graphene oxide

solution was stored at 4 °C for subsequent use.

2.4 High-temperature treatment of aptamers

Two aliquots of aptamers stored at −20 °C were thawed at room temperature. After complete melting, 990 µL of PBS buffer was added to each tube to obtain a final aptamer concentration of 1 µM. One tube was subjected to thermal treatment at 95 °C for 10 min, followed by immediate cooling in an ice bath for 10 min. The sample was then allowed to equilibrate to room temperature. Fluorescence intensity was subsequently measured and compared with that of untreated aptamers and aptamers subjected to high-temperature treatment.

2.5 Treatment of bacterial solution

The bacterial liquid culture was carried out according to the method described by Song et al. (2023). When the culture reached the logarithmic growth phase, 1 mL of bacterial suspension was transferred to a centrifuge tube and centrifuged at 10,000 rpm for 6 min. The supernatant was discarded, and the pellet was resuspended in 1 mL of PBS buffer. This centrifugation and washing step was repeated three times to remove residual culture medium. Finally, the bacterial suspension was resuspended in 1 mL of PBS buffer and serially diluted to obtain bacterial suspensions of different concentrations.

2.6 Detection of *Vibrio parahaemolyticus* with fluorescence aptamers and graphene oxide fluorescence resonance energy transfer

The VP was determined with fluorescence aptamers and graphene oxide fluorescence resonance energy transfer. The quencher amount in the reaction system was studied by adding an amount of graphene oxide solution with a final concentration of 200 nM fluorescent aptamer. The specific proportions are presented in Table 1. After a 30-minute reaction, the fluorescence intensity of the solution was measured to identify the amount of graphene oxide needed to quench the 200 nM fluorescent aptamer. Following quenching, 100 µL of *Vibrio parahaemolyticus* solution was added, and the mixture was shaken for 1 hour after centrifugation. The fluorescence intensity of the mixture was measured to assess the binding efficiency of the aptamer to *Vibrio*

152 *parahaemolyticus*.

153

154 **Table 1. Different volumes of GO**

1 μM apt (μL)	40				
Graphene oxide (μL)	6	8	10	12	14
PBS (μL)	154	152	150	148	146

155

3. Results and Discussion

3.1 Principle of the detection method

Fig 1 illustrates the modification of carboxyfluorescein at the 5' end of the aptamer. Graphene oxide's large specific surface area enables π - π stacking interactions with DNA bases when it contains carboxyfluorescein, leading to energy transfer between the fluorescent group and graphene oxide, resulting in fluorescence quenching (H. Zhao et al., 2020). In the presence of *Vibrio parahaemolyticus* in the reaction system, if binding strength exceeds its adsorption effect on graphene oxide, it causes fluorescence intensity restoration, allowing determination of target substance concentration based on fluorescence recovery. The underlying principle of the method for detecting *Vibrio parahaemolyticus* involves using fluorescent aptamers and graphene oxide.

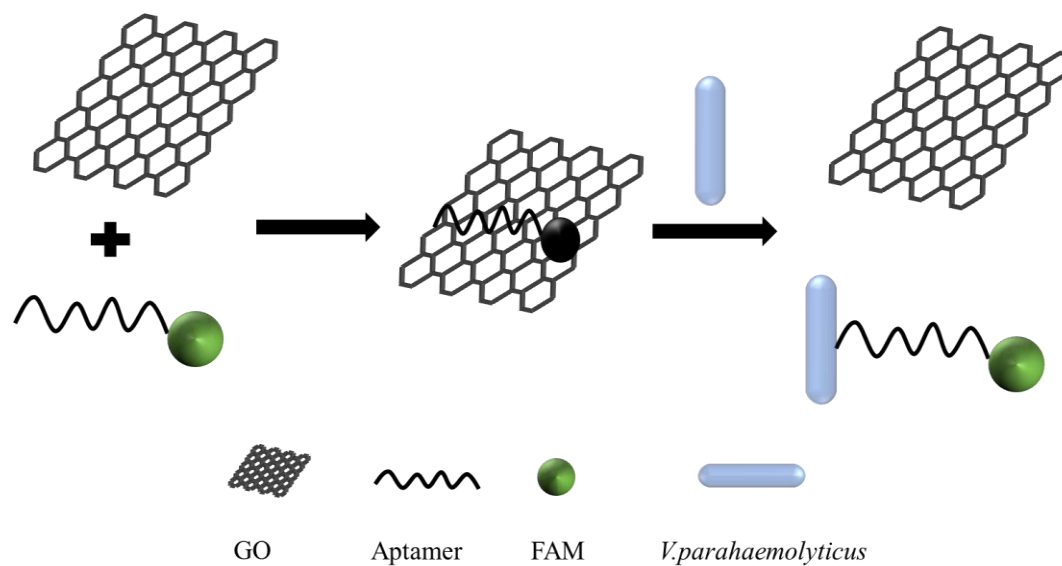


Fig. 1. Detection principle of *Vibrio parahaemolyticus* based on fluorescence resonance energy transfer

3.2 Optimization of the experimental conditions

3.2.1 Effect of high-temperature treatment on fluorescent aptamers

After reviewing the existing literature, it was found that some researchers use the aptamer solution directly, while others subject it to treatment at 95°C for ten minutes

176 before the experiment, followed by immediate placement in an ice bath (R. Liu et al.,
177 2022; Zhao et al., 2021). To understand the relationship between aptamer and *Vibrio*
178 *haemolyticus* under different conditions, a comparison was made of the binding
179 difference with its fluorescence intensity. Additionally, fluorescence recovery after
180 adding bacterial solution was evaluated. It was observed that under the excitation light
181 of 480 nm, carboxyfluorescein exhibited the highest fluorescence peak around 525 nm.
182 Consequently, the fluorescence value at 525 nm was utilized as the detection index.
183 (Fig 2) shows that the quenching efficiency reaches around 90% at an optimal GO
184 concentration of 10 µg/mL. This high quenching efficiency is due to the extensive
185 contact area and interactions between the GO and the fluorophore at this concentration.
186 The GO can effectively compete with other fluorescence deactivation pathways,
187 resulting in the observed 90% quenching.

188 While the 90% quenching efficiency is impressive, the data suggests that further
189 increasing the GO concentration beyond 10 µg/mL could potentially enhance the
190 quenching even more and lower the detection limit. The graph indicates that the
191 quenching continues to increase with higher GO loadings, even if the rate of
192 improvement begins to taper off. This suggests that high temperature does not affect
193 the fluorescence intensity of the fluorescent aptamer. When a final concentration of 200
194 nM fluorescent aptamer is present in a system with a total volume of 200 µL, varying
195 volumes of 1 mg/mL graphene oxide solution are added. The observed trend shows that
196 as the amount of graphene oxide added increases, the fluorescence value gradually
197 decreases, indicating its effective quenching effect on carboxyfluorescein. We observed
198 that heat treatment at 95°C significantly enhances the binding affinity between the
199 fluorescent aptamers and *Vibrio parahaemolyticus*. The increase in binding efficiency
200 is attributed to structural changes in the aptamer induced by heat.

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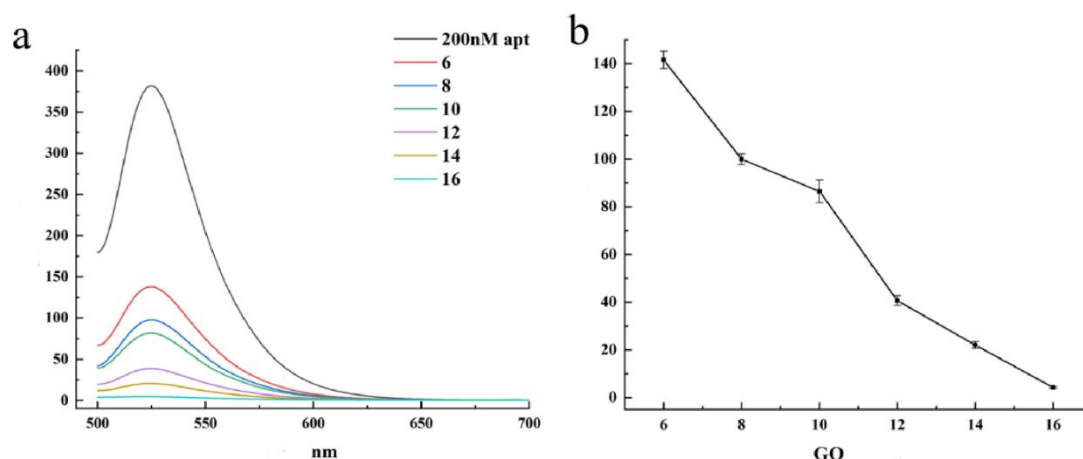


Fig. 2. Quenching efficiency of different concentrations of graphene oxide

3.2.2 Optimization of quenching time

To determine the time, it takes for the reaction between graphene oxide and carboxyfluorescein to completely quench the fluorescence, we reacted 14 μL of 1 mg/mL graphene oxide solution with a 200 nM aptamer modified with carboxyfluorescein for 10, 20, 30, and 40 minutes (Fig 3). After each reaction period, we measured the fluorescence intensity of the solution. Our results indicate that after just 10 minutes of reaction time, the fluorescence of carboxyfluorescein was almost entirely quenched. Further increasing the reaction time resulted in an only minimal additional reduction in fluorescence intensity; as such, we have established a fluorescence-quenching period of approximately ten minutes.

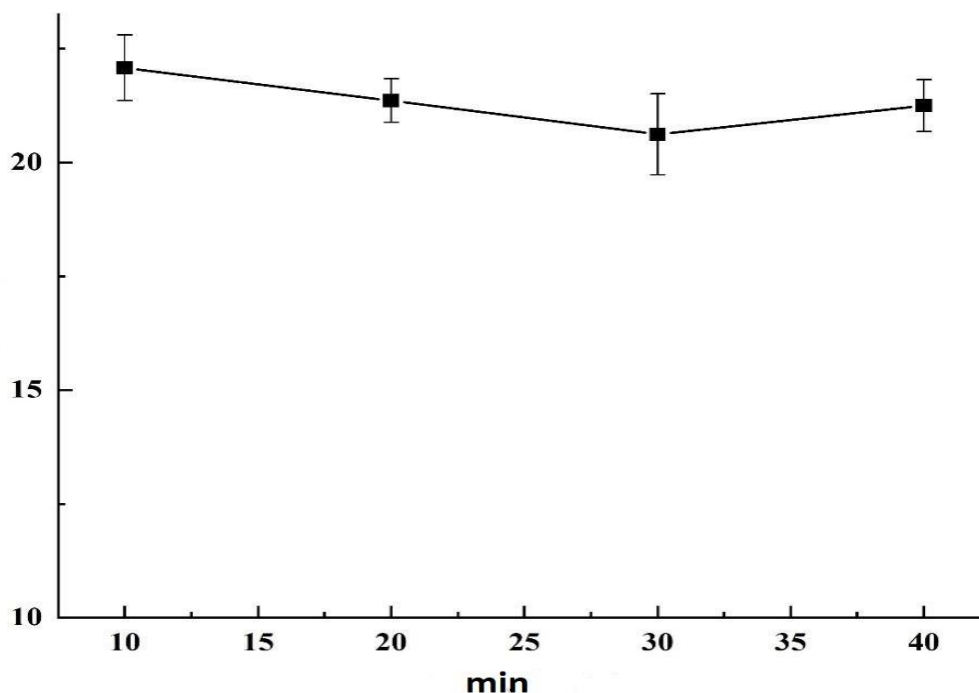


Fig. 3. Optimization of quenching time

3.2.3 Optimization of reaction temperature

Vibrio parahaemolyticus has an optimal growth temperature. To explore the effect of temperature on its binding with aptamer, the fluorescence of the reaction system was quenched, and *Vibrio parahaemolyticus* was then added at temperatures ranging from 25°C to 45°C for oscillation reaction. After completion, the fluorescence intensity was measured (Fig 4). Analysis of the fluorescence spectrum showed that as the reaction temperature increased under low-temperature conditions, there was a gradual increase in fluorescence recovery intensity. The highest recovery intensity occurred at 40°C. However, when the temperature reached 45°C, there was a decrease in fluorescence intensity; thus, indicating that 40°C was selected as the optimal temperature for this reaction.

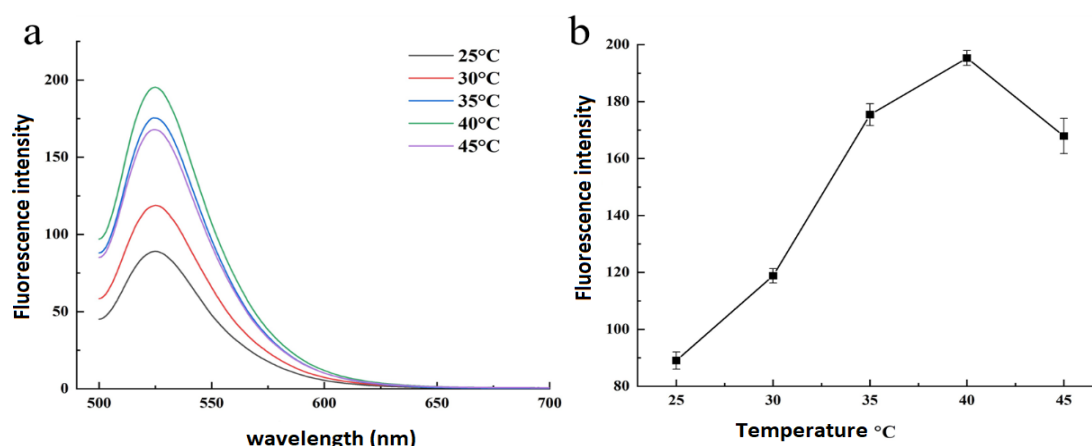


Fig. 4. Optimization of reaction temperature

3.2.4 Optimization of separation condition

The effect of separation speed on the binding efficiency between the aptamer and bacterial solution was investigated under otherwise constant conditions. After the addition of bacterial solution, different shaking speeds (static, 50 rpm, 100 rpm, 150 rpm, 200 rpm, and 250 rpm) were tested (Fig. 5). Following the reaction, fluorescence intensity was measured. The results demonstrated that reaction speed significantly influenced the interaction between the aptamer and *Vibrio parahaemolyticus*. Fluorescence recovery intensity increased with shaking speed and reached a maximum at 200 rpm. However, further increases in speed were not conducive to aptamer–bacteria interaction. Therefore, 200 rpm was identified as the optimal reaction speed for this system.

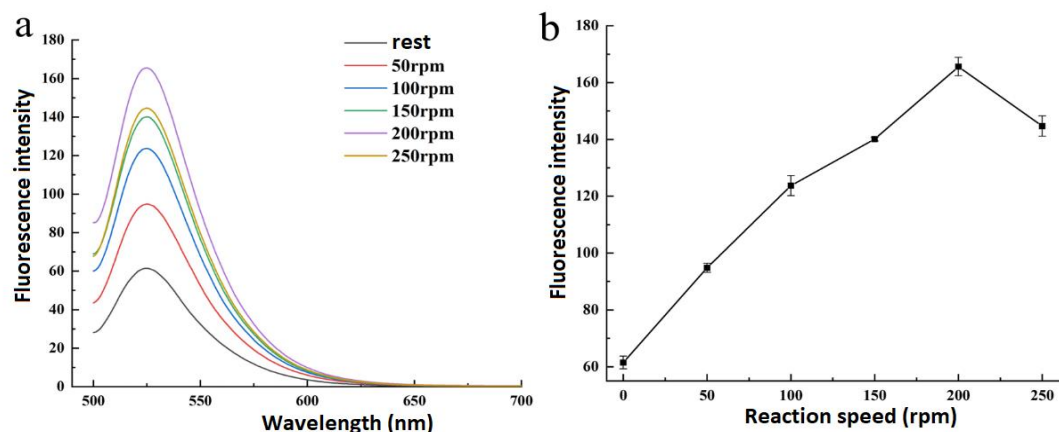


Fig. 5. Optimization of separation condition

3.2.5 Optimization of reaction time

If the reaction time is too short, the aptamer will not fully bind with *Vibrio parahaemolyticus*, leading to low fluorescence recovery intensity. On the other hand, if the reaction time is too long, it hinders rapid detection of *Vibrio parahaemolyticus*. Therefore, optimizing the reaction time was crucial. Based on our fluorescence results, a 10-minute reaction significantly improved fluorescence recovery (Fig 6). Extending this to 40 minutes led to stable fluorescence recovery intensity. Further prolonging the reaction did not notably increase fluorescence intensity; hence 40 minutes is deemed as the optimal reaction time.

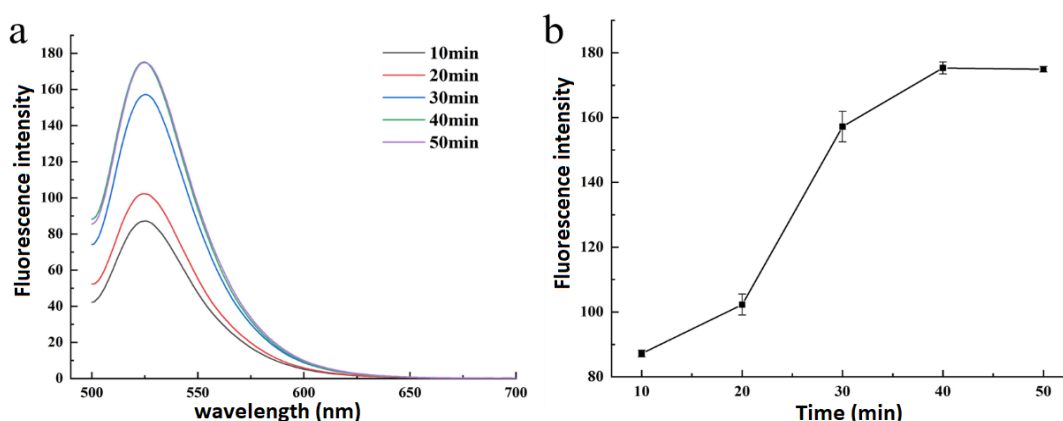


Fig. 6. Optimization of reaction time

3.3 Sensitivity of detection methods

Under optimized reaction conditions, 100 μ L of bacterial solutions at varying concentrations were added, and the resulting fluorescence intensity was measured and is presented in Fig. 7. The observed increase in fluorescence recovery intensity with higher bacterial concentrations demonstrates the effectiveness of our method in quantifying bacterial presence. To address the reviewer's concerns, we will expand the discussion in the revised manuscript to include a more detailed explanation of the fluorescence detection mechanism, specifically describing how the aptamer binds to

Vibrio parahaemolyticus and induces changes in the fluorescence signal. Additionally, we will elaborate on the sensitivity of our detection method, noting that fluorescence intensity remained similar to the blank control at concentrations of 10^4 CFU/mL or below. Nevertheless, our method successfully detects low-concentration bacterial solutions, as evidenced by the linear relationship between fluorescence intensity and bacterial concentration in the range of 10^5 to 10^9 CFU/mL ($y = 30.23x - 139.53$, $R^2 = 0.9685$). Furthermore, we will clarify that the determined detection limit of our method is 10^5 CFU/mL, underscoring its potential application in scenarios requiring early detection of *Vibrio parahaemolyticus*.

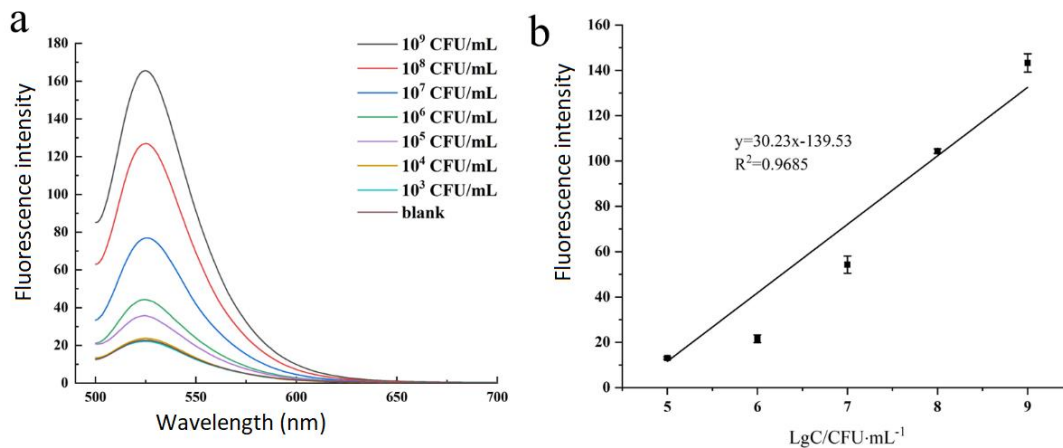


Fig. 7. Sensitivity of detection method

3.4 Specificity of detection method

To determine the specificity of this test method for detecting *Vibrio parahaemolyticus*, four other pathogenic bacteria (*E. coli* O157:H7, *Staphylococcus aureus*, enterotoxigenic *Escherichia coli*, and *Salmonella*) were added to the test instead of *V. parahaemolyticus*. The fluorescence intensity was then measured in reverse (Fig 8). Results

showed that after adding *Vibrio parahaemolyticus*, the fluorescence intensity was significantly higher compared to when other pathogenic bacteria were added. When other foodborne pathogenic bacteria were introduced into the reaction system, their fluorescence intensity remained lower than that of *Vibrio parahaemolyticus*. These consistent results demonstrate high specificity for detecting *Vibrio parahaemolyticus* using this method.

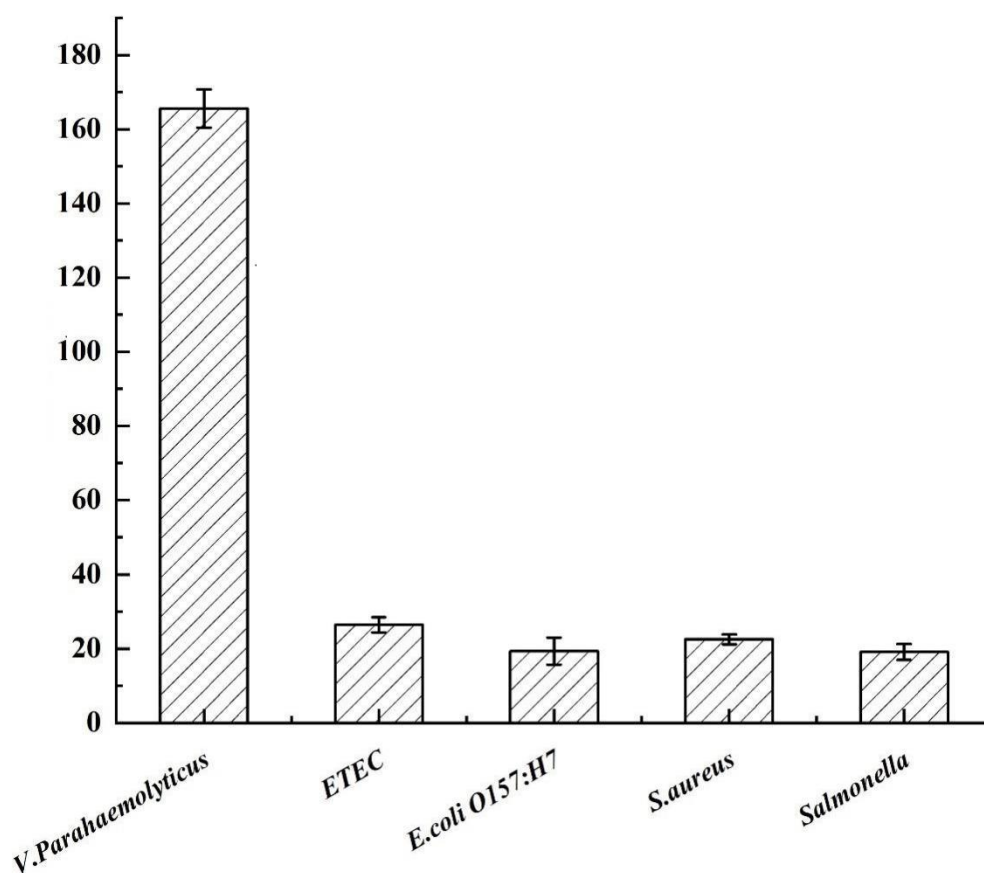


Fig. 8. Specificity of detection of method

4. Conclusion

In conclusion, a fluorescence resonance energy transfer method utilizing graphene oxide and carboxyfluorescein aptamers was developed for the specific detection of *Vibrio parahaemolyticus*. The presence of *Vibrio parahaemolyticus* in the system led to the detachment of the aptamer from the graphene oxide surface, resulting in

fluorescence recovery and allowing for the determination of bacterial concentration based on fluorescence intensity. Optimization of reaction conditions revealed that the method could only identify high concentrations of *Vibrio parahaemolyticus* and was insensitive to low concentrations. Increasing aptamer concentration did not improve sensitivity, indicating a need for further exploration in enhancing detection sensitivity. The high specificity of the method for *Vibrio parahaemolyticus* was confirmed by comparing with other foodborne pathogenic bacteria, demonstrating fluorescence restoration only in the presence of *Vibrio parahaemolyticus*. This method is capable of detecting *Vibrio parahaemolyticus* at concentrations of 10^5 CFU/mL and above.

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