Fluorometric Aptasensor for Determination of Escherichia coli O157:H7 by FRET Effect between Aminated Carbon Quantum Dots and Graphene Oxide

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A fluorometric aptasensor based on Escherichia coli O157:H7 (E. coli O157:H7) aptamer labeled aminated carbon quantum dots (NH$_2$-CQDs) and graphene oxide (GO) for the determination of E. coli O157:H7 was developed. In this research, carboxyl group (-COOH) terminated E. coli O157:H7 aptamer was steadily labeled to NH$_2$-CQDs by amidation reaction, and played the role of energy donor and was responsible for chemical recognition. Correspondingly, GO served as an energy acceptor. The introduction of NH$_2$-CQDs not only made the aptamer bond stably through covalent bond, but also significantly enhanced the fluorescence intensity compared with general CQDs. The NH$_2$-CQDs-aptamer is adsorbed on the surface of GO through π-π stacking and hydrophobic interaction. The fluorescence of NH$_2$-CQDs-aptamer was quenched via fluorescence resonance energy transfer (FRET) between NH$_2$-CQDs and GO. After adding E. coli O157:H7, the specific binding affinity between NH$_2$-CQDs-aptamer and E. coli O157:H7 lead to desorption of NH$_2$-CQDs-aptamer from GO, and recovery of the fluorescence intensity of NH$_2$-CQDs-aptamer. Under the optimal conditions, the increased fluorescence intensity showed a good linear relationship to concentrations of E. coli O157:H7 in the range $10^2$ – $10^7$ cells/mL, with a detection limit of 89 cells/mL. Furthermore, the developed method was successfully applied to the determination of E. coli O157:H7 in commercial milk samples.

Keywords Fluorometric aptasensor, aminated carbon quantum dots, graphene oxide, fluorescence resonance energy transfer

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Introduction

Escherichia coli O157:H7 (E. coli O157:H7) is a kind of main pathogenic strain of enterohemorrhagic Escherichia coli (EHEC) that causes a series of diseases in humans, such as intestinal edema, hemorrhage, spasmodic colic and so on. Moreover, E. coli O157:H7 is also the main cause of haemorrhagic enteritis and hemolytic uremia. Epidemiological studies indicate that E. coli O157:H7 is mostly related to food, such as beef, pork, milk, juice, vegetables, etc. In the United States, Japan, Canada and the United Kingdom, E. coli O157:H7 has erupted on a large scale, caused by the intake of organic spinach, Chinese cabbage, lettuce and potatoes. At present, there is no specific medicine or effective treatment to treat the diseases caused by E. coli O157:H7. Therefore, it is of great significance important to establish an effective method to detect E. coli O157:H7 for the prevention of associated diseases. In recent years, aptamer-based sensors have attracted more attentions for use in the detection of pathogenic microorganisms owing to the appreciable rapidity, high sensitivity and selectivity.

Aptamers are well-known as oligonucleotide sequences with high specificity and affinity for the target substances. They are selected in vitro by systematic evolution of ligands via exponential enrichment (SELEX). In recent years, aptamers have come to be considered as novel recognition elements that may replace antibodies since the time-cost of the aptamer screening cycle has been significantly reduced, in contrast to that for antibodies. Thus, the development of aptamer-based detection systems has gained tremendous.

The aptamer-based detection systems include the electrochemical method, spectrophotometry, fluorescence method, electrochemiluminescence method, etc. Among various methods, fluorescence spectrometry has been widely used owing to its simple operation and high sensitivity. The development of the quantum dots (QDs) fluorescent probe has further expanded the fluorescence spectrometry application. Compared to conventional organic fluorescent probes, QDs exhibit wider and continuous excitation spectrum. Moreover, the emission wavelengths can be simply adjusted by changing of the QDs. Carbon quantum dots (CQDs), which possess low toxicity and good biocompatibility, have been widely applied in the research on biological molecules, proteins and microorganisms. Thus, aptamer labeled CQDs based detection methods are expected to meet the requirements of high sensitivity and selectivity for biological targets, and play a supportive role in the early diagnosis, treatment and epidemic control of diseases.

Fluorescence resonance energy transfer (FRET) is a newly developed method and has been confirmed effective for...
improving the sensitivity of the fluorescence method. Generally, a FRET system is composed of a fluorophore and a quencher. Graphene oxide (GO) is a good choice for the quencher in a FRET system owing to its high quenching efficiency and good repeatability. Especially in aptamer-based FRET detection systems, GO has great application potential because it has a large electron conjugation structure that makes it an excellent anchor for aptamers through π-π stacking interactions.

Herein, a fluorometric aptasensor for E. coli O157:H7 was developed based on the FRET effect between aptamer labeled CQDs and GO. The -NH2 group modified CQDs was prepared by a one-step hydrothermal method and exhibited high fluorescence response. Subsequently, it was steadily labeled with -COOH group terminated aptamer by amide bond to prepare NH2-CQDs-aptamer. Afterward, the introduction of well-dispersed GO quenched the fluorescence intensity with high efficiency via FRET effect. Conversely, E. coli O157:H7 can interact with aptamer labeled CQDs to prevent the adsorption of GO, to further recover the fluorescence intensity. Finally, the developed FRET system was successfully applied to the determination of E. coli O157:H7 in milk samples.

Experimental

Chemicals

Citric acid monohydrate (C6H8O7·H2O), N-hydroxy succinimide (NHS) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) were purchased from Sinopharm Chemical Reagent Co. Ltd. (China). Polyethyleneimine (PEI) was purchased from Aldrich-Sigma Chemical Co. Ltd. (USA). E. coli O157:H7, Staphylococcus aureus (S. aureus) and Pseudomonas aeruginosa (P. aeruginosa) were purchased from China Center of Industrial Culture Collection (CICC). E. coli O157:H7 aptamer was purchased from Sangon Biotech Co. Ltd. (China) and its sequence was given as follows: 5’-COOH-GCA ATG GTA CGG TAC TTC CCC ATG AGT GTT GTG AAA TGT TGG GAC ACT AGG TGG CAT AGA GCC GCA AAA GTG CAC GCT ACT TTG CTA A-3’. All reagents were of analytical grade and used directly without any treatment. Ultrapure water (18.2 MΩ) was utilized throughout the experiment.

Bacterial culture

Bacterial cultures and experiments were executed in a biosafety level 2 laboratory designed and managed in accordance with safety regulations. All the bacterial strains were cultured at 37°C in liquid broth for 18 h. The cells were then harvested by centrifugation at 7000g for 15 min and washed with phosphate buffer (pH 7) four times.

Preparation of NH2-CQDs

Preparation of NH2-CQDs was conducted following the procedure described in Huang’s report. A 0.6 g of polyethyleneimine (PEI) and 0.6 g of citric acid monohydrate were dispersed/dissolved in 10 mL of ultrapure water, and then moved to autoclave to react at 180°C for 2 h. After that, the product was dried at 60°C and resuspended in ultrapure water (2.0 mg/mL) for further use.

Preparation of NH2-CQDs-aptamer

The conjugate of NH2-CQDs and carboxyl group (-COOH) terminated E. coli O157:H7 aptamer was prepared by well-established carbodiimide chemistry. In brief, freshly prepared EDC solution (100 μL, 60 mM) and NHS solution (100 μL, 60 mM) were successively added to 50 μL of 0.25 μM COOH-aptamer for activating carboxyl groups. Afterwards, 100 μL of the mixture was added to 100 μL of NH2-CQDs suspension (2.0 mg/mL) at 37°C with shaking for 30 min and the NH2-CQDs-aptamer was obtained.

Preparation of GO

A modified Hummers method was used to prepare GO in this experiment. First, 120 mL of concentrated H2SO4 (98% w/w) was taken in a flask, stirred and cooled to 0°C. Next, 3 g of natural graphite flakes and 15 g of KMnO4 was added gradually under stirring and the temperature of the mixture was kept to be below 20°C. Subsequently, the mixture was stirred at 35°C for 2 h, then 250 mL of deionized water was slowly added to this system. After 2 h, the mixture was diluted with 700 mL of deionized water and 20 mL of H2O2 (5% wt) was added. In doing this, the solution turned bright yellow. The mixture was filtered and washed with deionized water three times. Then it was purified by dialysis for one week to remove the remaining metal species. Finally, the solid GO was obtained and freeze dried for further use.

Determination of E. coli O157:H7

The determination of E. coli O157:H7 was conducted as follows: 100 μL of GO (2.0 mg/mL) suspension was added into the aforementioned NH2-CQDs-aptamer suspension and incubated for 15 min at room temperature. Afterwards, 50 μL of series suspensions containing different concentrations of E. coli O157:H7 were added into the above suspensions, respectively. Then the suspensions were incubated for 1 h at 20°C. The resulted mixture was then subject to fluorescence measurement with an excitation wavelength of 355 nm, and the fluorescence intensities of emission wavelength at 455 nm were recorded. The normalized intensity (F/F0) of E. coli O157:H7 was calculated from the fluorescence intensity of the suspension mentioned above in the presence (F) and absence (F0) of E. coli O157:H7. It is important to mention that the E. coli O157:H7 suspension should be shaken adequately during the experiment.

Preparation of milk samples

The pasteurized milk was pretreated according to the previously reported procedure. Briefly, 1.0 mL of acetonitrile, 1.0 mL of trichloroacetic acid, 2.0 mL of milk and 7.0 mL of water were added into the centrifuge tube. The mixture was then sonicated for 20 min and centrifuged at 4000 rpm for 30 min to remove the protein. Subsequently, the supernatant was transferred to another centrifuge tube, centrifuged at 12000 rpm for 20 min, washed twice with PBS, and then different concentrations of E. coli O157:H7 were added to make spiked samples.

Results and Discussion

Sensing principle of the proposed aptasensor for E. coli O157:H7 determination

As demonstrated in Scheme 1, the aptamer of E. coli O157:H7 was modified on the surface of NH2-CQDs to prepare the NH2-CQDs-aptamer complex according to the carbodiimide chemistry. When GO was introduced to the system, NH2-CQDs-aptamer was adsorbed on the surface of GO through π-π stacking and hydrophobic interactions. The FRET effect caused quenching of the fluorescence intensity of the NH2-CQDs-
aptamer. After adding *E. coli* O157:H7, the fluorescence intensity was recovered. The NH₂-CQDs-aptamer adsorbed on the surface of GO could be released from GO and bind to *E. coli* O157:H7, owing to the high binding affinity and specificity between *E. coli* O157:H7 and corresponding aptamer.

Meanwhile, the feasibility of the proposed system for detecting *E. coli* O157:H7 could be confirmed with Fig. 1. As shown in Fig. 1A, in the absence of GO, the fluorescence intensity of NH₂-CQDs-aptamer was around 90000 (curve a). After binding with GO, the fluorescence intensity significantly decreased to 43000 (curve b). After a certain concentration of *E. coli* O157:H7 was added into the suspension, the fluorescence intensity recovered to 62000 (curve c). It indicated that the GO and *E. coli* O157:H7 exhibited a good quenching and recovery effect on the fluorescence response of NH₂-CQDs-aptamer, respectively. In Fig. 1B, the ultraviolet-visible absorption of GO possessed a good overlap with the fluorescence emission band of NH₂-CQDs-aptamer, which also confirmed that an effective FRET was carried out between NH₂-CQDs-aptamer and GO.

**Morphology and microstructure of NH₂-CQDs-aptamer loaded GO**

The morphology of GO was investigated by transmission electron microscope (TEM). As shown in Fig. 2A, it was obvious that the plate-like morphology of GO with ripples and corrugations was well-defined, revealing the ultrathin structure of GO. The TEM image of NH₂-CQDs-aptamer loaded GO is shown in Fig. 2B. It was clearly observed that several dark dots with diameter of about 3 nm was uniformly dispersed on GO. It also indicated that the covalent combination of *E. coli* O157:H7 aptamer to NH₂-CQDs exhibited no negative effect on the surface morphology and dispersibility of NH₂-CQDs-aptamer. The NH₂-CQDs-aptamer loaded GO was further studied by high-resolution transmission electron microscope (HRTEM). As shown in the inset of Fig. 2B, the lattice spacing of 0.3 nm was observed, indicating the present of NH₂-CQDs-aptamer, which further confirmed that NH₂-CQDs-aptamer was successfully loaded on GO.

**Characterization of NH₂-CQDs**

As shown in Fig. 3A, the FT-IR spectrum of CQDs, the stretching vibration of C=O (1708 cm⁻¹) and broad stretching vibration of O-H (3453 cm⁻¹) were observed (curve a). In the
case of NH$_2$-CQDs, the peak that appeared at 1363 cm$^{-1}$ corresponded to the stretching vibration of C-N, the narrow and strong peak that appeared at 1554 cm$^{-1}$ corresponded to the stretching vibration of amino group (–NH$_2$), proving the binding of PEI on the surface of CQDs to acquire NH$_2$-CQDs (curve b).32

Furthermore, in the process of preparing CQDs using anhydrous citric acid as raw material, the addition of polyethyleneimine (PEI) greatly affected the fluorescence performance of CQDs. As shown in Fig. 3B, the CQDs prepared with anhydrous citric acid alone, exhibited a relatively low fluorescence intensity at 455 nm (curve a). In contrast, the introduction of PEI in the process of preparing CQDs significantly enhanced the fluorescence intensity of NH$_2$-CQDs at the same concentration. The results indicated that the introduced PEI not only effectively modified the NH$_2$ group on CQDs surface, but also improved the fluorescence intensity.

Optimization of experimental conditions

The concentration of NH$_2$-CQDs significantly affected the fluorescence intensity, thus the fluorescence intensity of NH$_2$-CQDs at different concentrations was studied. As shown in Fig. S1A (Supporting Information), the NH$_2$-CQDs at the concentration of 0.10 mg/mL exhibited the highest fluorescence intensity.

The effect of GO concentration on the detection of *E. coli* O157:H7 was also studied here. The concentration of aptamer was fixed at 12.5 nM and the GO concentration was varied from 0.02 to 0.10 mg/mL. After adding *E. coli* O157:H7 suspension (1.0 × 10$^7$ cells/mL) and incubating for 1 h, the recovered fluorescence intensities ($\Delta F = F - F_0$) were recorded. As shown in Fig. S1B, the proposed system provided the highest $\Delta F$ value at the GO concentration of 0.04 mg/mL. The $\Delta F$ value decreased rapidly when the concentration of GO was higher than 0.04 mg/mL because too much GO hindered the release of NH$_2$-CQDs-aptamer.33

Finally, the incubation time of *E. coli* O157:H7 was optimized. A certain concentration of *E. coli* O157:H7 (1.0 × 10$^7$ cells/mL) was added into the NH$_2$-CQDs-aptamer (0.10 mg/mL) and GO (0.04 mg/mL) mixture, and incubated for different times. The results are shown in Fig. S1C. The $\Delta F$ value increased rapidly with increasing incubation time, and achieved a highest value at the incubation time of 60 min. Thus, the incubation time of *E. coli* O157:H7 was fixed at 60 min in the following experiments.

Analytical performance

Under optimal experimental conditions, the relationship between normalized intensity ($F - F_0$)/$F_0$ and the *E. coli* O157:H7 concentration is shown in Fig. 4. The normalized intensity increased over a wide concentration range of *E. coli* O157:H7 from 10$^2$ to 10$^7$ cells/mL, with a high correlation coefficient of 0.961. And the limit of detection (LOD) for *E. coli* O157:H7 was 89 cells/mL based on 3σ/k ($\sigma$ was the standard deviation of blank measurements, and $k$ was the slope of calibration curve).
Selectivity of target cells is critical for real-world applications. Hence, the selectivity of the proposed aptasensor was evaluated using S. aureus and P. aeruginosa as interferences. As shown in Fig. 5, after adding E. coli O157:H7 at a concentration of $10^5$ cells/mL into the NH$_2$-CQDs-aptamer (0.10 mg/mL) and GO (0.04 mg/mL) mixture and incubating for 1 h, the $\Delta F$ value was 15454. While in the case of S. aureus and P. aeruginosa, a concentration of $10^7$ cells/mL, the $\Delta F$ value was only 1784 and 1489, respectively. The $\Delta F$ value for E. coli O157:H7 was much higher than other strains, thus highlighting the selectivity of the proposed aptasensor. The selectivity of the proposed aptasensor is attributable of the much higher binding affinity between E. coli O157:H7 and NH$_2$-CQDs-aptamer.34 The selectivity of the proposed aptasensor for E. coli O157:H7 was further investigated using an interference assay for a mixture sample. The mixture sample contained E. coli O157:H7 at a concentration of $10^5$ cells/mL, and S. aureus and P. aeruginosa both at a concentration of $10^7$ cells/mL. The results showed that the proposed aptasensor could discriminate the target strain from other types of strains.

Analysis of commercial milk samples

Under optimal conditions, the fluorescence recovery of the proposed aptasensor with E. coli O157:H7 in real samples was determined to evaluate the application of this fluorescence assay. Different concentrations of E. coli O157:H7 were added to commercial milk samples. As described in Table 1, the recovery of added E. coli O157:H7 ranged from 96.1 to 108.4%, with all RSD values below 4.30%. The results suggested that the proposed aptasensor has a potential applicability for the analysis of E. coli O157:H7 in real samples.

Conclusions

In this work, a fluorometric aptasensor for sensitive and selective determination of E. coli O157:H7 was effectively demonstrated by FRET effect between NH$_2$-CQDs-aptamer and GO. The competition mechanism between GO and E. coli O157:H7 for NH$_2$-CQDs-aptamer was newly introduced here for fluorescence intensity recovery. Meanwhile, the proposed aptasensor was successfully applied for actual sample analysis. Compared to general CQDs, the aminated CQDs not only provided sites for covalent binding with aptamers, but also significantly enhanced the fluorescence intensity. This work supports the application of FRET based sensor, and also provide prespects of many new possibilities in life science research.

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Supporting Information

This material is available free of charge on the Web at http://www.jsac.or.jp/analsci/.

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