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Whole cell FRET immunosensor based on graphene oxide and graphene dot for *Campylobacter jejuni* detection

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ABSTRACT

In this work a new fluorescence immunosensor with use of graphene oxide and graphene quantum dot for detection *Campylobacter jejuni* whole cell in food samples was designed. This biosensor was designed based on interaction of poly clonal antibody conjugated with graphene quantum dot with surface protein in *Campylobacter jejuni* cell membrane. Specific binding of graphene quantum dot with *Campylobacter jejuni* membrane leads to generate a distance among graphene dot and graphene oxide and fluorescence is ON. In lack of *Campylobacter jejuni* or in existence of other bacterial cells, distance between of graphene dot and graphene oxide is very low and graphene quantum dot fluorescence emission was OFF. Experiment revealed that step by step increase in bacterial target cells caused to gradually increased fluorescence emission and this process was linear. Limit of detection for this bacterial sensor was 10 CFU/ml and ability of this FRET immunosensor for *Campylobacter jejuni* sensing in comparison with other bacterial cells was significant. Also, this method for monitoring *Campylobacter jejuni* in poultry liver was applied and results revealed that this immunosensor could be used for analysis bacterial cell in food samples.

1. Introduction

Campylobacter jejuni is central origin of severe bacterial gastroenteritis that known as campylobacteriosis in developed world (Wei et al., 2007) and has the most widespread foodborne bacterial infection with an estimated 2.4 million cases of foodborne disease (Wang, Li, & Slavik, 2014). Commonly, break out of the campylobacteriosis occurs via consumption of contaminated food, mainly poultry-based food, unpasteurized milk or polluted water and keeping infected domestic animals (Yang, Jeffrey, & Aleksandr, 2013). Therefore, development of rapid and reliable diagnostic methods to inhibit the occurrence of *Campylobacter jejuni* and reduce its negative effects on human health and animal food source is essential (Masdor, Altintas, & Tothill, 2017). The culture method is a typical way for detection of *C. jejuni*. This technique, in addition to the need for a suitable culture medium and microaerophilic situation, takes a long time around several days to view colonies and to perform confirmation (Che, Li, & Slavik, 2001). Accordingly, study on development of fast and convenient method for detection of this bacteria has been under focus (Masdor et al., 2017) and other approaches such as ELISA, PCR, and Real Time PCR have been developed to detect *Campylobacter jejuni* (James et al., 2014). Although PCR and real time PCR have dependable results, expensive equipment and proficient operator are needed (Smith & Osborn, 2009). These problems, led to research on finding quick and easy techniques to detect target. One of the best approaches for achieving these goals is biosensor technology. Compared to previous approaches, biosensors have advantages such as high selectivity and sensitivity, and ability to perform at the minimum time (Masdor, Altintas, & Tothill, 2016).

As of yet, various biosensors like amperometric (Ivnitski, Wilkins, Tien, & Ottova, 2000), electrochemical impedimetric immunosensor (Huang et al., 2010), SPR (Wei et al., 2007; Masdor et al., 2017). and QCM (Masdor et al., 2016) sensors have been used for detecting *Campylobacter jejuni* (Huang et al., 2010). Although these types of sensors show sensitive performance in real-time tracking, they also produce heavy background signals. Additionally, they require a costly gold chip or electrode and also multiple phases of preparations like chemical functionalization of surface and immobilization of bio-receptor which are hard and time consuming (Drummond, Hill, & Barton, 2003;

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Ahmed, Wiley, Weidner, Bonnerup, & Mota, 2010; Taylor et al., 2006). Moreover, some researches were carried out on the basis of fluorescence methods. Bruno et al developed a sandwich assay biosensor based on fluorescence using aptamer labels alongside quantum dots and magnetic beads for Campylobacter identifying. Albeit these biosensors showed high sensitivity (Bruno, Phillips, Carrillo, & Crowell, 2009; Dadmehr et al., 2014; Naderi, Hosseini, & Ganjali, 2018), the toxicity of quantum dot to human and environment limits their use in bio-research (Bottrill & Green, 2011). Recently carbon-based material such as carbon quantum dot and nano diamonds because of unique properties such as high luminescent and low toxicity have been considered, but graphene quantum dot due to edge effect and quantum confinement exhibit strong photo luminescent (Dong et al., 2012).

So far, a few works for bacterial detection based on FRET (fluorescence resonance energy transfer) biosensor have been reported (Liu & Su, 2017; Morales-Narváez, Hassan, & Merkoçi, 2013; Shi et al., 2015), GQD due to the outstanding optical properties that mentioned above and tunable emission in FRET based biosensor has been used (Zhao et al., 2013). FRET is a technique based on photoexcitation energy transfer from a donor fluorophore to an acceptor molecule which are in proximity to each other (Borghei, Hosseini, & Ganjali, 2017; Borghei, Hosseini, Ganjali, & Hosseinkhani, 2018). Donor molecule such as GQD in conjugate with bio receptor can act as a probe in biosensing system (Nemati, Hosseini, Zare-Dorabei, & Ganjali, 2018; Zhao et al., 2013). Gold nanoparticle was used as acceptor molecule (quencher) in some FRET biosensors, (Sabet, Hosseini, Khabbaz, Dadmehr, & Ganjali, 2017; Shi et al., 2015) but recently carbon-based nanomaterial such as graphene and graphene oxide has been described to be a suitable quencher of fluorescence (Morales-Narváez, Hassan, Merkoçi, 2013; Zhao et al., 2013).

In this research we describe synthesis and application of a whole cell FRET immunosensor for specific detection of *Campylobacter jejuni*. For construction of this ON/OFF fluorescence biosensor, GQDs- antibody as specific probe for *Campylobacter jejuni* cells, were interacted with GO. In absence of bacterial cell, π-π stacking interaction happened between GQD and quencher (GO) and fluorescence state was OFF. But the presence of bacteria in reaction caused specific interaction among probe (GQDs- antibody) and *Campylobacter jejuni* cells and as a result of this, energy transfer among donor and acceptor was failed and fluorescence state was ON (Scheme 1). With assessment of this fluorescence state, a fast and highly sensitive approach for simple detection of *Campylobacter jejuni* whole cell was introduced.

2. Experimental

2.1. Apparatus

Absorption spectra were determined using Perkin -Elmer lambda 25 spectrometer. Fluorescence measurements were carried out by using a Perkin Elmer LS- 45 fluorescence spectrometer with slits 5 and 20 (Buckinghamshire, UK). TEM images were taken using a transmission electron microscope (Zeiss, EM10C, 80 KV, Germany) on a copper grid. SEM image of graphene oxide was obtained by TESCAN MIRA II (Czech). FT- IR spectra were recorded by Perkin –Elmer spectrometer at room temperature. XRD pattern of graphene oxide was done with use of Philips instrument (PW, 1730).

2.2. Reagents

Citric acid, sulfuric acid, graphite powder, $KMnO_4$, HCL and other buffer reagent were taken from Merck Millipore company. EDC and NHS were purchased from Sigma Aldrich. CCDA culture media for campylobacter was provided by ibresco (Iran). Anaerocult C (gas pack) for creating microaerophilic condition was purchased from Merck Company. *Campylobacter jejuni* antibody and general antibody conjugated with HRP were purchased from Fitzjerald company. BSA and buffered peptone water were obtained from Merck company. The poultry liver samples were supplied by Local market.

2.3. Synthesis of graphene oxide and graphene dot

Synthesis of graphene oxide was performed by improved Hummers method that introduced by Marcano in previous work (Marcano et al., 2010). Briefly 9:1 mixture of H_2SO_4/ H_3PO_4 was added to graphite powder and $KMnO_4$. In 40 °C temperature was increased to 50 °C and the mixture was shacked for 12 h. after that reaction product cooled in room temperature and was diluted with ice and 30% H_2O_2. The mixture was filtrated and centrifuged, then supernatant discarded and solid material rinsed with water, HCL and ethanol. Finally, obtained graphene oxide was filtrated with PTFE membrane and dried overnight under vacuum condition in room temperature.

Graphene quantum dot (GQD) synthesis based on pyrolysis of citric acid was performed (Dong et al., 2012). 2-gram citric acid powder was heated to 200 °C with use of heating mantle for 30 min, and at first created pale yellow liquid and then orange color was observed, that implied GQD. Finally GO and GQD were characterized with several methods such as UV–vis and FT-IR.

2.4. Conjugation GQD with anti- Campylobacter jejuni antibody

Bioconjugation of GQD and antibody was completed based on former study method (Zhao et al., 2013). 600 μL GQD solution, 200 μL EDC (10 mg/mL) and 150 μL NHS (15 mg/mL) were transferred in a 2 mL Eppendorf tube. After 20 min, 1 μL antibody (0.005 mg) was diluted in 600 μL PBS and mixed with previous reaction. The mixture was shaken with the speed of 800 rpm for 2 h at 25 °C in dark and then preserved overnight at 4 °C to complete covalent binding of antibody to GQDs surface. Then the sample was centrifuged at 4000 rpm for 5 min and filtrated via filtration membrane (300 kDa) to eliminate unreacted ingredients, the remains product on the membrane were diluted in 20 mM PBS (pH 8) and kept in 4 °C. Conjugation of antibody and GQDs was characterized with FT-IR and fluorescence analysis.

2.5. Optimization GO concentration and time for interaction with GQD-antibody

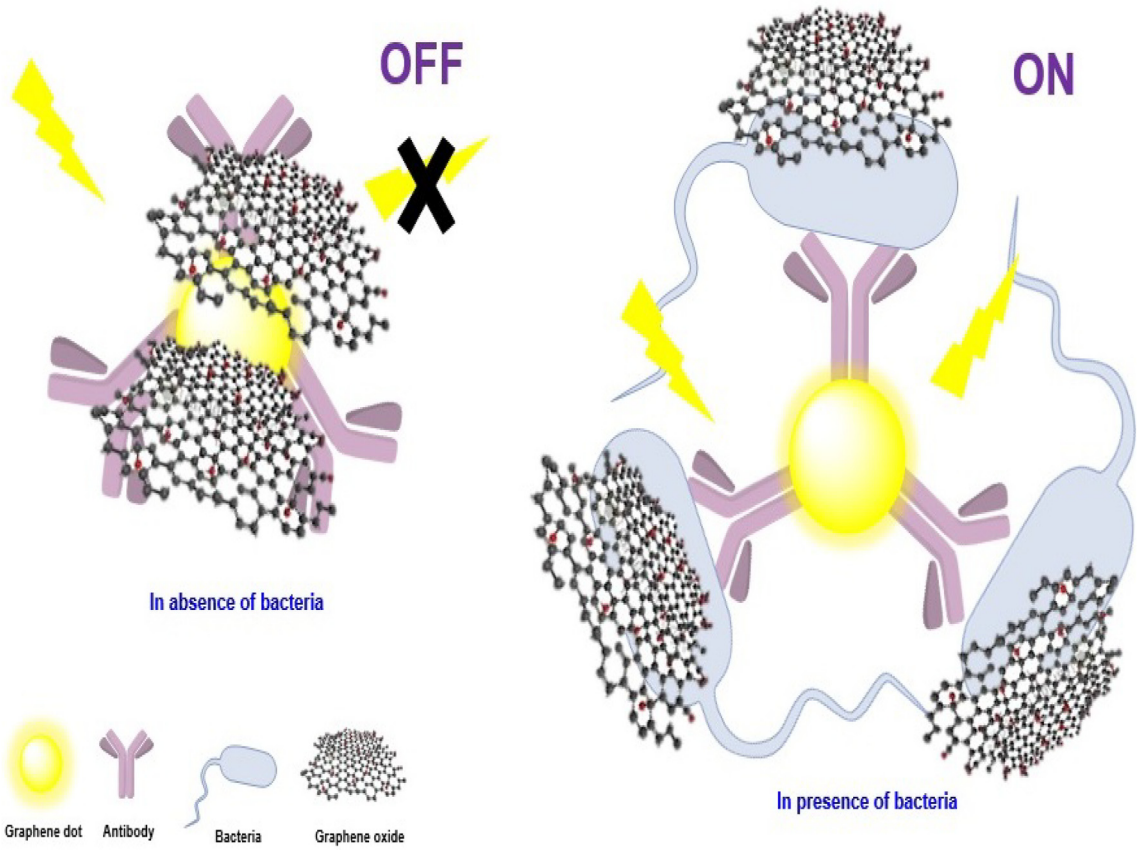
For fluorescence quenching test a series of various concentration of GO (0, 25, 50, 75, 100 μg) was incubated with 100 μL GQD- antibody in 20 mM PBS (pH 7.5) buffer in room temperature under gentle shaking for 5 min. Then optimum concentration of GO was chosen for identifying best interaction time among GO and GQD- antibody.

2.6. GQD- antibody/GO interaction with Campylobacter jejuni

7 mini tube containing 100 μL GQD- antibody were incubated with 10 μL GO (75 μg) and shaked for 15 min in room temperature. After that 100 μL of 6 bacterial dilutions (10–10^6), was added to each tube and one tube was without bacteria as negative control. All tubes were incubated in 37 °C under slow stirring for 45 min. After that fluorescence intensity in 450 nm was measured.

2.7. Bacteria cell culture and serial dilution preparation

C. jejuni and *C. coli* bacteria were isolated from the patient's stool samples that suffered from campylobacteriosis and were cultured on CCDA medium for 48 h at 37 °C in microaerophilic condition using jar contain Gas Pak. Several colonies of fresh bacteria were dispersed in 1X PBS buffer (pH 7) and their concentration determined by spectrophotometer at 620 nm. Then a dilution series of bacteria from 10 to 10^6 CFU was prepared for subsequent experiments. Also, *H. pylori* and E. coli needed for selectivity evaluation, were cultured in LB and Brucella media.



Scheme 1. Schematic representation of the antibody-GQDs/GO-based biosensor for *Campylobacter jejuni* whole cell detection.

2.8. *Campylobacter jejuni* detection by ELISA

ELISA experiment was completed based on direct method (Verma, Saxena, & Babu, 2013). In summary, 100 μL of each *Campylobacter. jejuni* cell concentration (10–10^6 CFU/ml) transferred into one well of ELISA plate and protected overnight at room temperature. Afterward, the sample was discarded and plate were washed 2 times with 200 μL of 1X PBS buffer. Then 200 μL BSA 5% was injected and incubated at room temperature for 2 h. All wells were clean-washed several times with 1X PBS buffer. 100 μL of *Campylobacter jejuni* antibody (10 μg), was added to all wells for interaction with *Campylobacter. jejuni* surface antigen at room temperature for 2 h. After rinsing of wells with 1X PBS buffer for 3 times, 100 μL general antibody labeled with HRP enzyme was added and incubation was done similar to former step. After cleaning the wells with 1X PBS for 3 times, 100 μL TMB (10 μM) and 50 μL H_2O_2 (10 μM) was put in each well and UV–vis absorption at 650 nm was measured.

3. Results and discussion

3.1. Characterization of GQDs and GO

TEM image shown in Fig. 1(a) imply spherical morphology of GQDs with size of nanoparticle around 1 to 2 nm. UV– vis spectrum of GQDs nanoparticle shows (Fig. 1b) two absorption peak at 250 and 350 nm that absorption band around 250 nm was related to $\pi \rightarrow \pi^*$ transition of C═C and a shoulder at 320–350 nm in UV spectrum that was attributed to the $n \rightarrow \pi^*$ transition of C═O (Qu, Zheng, Li, Xie, & Sun, 2015). FT-IR spectra of GQD showed two absorption band around 1381 and 1571 cm^{-1} that were attributed to COO^- symmetric stretching vibration and COO^- antisymmetric stretching vibration, respectively (Yuan et al., 2014). Also absorption peak at 1720 cm^{-1} was assigned to C═O and 1560 cm^{-1} was related to C═C groups of GQD (Lu, Zhang, & Liu, 2015). Solution of GQDs emitted blue light (Fig. S1) in 450 nm when excited at 350 nm. The blue photo luminescence of GQDs was related to SP^2 structure and functional groups containing oxygen (Dong et al., 2012).

SEM image of graphene oxide has been illustrated in Fig. 1c. The dimension of sheets around micrometer and thickness of nano flakes was estimated around 23 nm. UV spectrum of graphene oxide showed an absorption peak centered at 235 nm and a shoulder at ~300 nm (Fig. 1b), which could be assigned to the $\delta \rightarrow \delta^*$ transition of aromatic C−C bonds and the $n \rightarrow \delta^*$ transitions of C═O bonds, respectively (Morales-Narváez, Hassan, Merkoçi, 2013). Analysis of graphene oxide structure by means of FT- IR exhibited in Fig. S2a. The peaks at 1000 cm^{-1}, 1150 cm^{-1}, 1650 cm^{-1}, 1750 cm^{-1}, and 3350 cm^{-1} were attributed to C−O, C–OH, C═C, C═O, and O−H, respectively (Morales-Narváez, Hassan, Merkoçi, 2013). Also, XRD analysis (Fig. S2b) displayed $2\Theta \sim 11$, this result explained crystalline structure and interlayer space that is in agreement with a previous study (Zhong & Yun, 2015).

3.2. Conjugation of antibody to GQDs

Direct linkage among carboxyl groups on carbon based material and amino groups of antibody via EDC/NHS coupling reported in previous work (Zhao et al., 2013; Singh, Hong, & Jang, 2017). In order to confirm the proposed strategy for conjugation of antibody to GQDs, Covalent binding between free NH_2 of antibody and carboxyl groups of GQDs are demonstrated with FT-IR analysis. As indicated in Fig. 2a, intensity and location of absorption band in 1381 and 1571 cm^{-1} that are related to carboxyl groups of GQDs, in presence of antibody have changed. Also, fluorescence intensity of GQD before and after antibody conjugation was measured that result in Fig. 2b, showed that slight decrease in emission intensity in presence of antibody on GQD surface.

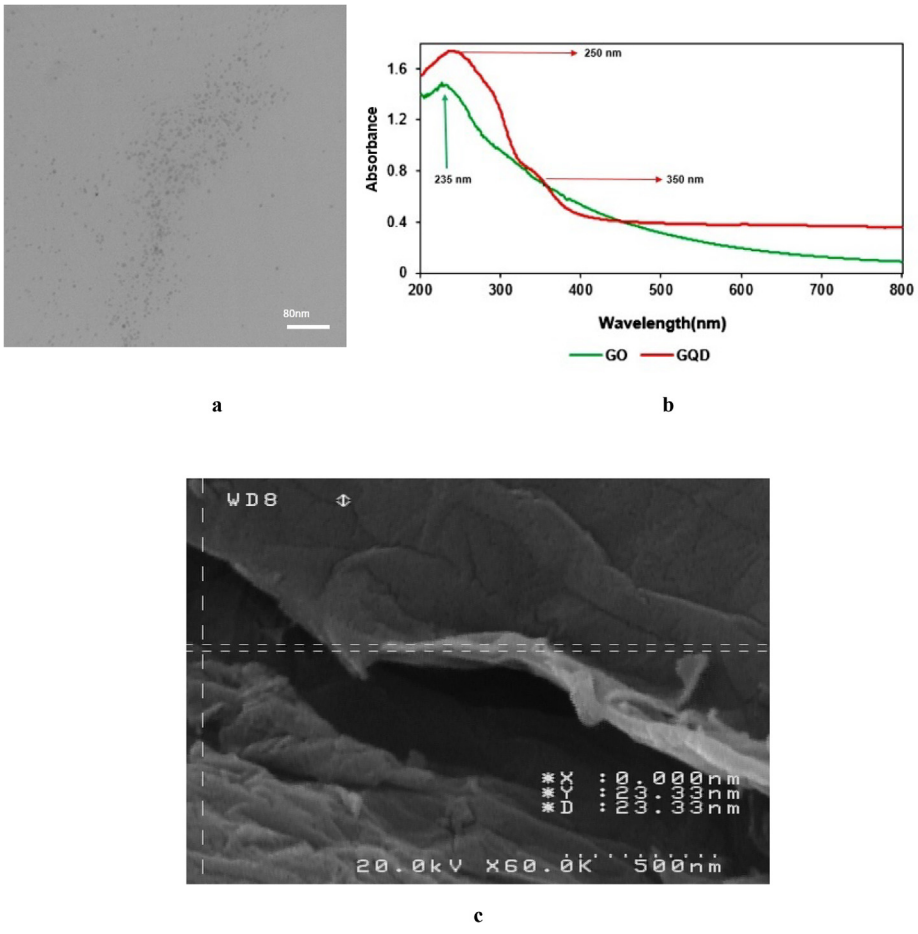


Fig. 1. (a) TEM image of GQD (b) UV–vis absorption spectrum of GQD and GO (c) SEM image of GO.

Furthermore, in order to confirm for conjugation of antibody to GQDs, TEM imaging was used. As can been seen in Fig. 3, the GQDs was appeared on the surface of bacteria which can support the strategy of binding of graphene quantum dot with *Campylobacter jejuni* membrane.

3.3. Optimization of GO concentration and incubation time

To consider effect of GO nano sheet as acceptors of GQD- antibody emission in FRET bio sensor several concentrations of GO was examined. In FRET system with increase of number of acceptor molecule, a larger portion of the photons that emitted by donor molecule is quenched (Dong et al., 2012). As presented in Fig. S3 (a), with increase of GO from 0 to 75 µg, emission of free GQD- antibody was decreased gradually. At a concentration of 75 µg, approximately 80% of GQD-antibody blue emission was quenched, but in higher concentrations, emission is generally turned off. Thus, based on the results of this step, 75 µg of GO was selected for optimization of reaction time. In order to evaluate optimum time for GQD-antibody interaction with GO, reaction in different times (5, 10, 15 and 20 min) was completed and results are shown in Fig. S3 (b). The efficiency of GO (75 µg) in fluorescence quenching was about 90% at a time of 15 min. Therefore, duration of incubation time for next experiment (sensitivity and selectivity test) were considered fifteen minutes.

3.4. Principle of the whole cell FRET immunosensor

The principles of the fluorescence approach for bacteria cell detection based on antibody and FRET between GQDs and GO are shown in Scheme 1. The specific interaction of the GQDs conjugated antibody to bacteria cells (Fig. 4), caused inhibitory effect on π-π interaction among GQDs and GO. *Campylobacter jejuni* is selectively captured by GQD-antibody, which emits blue fluorescence when excited at 350 nm, and then GO was added. When the GQDs- antibody was incubated with *Campylobacter jejuni* cells, the binding of the antibody to the cell membrane was happened and GQD weakly interact with GO, therefore GQD- antibody fluorescence emission was slightly quenched. But in the absence of *Campylobacter jejuni*, GQD- antibody have wide interaction with GO through π-π stacking, thus GQD fluorescence emission was quenched by GO.

3.5. Sensitivity of experiment

As discussed about principle of fluorescence immunosensor sensor, GO could interact with GQD that leads to quenching of fluorescence property. Based on this fact serial dilution of *Campylobacter jejuni* cells from 10-10^6 CFU to identify the ability of this technique in quantitative analysis was tested. As revealed in Fig. 4, with increasing number of bacterial cells specifically the intensity of fluorescence emission in 450 nm increases. With raising the number of bacteria, interaction of GQDs- antibody with bacteria increased thus π-π interaction of GO with GQDs decreased consequently and GO quenching effect on fluorescence property was lowered and intensity of blue emission in 450 nm increased. The increase in fluorescence intensity in 450 nm are linearly related to the number of bacterial cells. The regression coefficient was obtained 0.996 by plotting the fluorescence intensity ratio against the number of bacterial cells. Detection limit of this FRET immunosensor for *Campylobacter jejuni* cells was obtained 10 cells that was comparable to previously reported studies in Table 1 (Wei et al., 2007; Wang et al., 2014; Masdor et al., 2016; Huang et al., 2010; Che et al., 2001). With regard to prior studies, our proposed FRET immunosensor displayed to

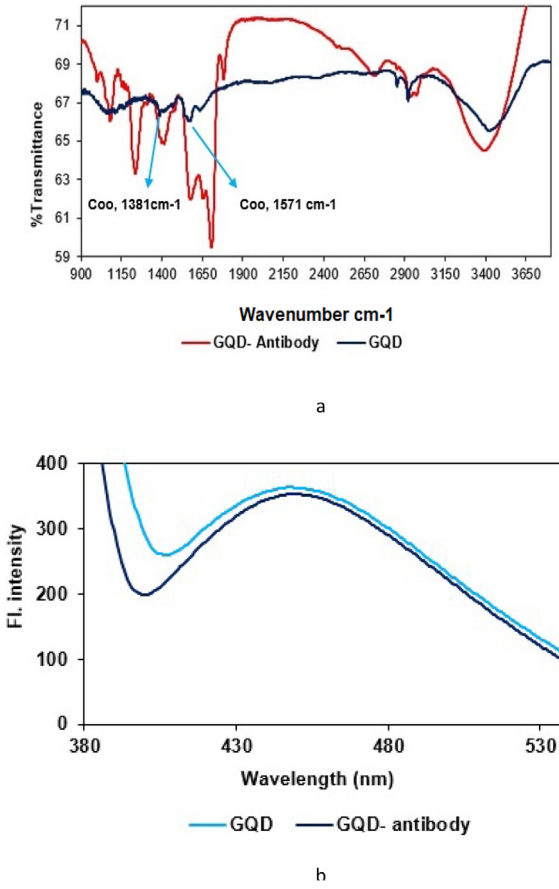


Fig. 2. (a) FT-IR spectrum and (b) Fluorescence intensity of GQD and GQD conjugated with antibody.

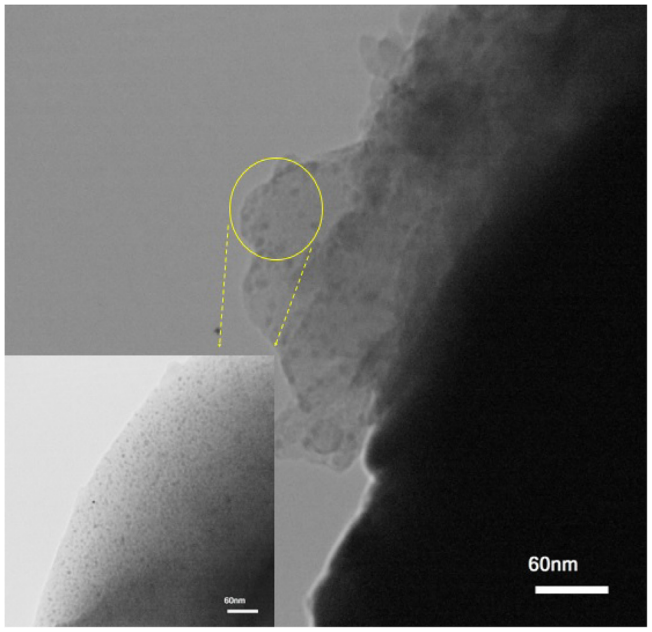


Fig. 3. TEM image of GQD – antibody interaction with *Campylobacter jejuni.*

be very sensitive, specific and less time-consuming for *Campylobacter jejuni* whole cell detection.

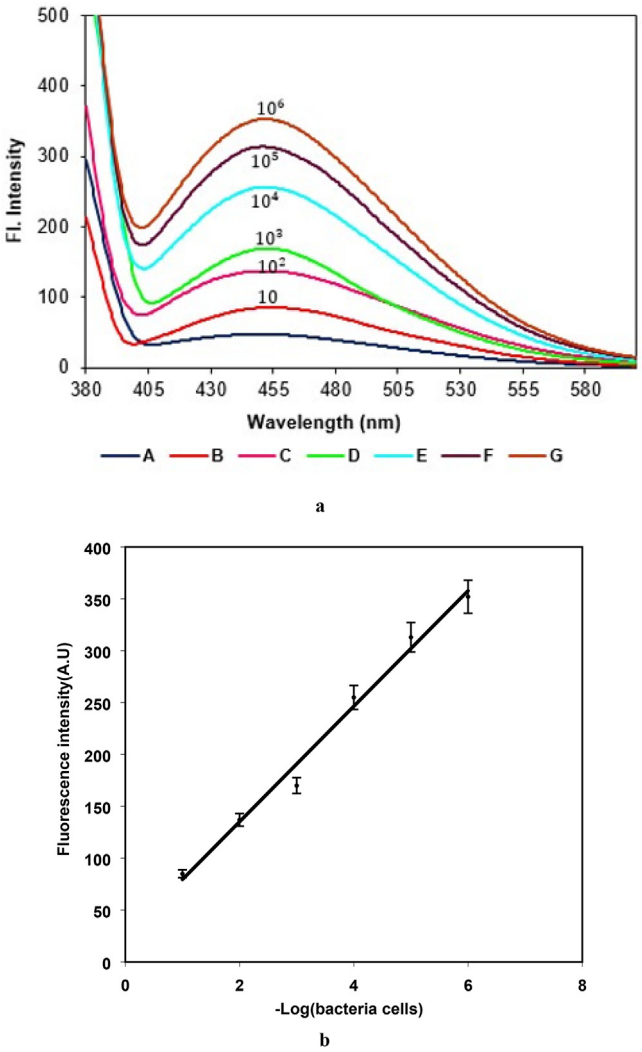


Fig. 4. (a) Fluorescence spectra of GQDs- antibody/ GO, in the presence of various number of *Campylobacter jejuni* cell, A: Control, B: 10^1 CFU, C: 10^2 CFU, D: 10^3CFU, E: 10^4 CFU, F: 10^5 CFU, G: 10^6 CFU (b) the corresponding plot of Fluorescence spectra of GQDs- antibody/ GO in the existence of various log. number of *Campylobacter jejuni*.

Table 1
Comparison of different techniques for revealing Campylobacter jejuni bacterial cell.

Method	Linear range/ cells/ml	LOD	Reference
QCM sensor	–	150	[Masdor, N. A]
electrochemical impedimetric immunosensor	1.0×10^3 to 1.0×10^7		[Huang. J]
ELISA	10^2–10^7	2.1×10^4	[Che. Y]
SPR	–	10^3	[Wei. D]
Fluorescent Immunoassay	–	20–30	[Wang. H]
FRET immunosensor based on graphene oxide and graphene dot	10–10^6	10	This work

3.6. Selectivity of experiment

Potential of this FRET immunosensor in selective detection of *Campylobacter jejuni* toward *Campylobacter coli*, *Helicobacter pylori* and *E. coli* was assessed, and results is accessible in Fig. S4. Non–*Campylobacter jejuni* cells were interacted with the GQD-antibody/GO

at the equal concentration of *Campylobacter jejuni* (10^6 CFU) and just *Campylobacter coli* show slight cross reactivity but other three bacterial cell have no similar fluorescence intensity with that of *Campylobacter jejuni* at the same number of bacterial cells. This phenomenon was due to specific interaction and binding between outer membrane protein of *Campylobacter jejuni* and antibody. But slight interaction among GQD-antibody with *Campylobacter coli*, resulted from the use of polyclonal antibody in making the FRET immunosensor. This antibody does not have affinity to other bacterial cell due to dissimilar antigens existing in various cell membrane. π- π stacking interaction between GQD-antibody and GO was high and fluorescence quenching increased.

3.7. Campylobacter jejuni detection in real sample

For evaluating ability of suggested FRET immunosensor in real sample under the effect interfering factors, poultry liver sample was chosen. Sample preparation was performed based on the work of Che et al. (2001) with few small modifications. Samples were put in to nylon bag and 100 mL buffered peptone water (0.1%) added followed by shaking for 5 min at room temperature. Then, nylon bag content was filtrated through whatman filter paper, and filtrate solution were preserved in clean beaker until use. Now, various numbers of the *Campylobacter jejuni* cells (from 10 to 10^6 CFU) were spiked to the prepared poultry liver samples. In the next step, these real samples were incubated with GQD-antibody/ GO in 20 mM PBS buffer (pH 7.0) for 45 min. Finally, fluorescence intensity of samples was measured and data were collected. As shown in Fig. S5, fluorescence peak in 450 nm associated with number of bacteria (10^2 to 10^6 CFU) have logical increase and the recoveries were in the range of 93.3–104.1% which indicate that the species in the matrix of the poultry liver samples didn't interfere with the assay. These results indicate that, the suggested FRET immunosensor is capable for the sensing of *Campylobacter jejuni* in food and other biotic samples.

3.8. Campylobacter jejuni detection by ELISA

The serial dilution of 6 concentrations of *Campylobacter. jejuni* bacteria was performed for evaluating ability of ELISA test in revealing bacteria. Results showed that 10 and 100 CFU have no absorption peak in UV–vis spectrum (Fig. S6). Therefore, ELISA technique did not have much of success in detecting low concentrations of bacteria, which is in accordance with former study by Che et al. (Che et al., 2001), but our recommended FRET immunosensor, not only was completed in simple steps compared to ELISA test, but also acts with greater sensitivity.

4. Conclusions

In this study we investigated a FRET immunosensor based on GQDs and GO for the selective and sensitive identifying of *Campylobacter. jejuni*. The detection limit of this method in PBS buffer was 10 CFU/mL and in poultry liver sample around 100 CFU/mL. Compared to other reported approaches that require several steps such as labeling tag, this method was simple. Pathogen detection with this method can be completed within 1.5 h, that is faster than SPR, ELISA, electrochemical and conventional systems. This technique can be proposed in other filed cell capturing. Additionally, separation of different pathogens in food sample could also be studied.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

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