Validity of a single antibody-based lateral flow immunoassay depending on graphene oxide for highly sensitive determination of *E. coli* O157:H7 in minced beef and river water

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Abstract

Considering the health risks of *E. coli* O157:H7 presence in food and water, an affordable and highly sensitive detection method is crucial. Herein, we report the first use of a single antibody-based fluorescent lateral flow immunoassay (FLFIA) depending on non-radiative energy transfer between graphene oxide and quantum dots for determination of *E. coli* O157:H7 in beef and river water. FLFIA showed a high sensitivity rate thousand-fold better than the conventional lateral flow (LF). In inoculated minced beef and river water samples, the limits of detection were 178 and 133 CFU g^{-1} or mL^{-1}, respectively. Besides, it presented a high selectivity in the presence of other possible interfering bacteria. The single antibody approach reduced the assay cost to 60% less than the conventional LF. Alongside, the results could be read by portable LF readers or smartphones. These advantages offer FLFIA as a promising technology for pathogen detection in food and water.

Keywords

Graphene oxide, fluorescent lateral flow, *E. coli* O157:H7, food safety, minced beef, water quality

Chemical compounds studied in this article

Phosphate buffered saline (PubChem CID: 24978514); Tween-20 (PubChem CID: 443314); Graphene Oxide (PubChem CID: 124202900); Streptavidin-conjugated CdSe/ZnS quantum dots (Qdot ^{TM} 655) (PubChem CID: 121237577)

1. Introduction

Foodborne and waterborne pathogens, mostly bacteria, may get into our bodies through contaminated food and water leading to several health disorders varying from mild diarrhoea to death, and great losses in productivity as well. *E. coli* O157:H7 is the most frequently reported serotype of Shiga toxins-producing *E. coli* (STEC) in foodborne-linked hospitalizations and deaths in the United States (Scallan et al., 2011). Beef has been incriminated in most food infection outbreaks by *E. coli* O157:H7 (CDC, 2009). However other sources such as drinking water, dairy products and vegetables were previously reported as well (Olsen et al., 2002, Islam, Doyle, Phatak, Millner and Jiang, 2004, Lorusso et al., 2011, Tsiraki, Yehia, Elobeid, Osaili, Sakkas and Savvaidis, 2018). The health problems that could be induced by *E. coli* O157:H7 infection range from mild watery diarrhoea to life-threatening conditions such as haemolytic uremic syndrome and haemorrhagic colitis especially in children and the elderly (Jay, 2000). Considering the health risks of *E. coli* O157:H7 and its impact on food safety, rapid, affordable and highly sensitive methods of detection are necessary to monitor food and water contamination to protect the consumers from the danger of that foodborne hazard.

The currently available methods of *E. coli* O157:H7 detection that depend on culturing and then biochemical and serological examination usually take a couple of days to be completed, while molecular biology-based techniques might be required for confirmation of the results. Nevertheless, such conventional methods are reliable and quite accurate, they are not user-friendly as they require well-trained technicians, and relatively sophisticated laboratory equipment, as well as their high costs (Johnson, Brooke and Fritschel, 1998, Ngwa, Schop, Weir, León-Velarde and Odumeru, 2013, Zhou, Zou, Li, Sun, Ren, and Li, 2018). Immunoassays became one of the most popular approaches in analytical determination of countless kinds of pathogens in various samples, since they are moderately sensitive and selective. Nonetheless, immunoassays such as ELISA and microarrays are laboratory-based techniques that require multiple complex procedures to be done by well-trained operators, as well as they detect *E. coli* O157:H7 in food at a limit of detection (LOD) ranges from

10^5 to 10^6 colony forming units per mL or g (CFU mL^{-1} or g^{-1}) or even higher (Firstenberg-Eden and Sullivan, 1997, Arbault, Buecher, Poumerol, and Sorin, 2000, Shen et al., 2014, Zhaohui, Chunyang, Yingchun, and Yanbin, 2017, Kim, Jo, Mun, Noh, and Kim, 2018). Conversely, lateral flow (LF) immunoassays are one of the most important analytical tools nowadays, since they are simple, robust, portable, and rapid devices. Though, those conventional LF immunoassays -which based on gold nanoparticles, latex beads, or quantum dots as labels- always need three antibodies; one for capturing the bacteria (conjugate pad antibody), a second one for detecting the bacteria (test line antibody), and a third one as a control line antibody, which means extra costs spent by such devices (Berg et al., 2015, Zhang et al., 2015, Kim et al., 2018). Moreover, their LOD of *E. coli* O157:H7 in water and minced beef is about 10^5 CFU mL^{-1} or g^{-1} (Hassan, de la Escosura-Muñiz and Merkoçi, 2015). However, it has been assumed that exposure to < 100 cells of *E. coli* O157:H7 is enough to induce infection in humans. As the Food and Agriculture Organization of the United Nations and the World Health Organization (FAO/WHO, 2018) reported numerous food poisoning outbreaks by *E. coli* O157:H7 at doses as low as 5 CFU / g of food. So, they stated that the presence of *E. coli* O157:H7 at or above one CFU / 25 g constitutes a risky food commodity. Accordingly, the detection of this dangerous pathogen by conventional LF assays might result valueless in particularly demanding contexts. Consequently, the food and environment hygienists are in need to another simple, portable and rapid device that must be affordable, highly sensitive, and highly specific for rapid *in-situ* determination of *E. coli* O157:H7 in complex food matrices under the field conditions.

Our group has been studying the quenching capabilities of graphene oxide (GO) based on the fluorescence resonance energy transfer (FRET), and its interaction with photoexcited quantum dots (QDs) (Morales-Narváez and Merkoçi 2012, Morales-Narváez, Hassan and Merkoçi, 2013, Morales-Narváez, Naghdi, Zor, and Merkoçi, 2015; Cheeveewattanagul et al., 2017, Zamora-Gálvez, Morales-Narváez, Romero and Merkoçi, 2018). We had previously patented a highly sensitive pathogen-detection device for the sensing of *E. coli* in a standard buffer (Morales-Narváez

et al., 2013, Patent: EP 13188693.9, 2015). However, using a traditional glass slide-based microarray system as a biosensing platform was quite expensive and not suitable for portability. Therefore, paper-based lateral-flow assay was another low-cost option in another study done by our group (Morales-Narváez et al., 2015). While, that study was limited to the detection of general *E. coli* in buffer and bottled water by using QDs/anti-*E.coli* antibody. Although, assessment of the validity of this GO-based LF immunoassay for detection of pathogenic *E. coli* O157:H7 in real samples of highly complex matrices such as minced beef and river water is another hot topic worthy to be investigated, since those samples are the main source of human infections by that pathogen. Herein, we report the first exploit of FRET-based quenching properties of GO, and their interaction with QDs for development of a fluorescent lateral flow immunoassay (FLFIA) for determination of the highly pathogenic *E. coli* O157:H7 in minced beef and river water. The detection part of that strip has two lines; a test line (TL) which composed of CdSe@ZnS QDs/anti-*E.coli* O157:H7 antibody that works as a fluorescent probe and a control line (CL) that composed of only bare QDs. GO is added to the LF strip as a quencher for the fluorescent QDs after adding the sample to divulge the presence of bacteria. If the sample does not have *E. coli* O157:H7, the test line will be efficiently quenched when adding GO by FRET, since the distance between QDs/Abs (donor) and GO (acceptor) is few nanometres (Gaudreau, Tielrooij, Prawiroatmodjo, Osmond, de Abajo, and Koppens, 2013, Lin et al., 2013). On the other hand, if the sample has *E. coli* O157:H7, it will be selectively captured by the specific QDs/Abs probe on the test line, then after adding GO, resonance energy transfer is hindered or minimally occurs since the distance between GO and QDs exceeds to more than 20 nm by the bacteria interference (Gaudreau et al., 2013, Lin et al., 2013). Consequently, the fluorescence of QDs on the test line is maintained, and its intensity is correlational to the concentration of the *E. coli* O157:H7 in the sample. Instead, the control line QDs will be always quenched by GO because this line has not any antibodies to the target pathogen. The principle and reading of FLFIA is fully illustrated in Figure 1.

2. Materials and methods

2.1. Reagents and equipment

All commercial reagents were of analytical grade and they were handled according to the safety data sheets provided by the suppliers. Goat polyclonal Anti-*Escherichia coli* O157:H7 antibody (conjugated with biotin) (LS-C525826-100) was purchased from LifeSpan BioSciences (Seattle, WA, USA), and streptavidin-conjugated CdSe/ZnS quantum dots 655 (QDs) (Cat. No. Q10121MP) were obtained from Life Technologies (Carlsbad, CA, USA). Phosphate buffered saline (PBS) (PubChem CID: 24978514), bovine serum albumin (BSA), and Tween-20 (PubChem CID: 443314) were purchased from Sigma-Aldrich (Madrid, Spain). Graphene oxide (GO) was bought from Angstron Materials (Ohio, U.S.A.). *Escherichia* coli O157:H7 (CECT 4783, *E. coli* O157:H7) and *Salmonella* enterica subsp. enterica serovar Typhimurium LT2 (CECT 722T, *S.* Typhimurium) strains were obtained from the Colección Española de Cultivos Tipo (CECT, Valencia, Spain). TS-100 Thermo-Shaker (Biosan, Riga, Latvia) was used as a stirrer for modification of QDs with antibodies. Laminated cards (HF000MC100), nitrocellulose membranes (SHF1800425), and cellulose fibre (CFSP001700) that were used for fabricating FLFIA strips were purchased from Millipore (Billerica, MA, USA). An IsoFlow reagent dispensing system (Imagene Technology, Hanover, NH, USA) was used for dispensing the TL and CL onto the nitrocellulose membrane. A Dahle 533 guillotine (Dahle, Peterborough, NH, USA) was used to cut the FLFIA strips into 6 mm width. JP Selecta 2000210 oven from JP selecta (Barcelona, Spain) was used to dry the strips. A portable ESEQuant lateral flow reader with its software LF-Studio Version 3.3.6 from Qiagen GmbH (Stockach, Germany) were used to measure the intensities of the TL and CL of FLFIA strips. As well as, fluorescent images of FLFIA strips were produced using a Typhoon 9410 Variable Mode Imager (GE, Freiburg, Germany). The intensities of the lines of those fluorescent images were measured using ImageJ 1.46r (Wayne Rasband, National Institutes of Health, Bethesda, MD, USA). PBS (10 mM, pH 7.4) with 0.5% (v/v) Tween-20 containing 1% of BSA fraction V (w/v) was employed as a standard buffer for preparation of bacterial inocula. While, PBS (10 mM, pH 7.4) with 0.05% (v/v) Tween-20 was used as a washing buffer. All aqueous solutions were freshly

157 prepared in Milli-Q water produced using a Milli-Q system (>18.2 $M\Omega cm^{-1}$) purchased from
158 Millipore (Billerica, MA, USA). Scanning Electron Microscopy (SEM) images were obtained by a
159 Magellan 400L High-Resolution SEM (FEI, Hillsboro, OR, USA).

160 **2.2. Preparation of minced beef extract and bacterial inocula**

161 Minced beef was purchased from a local retail market in Barcelona and analysed by the standard
162 culturing method for the presence of *E.* coli O157 (ISO 16654:2001). Only negative samples of beef
163 and water were selected to be inoculated with bacteria. Twenty-five g of *E. coli* O157-free minced
164 beef were homogenized in a sterile stomacher bag with 225 mL of sterile PBS (10 mM, pH 7.4)
165 using a stomacher (Lab Blender 400, Seward, UK) for 3 min. Then the filtrate was used as a diluent
166 for preparation of bacterial suspensions.

167 For preparation of bacterial inocula, freeze-dried cultures of *E. coli* O157:H7 and *Salmonella*
168 Typhimurium were revived in a sterile tryptone soy broth (TSB, Oxoid Ltd., UK) and incubated at
169 37 ºC for about 24 h, then transferred onto sterile tryptone soy agar (TSA, Oxoid Ltd., UK) plates
170 for another 24 h at 37 ºC. Stock cultures of both strains were kept on TSA slope tubes for future use.
171 Bacterial cell suspensions were prepared directly from bacterial colonies of TSA plates, during the
172 logarithmic phase, in sterile standard buffer and river water to obtain a bacterial load of 1.5×10^8
173 CFU mL^{-1} according to McFarland standards (McFarland, 1907) using Densimat densitometer
174 (Biomerieux, Brazil). Afterwards, ten-fold decimal bacterial dilutions (10 to 10^8 CFU mL^{-1}) were
175 prepared from the original one. Finally, heat killing of the bacteria was done by putting the bacterial
176 suspension in tightly sealed tubes to be placed in a water bath at 80 ºC for 15 min to stop bacterial
177 replication. Regarding minced beef, a suitable volume of heat-killed bacterial suspension in a sterile
178 standard buffer (1.5×10^8 CFU mL^{-1}) was used to prepare ten-fold decimal dilutions of *E. coli*
179 O157:H7 in minced beef homogenate (10 to 10^8 CFU g^{-1}). The prepared bacterial dilutions were
180 stored at 4 °C until being used for the assay within two weeks in case of standard buffer and river
181 water. Whereas inoculated minced beef was used without delay to avoid sample deterioration.

182 **2.3. Fabrication of FLFIA**

The proposed lateral flow strips were prepared as follows: (a) assembling of the nitrocellulose membrane on the laminated card. (b) Dispensing the QDs/anti-*E. coli* O157:H7 as a TL and bare QDs as a CL using an IsoFlow reagent dispensing system on the nitrocellulose membrane. For TL, we used a conjugate composed of 4 nM streptavidin-quantum dots 655 and 300 µg/mL biotinylated anti- *E. coli* O157:H7 polyclonal antibody in standard buffer. The conjugate was prepared through mixing them at 650 rpm/4 °C /30 min. Whereas for CL, we used only 4 nM of streptavidin-quantum dots 655. After line dispensing, the detection pad was kept overnight inside a tightly closed container in the fridge at 4 °C temperature. In the second day, the nitrocellulose membrane (2.5 × 20 cm) was homogenously treated with 5 mL of standard buffer, then kept in the fridge for 15 min before drying in the oven at 37 °C for about 3 h. (c) Some pieces of cellulose sample and absorbent pads (≈20 cm each) were saturated sequentially with Milli-Q water, and standard buffer, then they were kept in the oven at 37 °C for overnight until complete dryness. (d) Afterwards, assembling the sample and absorbent pads on the same laminated card. (e) Ultimately, cutting the assembled card using a clean guillotine into strips of 6 mm in width. The strips were kept in a tightly closed plastic container with some drying pearls in the fridge until use for bacteria determination.

2.4. Using FLFIA for *E. coli* O157:H7 detection in standard buffer

In order to use the prepared FLFIA strips for detection and quantification of *E. coli* O157:H7 in various samples, the initial photoluminescence intensities (I_1) of both TL and CL were measured using a portable lateral flow reader (Figure 1C). Then 100 µL of previously prepared *E. coli* O157:H7 suspension of various concentrations in standard buffer was added onto the sample pad of the fabricated strip, the strips were left for about 15 min until complete flow of the sample to the absorbent pad. Afterwards, 100 µL of PBS with 0.05 % tween 20 (v/v) was dispensed on the sample pad as a washing buffer, to remove any kind of intervention. Then, they were left at room temperature for around 10 min until complete flow of the washing buffer. Subsequently, 100 µL of aqueous solution of graphene oxide (GO) 150 µg mL^{-1} contains 0.1 % Tween-20 (v/v) was dispensed on the sample pad, for revealing the presence of bacteria. A final step of dryness was

done before reading the final photoluminescence intensities (I_2) of both lines using the LF reader. The ratio of the final intensity to the initial one (I_2/I_1) of the test line (R_{TL}) was used as an estimation for the concentration of the target bacteria in the sample.

2.5. Validation of FLFIA in real samples

To evaluate the overall performance of the proposed assay in real samples, artificially inoculated minced beef and river water with serial concentrations of *E. coli* O157:H7 (0, 50, 10^2, 10^3, 10^4, 10^5, and 10^6) CFU g^{-1}/mL^{-1} were used. The same abovementioned procedure used with standard buffer was conducted with real samples as well. Calibration curves were created for each sample type at decimal concentrations of bacteria. The linear regression and coefficient of determination (R^2) were calculated for both minced beef and river water.

Furthermore, spike and recovery experiment was conducted to distinguish how much the complex matrix of real sample could affect the performance of our FLFIA. Two concentrations of *E. coli* O157:H7 (10^3 and 10^4 CFU mL^{-1} or g^{-1}) were spiked in each of standard buffer, minced beef, and river water. At least 3 replicates were used in each concentration. The average R_{TL} of spiked minced beef and river water was compared to that of standard buffer at the same concentration to estimate the recovery percentage according to the following equation; recovery %= R_{TL} of real sample/R_{TL} of standard buffer.

As well as, the specificity of FLFIA against non-specific pathogen was tested. *Salmonella* enterica subsp. enterica serovar Typhimurium (*S.* Typhimurium), a Gram-negative pathogen from the same *Enterobacteriaceae* family of *E. coli* O157:H7, was used to conduct the specificity test. In this experiment, we evaluated the response of FLFIA to the presence of *S.* Typhimurium either alone or in a mixture with *E. coli* O157:H7, as well as it was compared with blank buffer. Blank (0 CFU mL^{-1}), single *S.* Typhimurium (10^4 CFU mL^{-1}), single *E. coli* O157:H7 (10^2 and 10^4 CFU mL^{-1}), and two mixtures of both bacterial species (*E. coli* O157:H7 10^2 + *S.* Typhimurium 10^4 and *E. coli* O157:H7 10^4 + *S.* Typhimurium 10^2 CFU mL^{-1}) were prepared in standard buffer to conduct such experiment.

Additionally, the reproducibility of the assay was evaluated by estimating the variation coefficient through calculating the relative standard deviation (RSD %) along different batches of FLFIA strips used throughout the study.

3. Results and discussion

3.1. Optimization of fluorescence and quenching process

Since the currently available conventional LF immunoassays based on gold nanoparticles or latex beads used for *E. coli* O157:H7 detection in various food and water samples are of high costs (≈ 0.30 USD / test strip) and low sensitivity (≈ 10^5 CFU mL^{-1} or g^{-1}) (Karakus and Salih, 2013; Hassan, et al. 2015; Luo et al., 2017; Han et al., 2018), the food and water monitoring may require another simple, portable, affordable and highly sensitive device. Herein, we designed a novel fluorescent lateral flow immunoassay based on the interaction between photoexcited molecules and quencher. We exploited streptavidin functionalized CdSe@ZnS QDs, of an approximate diameter of 14±2 nm and a maximum emission wavelength at ≈ 665 nm, as donors of non-radiative energy that makes them a powerful fluorescence agent. As well as, we used GO sheets in the form of water-based dispersion of an average lateral dimension range of 500 nm, an average thickness of approximately 1.1 nm and C/O ratio of about one unit (according to manufacturer`s data), as acceptors for the non-radiative energy leading to highly effectual quenching of fluorescence (Morales-Narváez et al., 2013). SEM images illustrated in Figures 2C and $2C_1$ show GO sheets in water suspension surrounding to *E. coli* O157:H7 cells. In addition, Figure $2C_2$ shows bare GO suspension of the same concentration. Since the distance between the QDs and GO is very crucial for non-radiative energy transfer between them as Lin et al. (2013) recorded that quenching is not strongly observable at distances greater than 20 nm, so here the target bacteria (≈ 0.5×2 µm size) acts as a spacer between the donor and the acceptor hindering the photons transfer and keeping the fluorescence of QDs. Figure 2D shows a SEM micrograph of QDs-Ab conjugates are capturing to bacterial cells of *E coli* O157:H7.

To get the most suitable photoluminescence, different concentrations of QDs (1.5, 3, 4, 6, 8, 9, and 10 nM) were dispensed on nitrocellulose membranes and their intensities were measured by a portable LF reader (data not shown). The LF reader used in this study has an excitation wavelength of 365 nm, and an emission filter of about 670 nm. Hence, 4 nM was chosen as the appropriate concentration that gives about 80% of the dynamic range of the reader (Figure 1C). Additionally, since the concentration of the acceptor molecules should affect the rate of photons transfer from the donor to the acceptor, so, different concentrations of GO suspension in Milli-Q water (60, 70, 80, 100, 150 and 200 µg mL^{-1}) with two concentrations of Tween-20 (0.05 and 0.1 %) were investigated to optimize the most suitable quenching conditions. The presence of Tween-20 in the GO suspension aids the process of GO flow through the nitrocellulose. A hundred µL of each concentration was added onto a blank strip, the TL and CL intensities were measured before and after addition of GO. The degree of quenching (I_2/I_1) was calculated by dividing the final intensity (I_2) by the initial one (I_1). GO 150 and 200 µg mL^{-1} with 0.1 % Tween-20 (v/v) achieved the highest quenching rates ($I_2/I_1 \approx 0.3$-0.4) (Figure 3A). However, GO 150 µg mL^{-1} with 0.1 % Tween-20 (v/v) was preferred because it achieved the most reliable results afterwards, in terms of steady performance and error rate. In conclusion, 4 nM QDs and GO 150 µg mL^{-1} with Tween-20 (0.1 % v/v) were the most appropriate condition for proper photoluminescence and quenching of blank strips.

Since bacterial cells are much bigger (≈ 0.5 ×2.0 µm, Figure 2A) than other analytes like proteins, a nitrocellulose membrane with big pore size (the diameter of the largest pore in the filtration direction) was essential for our proposed assay. Moreover, there is an inverse relationship between the flow rate and sensitivity of the assay, that means slow flow rate should give highly sensitive assays, because it allows longer time of interaction between the antibody and the target analyte, while fast flow rate reduces the sensitivity. Thus, to develop a highly sensitive assay for a big analyte like bacteria, Hi-Flow 180 nitrocellulose membrane (SHF1800425) of slow flow rate (≈ 180 seconds/4 cm) was chosen out of others to develop our assay. Figure 2B demonstrates SEM image

of HF 180 nitrocellulose membrane used in this study, it proves that the pore size (≈ 8 - 20 µm) is big enough to allow the proper flow of bacteria. Besides, it shows the difficulty of distinguishing between bacterial cells and nitrocellulose tissue by SEM. So, all SEM images of bacterial cells (Figures 2A, 2C and 2D) were prepared on silicon discs not on nitrocellulose. The total cost of each strip of this fluorescent lateral flow assay was previously estimated to be ≈ 0.12 USD (Zamora-Gálvez et al., 2018), which is considered about 60% less than that of conventional lateral flow strip.

3.2. Optimization in standard buffer

To evaluate the overall performance of the proposed FLFIA, serial concentrations of *E. coli* O157:H7 (0, 10^2, 10^3, 10^4, 10^5 and 10^6 CFU mL^{-1}) in standard buffer were investigated. A hundred µL of each concentration was loaded onto the sample pad of FLFIA strips, then followed by 100 µL of GO 150 µg mL^{-1} with Tween 20 (0.1 % v/v). A drying step of the strips for almost an hour in an oven at 35 °C before reading them using a portable lateral flow reader was essential because QDs have better photoluminescence capabilities in the solid phase than the liquid one (Shi et al., 2010). Afterwards, the ratio of the final intensity of TL (I_2, after addition of GO) to the initial one (I_1, before addition of the sample) was calculated and used as an indicator to the presence or absence of *E. coli* O157:H7. We refer to it in this paper as R_{TL}= I_2/I_1 of TL. As high R_{TL} (close to one) indicates low quenching rate and high concentration of bacteria, whereas low R_{TL} (close to zero) indicates high quenching rate and low concentration or absence of bacteria. On the other hand, I_2/I_1 of CL = R_{CL} should be unchangeable with varying bacteria concentrations, since there are not any antibodies on the CL. However, CL is essential to prove the successful flow of GO along the strip. The obtained results showed an elevation in R_{TL} with increasing the concentration of bacteria in the standard buffer, which means that the target *E. coli* O157:H7 is captured by the specific antibody of TL (anti-*E. coli* O157:H7). However, a similar phenomenon was observed in R_{CL} as well. That indicates some bacterial cells halt over CL and act as a spacer between GO and QDs there, thus leading to non-specific response of CL (Figure 3B). Therefore, a washing step with 100 µL of PBS with 0.05 % Tween 20 (v/v) by dispensing onto the sample pad after complete flow of the bacteria-

containing buffer along the strip (approximately after 15 min) was suggested to remove any kind of non-specific response before addition of GO.

Obviously, this washing step has improved greatly that issue of non-specific response of CL, leading to almost constant R_{CL} with varying concentrations of *E. coli* O157:H7 in the buffer (Figure 3C), while R_{TL} increased progressively with increasing the concentration of bacteria (from zero to 10^5 CFU mL^{-1}) and in a logarithmic manner from 50 to 10^5 CFU mL^{-1} with R^2 equals 0.9874. Then this response slightly decreased in concentrations higher than 10^5 CFU mL^{-1} (Figure 4A, 4B). This decline behaviour in response could be attributed to blocking the nitrocellulose membrane by the enormous number of bacteria that lead to hindering the bacterial flow. A similar phenomenon was previously reported by some literatures such as Hassan et al. (2015) who reported a decline behavior in commercial gold nanoparticles-based lateral flow kits for *E. coli* O157:H7.

To estimate the sensitivity of FLFIA for detection of *E. coli* O157:H7 in standard buffer, the mean R_{TL} of blank samples plus 3 times its standard deviation (SD) was calculated and used to determine the limit of detection (LOD) of the assay. Fascinatingly, the estimated LOD of FLFIA was calculated to be 57 CFU mL^{-1} of *E. coli* O157:H7 in standard buffer (Figure 4A). This achieved LOD by our assay was about thousand-fold better than the conventional lateral flow assays (LOD ≈ 10^5 CFU mL^{-1} or g^{-1}) that depend on a sandwich-type immunoassay on the TL and a third Ab on the CL (Karakus and Salih, 2013; Hassan, et al. 2015; Luo et al., 2017; Han et al., 2018). However, the proposed FLFIA requires only one antibody on the TL and without any antibodies on the CL.

3.3. Specificity of FLFIA

The specificity of immunoassays is another crucial parameter of any innovative approach. The obtained results summarized in Figure 4C showed that the average R_{TL} produced by *S.* Typhimurium (10^4 CFU mL^{-1}) was lower than the blank`s one. Equally, the mixture of *E. coli* O157:H7 10^2 + *S.* Typhimurium 10^4 CFU mL^{-1} gave a response similar to *E. coli* O157:H7 10^2 CFU mL^{-1}. Likewise, *E. coli* O157:H7 10^4 + *S.* Typhimurium 10^2 CFU mL^{-1} and *E. coli* O157:H7 10^4 CFU mL^{-1}. That experiment proved the high selectivity of the proposed FLFIA to the target

pathogen (*E. coli* O157:H7), without any interferences from non-specific bacteria present in the same medium.

3.4. Using FLFIA for determination of *E. coli* O157:H7 in real samples

Even though, investigation of the performance in standard buffer is quite important for the optimization process, the evaluation in complex matrices is vital for validation of new methods. The data illustrated in Figure 5 summarize the performance in real samples. The obtained results in minced beef and river water showed a similar scenario to that of standard buffer. As R_{TL} elevated regularly in a logarithmic response in concentrations from 50 to 10^5 CFU g^{-1}/mL^{-1}, with R^2 equals 0.9592 and 0.9542 in minced beef and river water samples, respectively. Then the response slightly declined in concentrations higher than 10^5 CFU g^{-1}/mL^{-1} in both sample types, however, it is still within the positive range. The same decline behaviour in response to high concentrations happened with standard buffer, which confirms that this behaviour is due to the blockage of flow by the vast bacterial number in higher concentrations.

LOD of FLFIA in minced beef and river water was estimated by calculating the averages R_{TL} of FLFIA strips tested with at least 3 replicates of blank minced beef and blank river water plus 3 times their SD. The obtained LOD in minced beef samples was *ca.* 178 CFU g^{-1}, while it was *ca.*133 CFU mL^{-1} in river water ones (Figure 5A). The reduced sensitivity in minced beef and river water than standard buffer is attributed to the matrix effect of real samples. Similar effect of the sample matrix on immunoassays were previously reported by Aydin, Herzig, Jeong, Dunigan, Shah, Ahn (2014), Hassan et al. (2015), Luo et al., (2017), Han et al. (2018) and Kim et al. (2018). However, our achieved LODs in real samples do not affect the reliability of FLFIA and confirm its high sensitivity in comparing with conventional immunoassays. The ability to detect *E. coli* O157:H7 at such low concentrations without broth enrichment designates that FLFIA could be used to determine as low as one CFU g^{-1} or mL^{-1} of *E. coli* O157:H7 in minced beef and water samples after about 3 h of broth enrichment of the sample, since *E. coli* O157: H7 could duplicate by mitotic division every 15-20 min under favourable conditions (Buchanan and Klawitter, 1993).

By comparing FLFIA in terms of LOD with other reported rapid methods, which were depending on sandwich antibody formats, more complicated techniques and/or more expensive approaches for determination of *E. coli* O157:H7 in various food samples, we noticed the high sensitivity of our costless approach over those more complicated and expensive technologies. For instance, Aydin et al. (2014) reported 250 CFU g^{-1} as a LOD of *E. coli* O157:H7 in ground beef using magnetic bead–based immunoassay coupled with tyramide signal amplification after 3 h of enrichment. Hassan et al. (2015) reported *E. coli* O157:H7 LODs of 457 and 309 CFU g^{-1} or mL^{-1} in minced beef and tap water samples, respectively, through using gold nanoparticles-labelled antibody sandwich-based electrochemical detection. Additionally, Song, Li, Liu, Liu (2016) reported an *E. coli* O157:H7 LOD of 10^5 CFU g^{-1} or mL^{-1} in bread, milk and jelly samples using Fluorescein isothiocyanate-based immunosensor. As well as, Luo et al. (2017) compared different immunochromatographic labels for lateral flow assays for *E. coli* O157:H7 determination in milk. In that study, they reported LODs accounted for 1×10^5, 2.5×10^4, 1×10^3, 5×10^2 CFU mL^{-1} using gold nanoparticles, quantum dots, fluorescent nanoparticles, and europium chelate nanoparticles as labels, respectively. Eventually, Han et al. (2018) mentioned that the sensitivity of the nanozyme-based LFA depending on a sandwich antibody format developed by them for *E. coli* O157:H7 was 900 CFU mL^{-1} in milk.

3.5. Spike and recovery test in real samples

The results of spike and recovery experiment are summarized in Table (1). The recovery percentages from minced beef ranged from 92.86 to 95.02 %, while those of river water ranged from 95.11 to 97.98 %. Obviously, the extreme complex matrix of beef affects the assay performance more than that of river water. However, it still performs in an admirable way, adequate for real application requirements. Accordingly, these recovery rates demonstrate that this novel approach is a promising device for determination of *E. coli* O157:H7 in food and water without any interferences from the complex food and water matrices nor other competing microorganisms.

3.6. Reproducibility

Another important parameter for evaluating new analytical technologies is the reproducibility. In this study, for executing all experiments mentioned above, we used different fabrication batches of FLFIA. Among the working range of bacterial concentrations, the FLFIA strips exhibited variation coefficients below 16 % in minced beef and river water. This meets the validation criterion of reproducibility of new immunoassays that was stated by Findlay et al. (2000), who recommended a RSD below 20 % for acceptance of new procedure in terms of reproducibility.

3.7. Possibility of smartphone integration

To prove the possibility of integration of proposed FLFIA into smartphones without the need to a portable lateral flow reader, another device for reading the line intensities was tried. A fluorescence image Typhoon scanner was used to take pictures of the strips. Then those scanned pictures were analysed using ImageJ 1.46r software to determine line intensities (Figure 1B). A similar procedure with smartphones could be used through a 3D-printed cassette containing an excitation LED for holding the FLIFA strip to enable smartphone camera to capture the fluorescence and then an ImageJ application (smartphone version) be used for analyzing the picture. This proof of concept makes it a highly promising device for automation, portability, and field applications without the need for a highly equipped laboratory.

3.8. FLFIA versus traditional methods for detecting *E. coli O157* in random food samples

Herein, we summarize the whole procedure of using FLFIA to analyze a random unknown food sample for the presence of *E. coli* O157:H7, in comparing with the traditional method in terms of procedure and assay time. In case of FLFIA, firstly, 25 g or mL of the food sample is homogenized or mixed with 225 mL of a pre-warmed modified tryptone soya broth plus novobiocin (mTSB+N) at 41.5 °C ± 1°C and then incubated for 3 h. Meanwhile, the TL initial intensity of FLFIA strip being recorded using a portable LF reader. Then a 100 µL of the incubated sample broth is added onto the sample pad. Wait for 10 minutes to allow sample flow. Subsequently, a 100 µL of washing buffer, followed by a 100 µL of GO solution are added. After strip dryness, record the TL final intensity. If the R_{TL} is < 0.4 indicates a negative sample, while if it is ≥ 0.4 indicates a positive sample. Though,

CL should be quenched in both positive and negative samples to confirm the successful flow of solutions through the strip pads. Accordingly, the total assay time of FLFIA is only 5 h, including 3 h of sample enrichment.

On the other hand, in order to detect *E. coli* O157 in food samples, using the traditional horizontal method stated by the International Organization for Standardization (ISO 16654:2001) more than 60 h of sample examination were required to confirm the presence of this pathogen. The detection of *E. coli* O157 by ISO`s method necessitates four successive stages: a) enrichment, b) separation and concentration, c) isolation and d) confirmation. Briefly, the sample was enriched in nine times the weight in mTSB+N for 6 h and subsequently for a further 12 - 18 h. Then *E. coli* O157 were separated and concentrated using immunomagnetic beads coated with anti-*E. coli* O157 antibodies after 6 h and again, if necessary, after a further 12 - 18 h incubation. Afterwards, *E. coli* O157 captured with immunomagnetic particles were subcultured onto cefixime tellurite sorbitol MacConkey agar (CT-SMAC) and the agar plates were incubated at 37 °C /18 - 24 h. Subsequently, typical *E. coli* O157 colonies (sorbitol negative) were streaked onto nutrient agar (NA) and incubated at 37 °C /18 - 24 h. Eventually, *E. coli* O157 on NA was confirmed by indole production and agglutination with *E. coli* O157 antiserum. Thus, the traditional method is laborious, time consuming and of high cost, as well as, it requires well-trained operators and highly equipped facilities. That confirms the advantages of FLFIA over standard traditional methods.

4. Conclusions

In conclusion, we exclusively developed a fluorescent lateral flow immunoassay based on quantum dots as donors of non-radiative energy and graphene oxide as an acceptor for such energy. We used only a single antibody on the test line to capture the target pathogen, which reduced the total assay cost per strip to be 60% less than the conventional LF. This study is the first report of using that principle for *E. coli* O157:H7 detection in minced beef and river water. FLFIA achieved outstanding LODs of *E. coli* O157:H7 (≈133 and 178 CFU mL^{-1} or g^{-1} in river water and mined beef, respectively). Theoretically, this indicates the possibility of detecting as low as one CFU mL^{-1} or g^{-1}

441 of *E. coli* O157:H7 after about 3 h of food sample enrichment in a suitable broth. The detection of
442 *E. coli* O157:H7 by FLFIA in beef complex matrix designates the ability of their using for other
443 food commodities, as well as for other similar bacterial species with changing the antibody. A
444 portable lateral flow reader was used for reading and quantifying the results. Alongside, analysing
445 the images with ImageJ software was proved to be an alternative way for reading the results with
446 smartphones. FLFIA showed numerous advantages in comparing with the standard traditional
447 method of *E. coli* O157 detection, as well as against other previously reported rapid methods.

448 **Declaration of Competing Interest**

449 The authors declare that there is no any conflict of interest in this work.

450 **Acknowledgement**

451 A.H.A. Hassan gratefully acknowledges the financial support from the Science and Technology
452 Development Fund (STDF), Egypt (Grant 25347). E. M.-N. acknowledges the financial support
453 from CONACYT (Mexico, Grant 293523) and National System of Researchers, CONACYT
454 (Mexico, Grant 74314). ICN2 is supported by the Severo Ochoa program from Spanish MINECO
455 (Grant No. SEV-2017-0706 and MAT2017) and by CERCA Programme/ Generalitat de Catalunya.

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Figures:

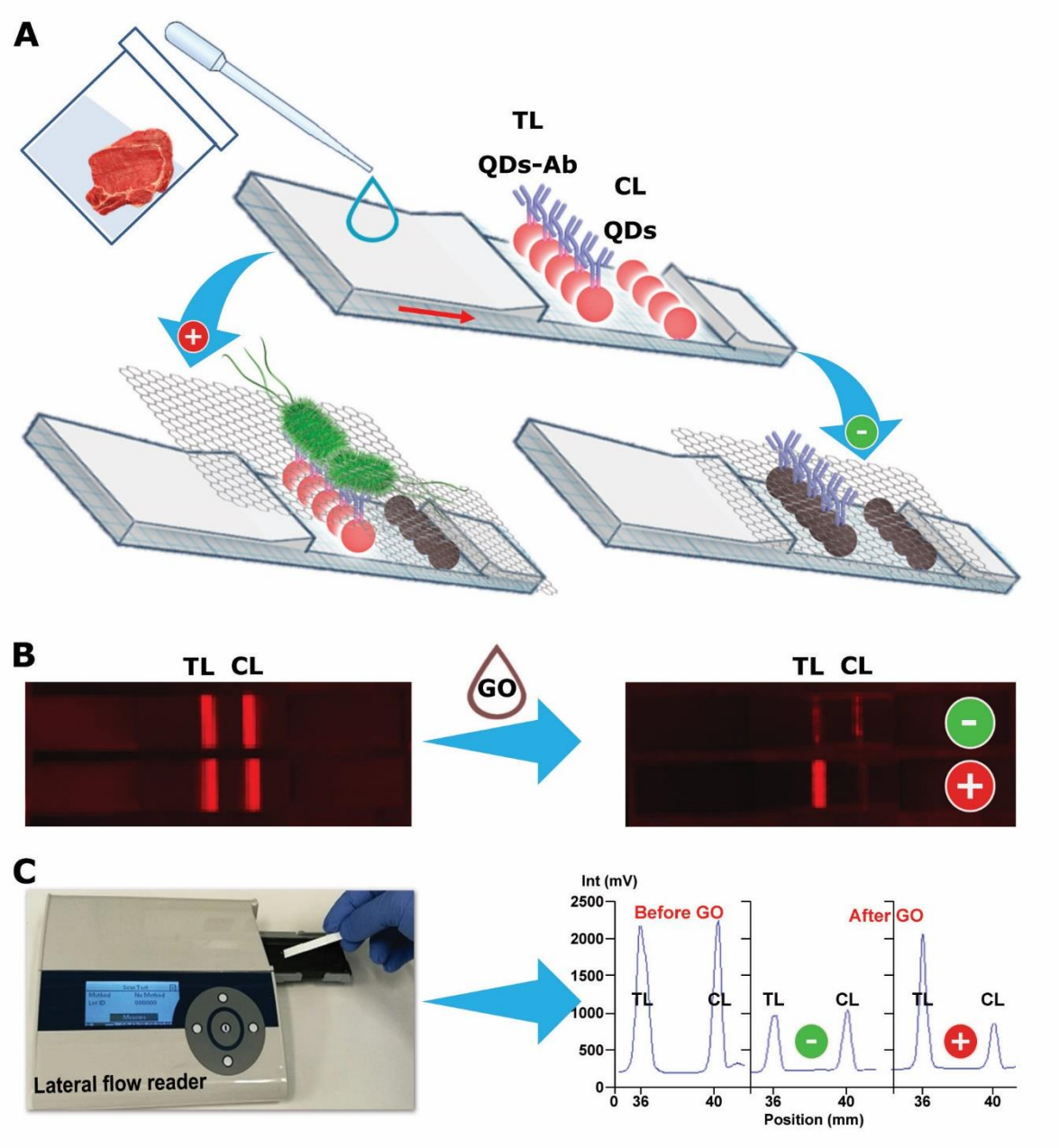


Figure 1. Fluorescent lateral flow immunoassay (FLFIA) principle and reading. A) FLFIA strip is composed of a sample pad, detection part and an absorbent pad. The detection part of FLFIA strip is composed of a test line TL (Streptavidin-Quantum dots conjugated with biotinylated anti-*E. coli* O157:H7 antibody "QDs-Ab") and a control line CL (bare quantum dots "QDs"). When a beef extract or water sample is added to the sample pad of FLFIA strip, it flows by capillary force towards the absorbent pad. If the sample contains *E. coli* O157:H7, the bacteria will be captured by specific antibody-QDs conjugate on the TL. Afterwards, graphene oxide GO is added onto the

sample pad. *E. coli* O157:H7 captured on the TL acts as a spacer between GO and QDs and interrupts the non-radiative energy transfer between them, and this keeps the fluorescence of QDs. On the other hand, the absence of the target bacteria allows the non-radiative energy transfer between GO and QDs on the TL and consequently, quenches the fluorescence of QDs. B) Scanned images with a fluorescence Typhoon reader of two FLFIA strips. Before addition of GO, both TL and CL are fluorescing in both strips. However, after addition of GO, both TL and CL are quenched in negative sample, while, TL of positive sample is still fluorescing. C) Another option of reading the assay is measuring TL and CL intensities by a portable fluorescence lateral flow reader. The measured fluorescence intensities (mV) of both TL and CL, before and after addition of GO to the strip clarify the difference between positive (+) and negative (-) samples.

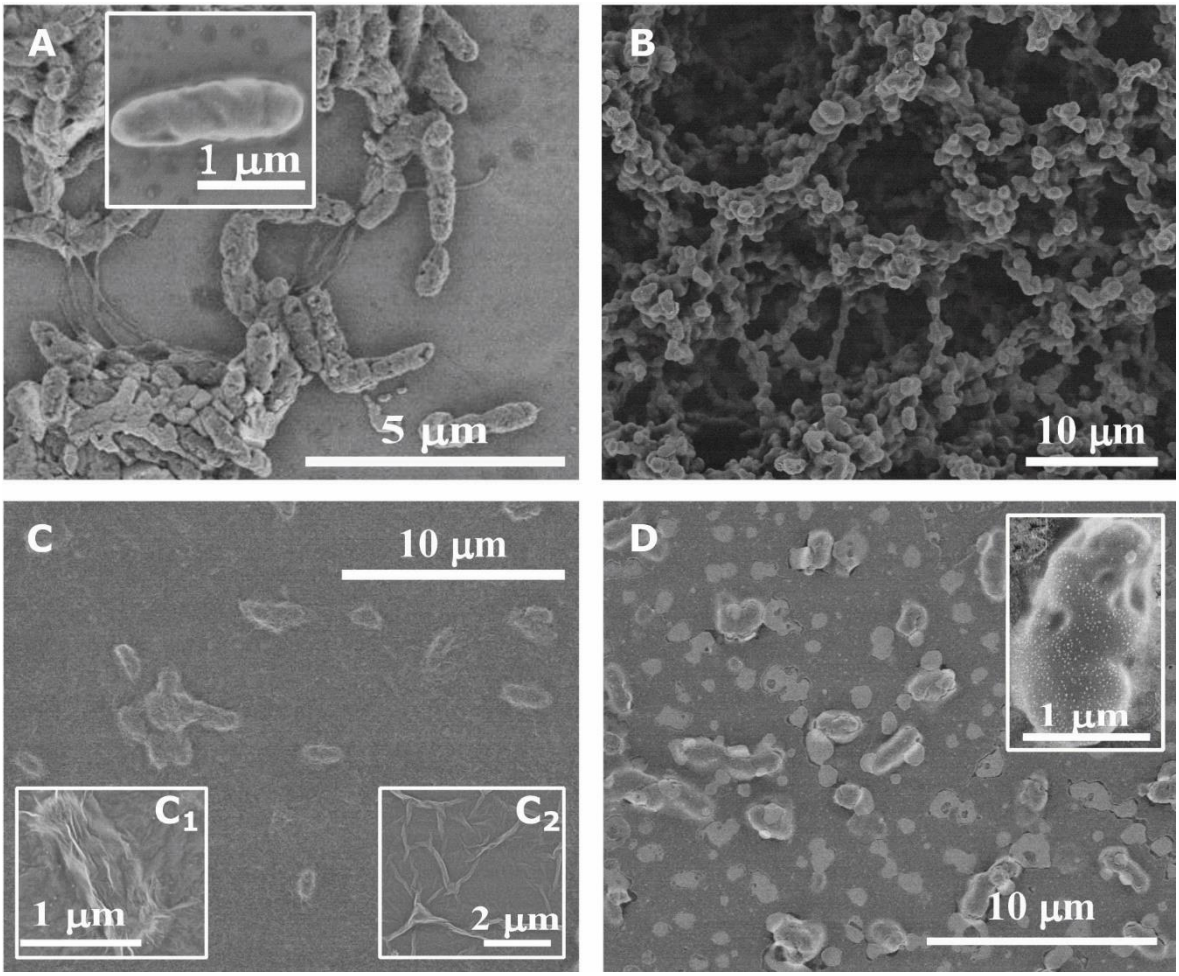
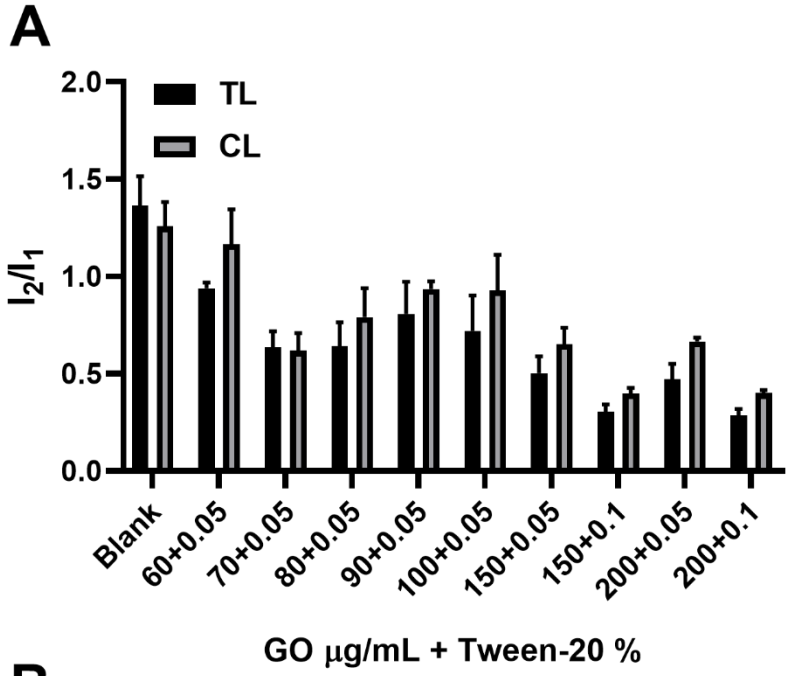
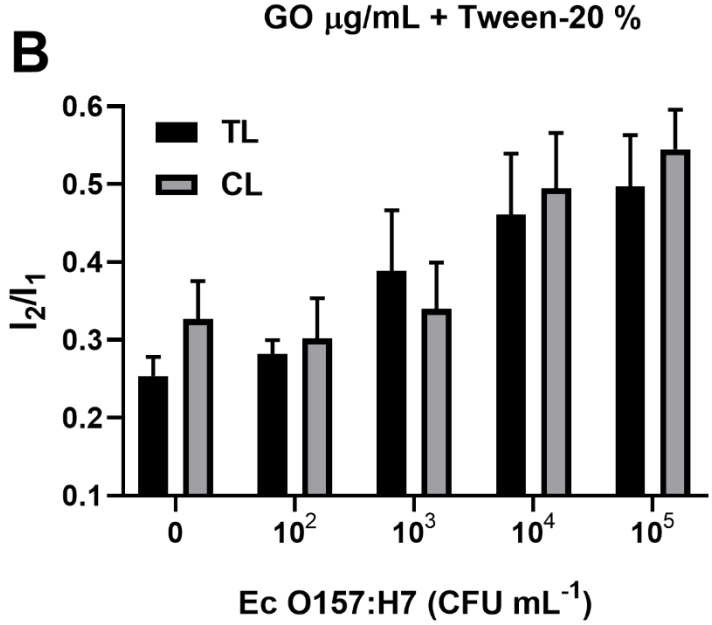
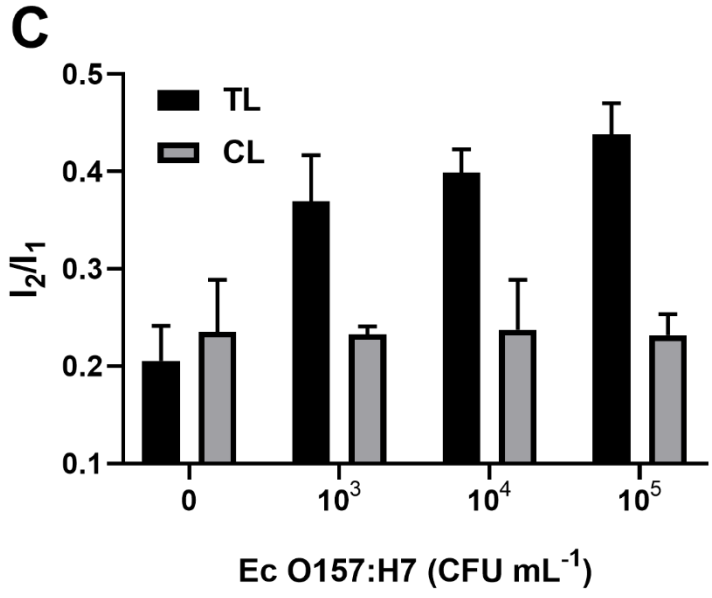


Figure 2. Scanning electron micrographs. A) Heat-killed *E. coli* O157:H7 (10^5 CFU mL^{-1}) in standard buffer (10 mM PBS with 0.5 % Tween-20 and 1 % BSA). B) Bare nitrocellulose membrane (Hi-Flow 180, SHF1800425) used for development of detection part of strip. C) GO sheets (150 µg mL^{-1} with 0.1 % Tween 20) suspended in Milli-Q water, coating *E. coli* O157:H7 cells. C_1) A magnified SEM image of *E. coli* O157:H7 cell is surrounded by GO sheets, C_2) Bare GO sheets. D) QDs-anti-*E. coli* O157:H7 antibody conjugates are capturing to heat-killed *E. coli* O157:H7 cells in standard buffer.


A
TL
CL
I_2/I_1
2.0
1.5
1.0
0.5
0.0
Blank
60+0.05
70+0.05
80+0.05
90+0.05
100+0.05
150+0.05
150+0.1
200+0.05
200+0.1
GO μg/mL + Tween-20 %


B
TL
CL
I_2/I_1
0.6
0.5
0.4
0.3
0.2
0.1
0
10^2
10^3
10^4
10^5
Ec O157:H7 (CFU mL^{-1})


C
TL
CL
I_2/I_1
0.5
0.4
0.3
0.2
0.1
0
10^3
10^4
10^5
Ec O157:H7 (CFU mL^{-1})

639

Figure 3. A) Optimization of the quenching process of QDs. Different concentrations of graphene oxide (GO) suspension in Milli-Q water with Tween-20 were investigated to achieve the optimum quenching conditions of both test line (TL) and control line (CL) by measuring the ratio of the final intensity to the initial one (I_2/I_1) of lines. It shows that GO 150 and 200 μg mL^{-1} with 0.1 % Tween-20 are the most quenching conditions. B) and C) The significance of using a washing buffer after sample loading to remove nonspecific reaction. B) Without washing step, the initial optimization process exhibited a nonspecific accumulation of the bacterial cells on the CL. The I_2/I_1 of CL is increasing with bacterial concentration like the TL. C) Conversely, with a washing step, nearly constant I_2/I_1 of control lines were obtained regardless the bacterial concentrations in the sample. The error bars represent the standard deviation of at least 3 replicates.

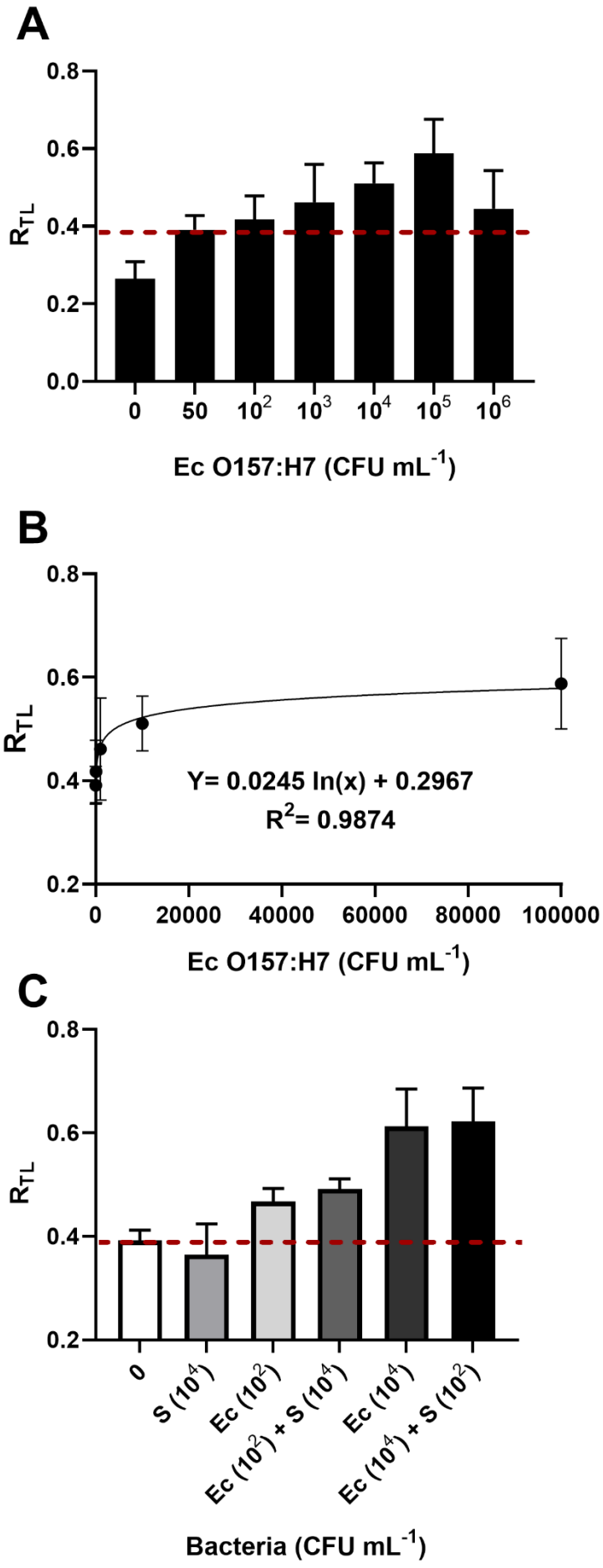
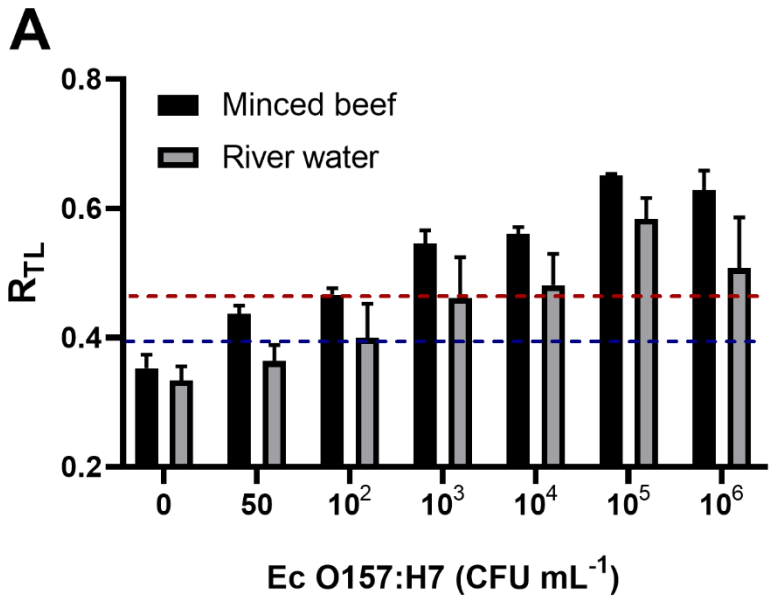
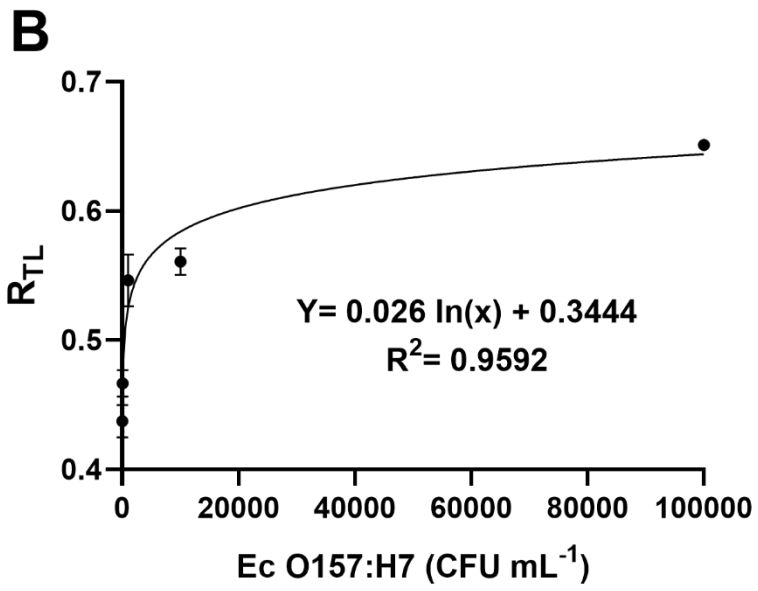
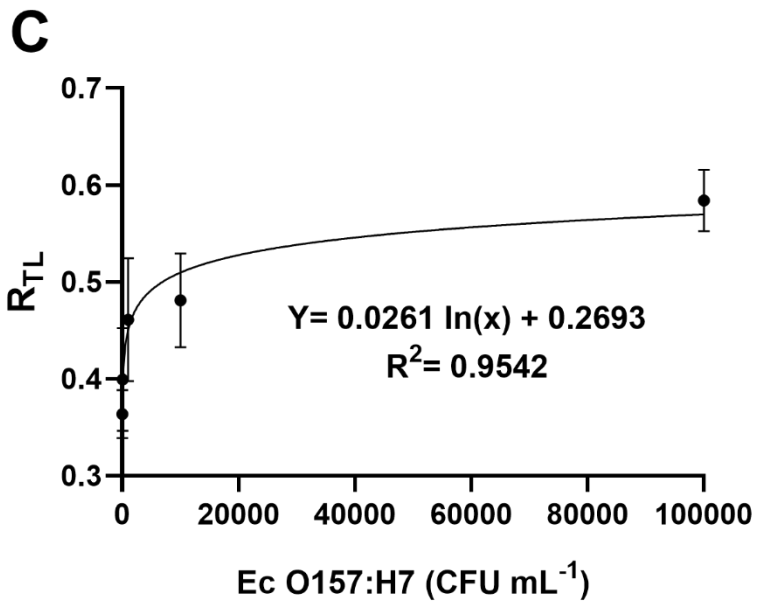

A
0.8
0.6
0.4
0.2
0.0
R_{TL}
0
50
10^2
10^3
10^4
10^5
10^6
Ec O157:H7 (CFU mL^{-1})
B
0.8
0.6
0.4
0.2
R_{TL}
Y= 0.0245 ln(x) + 0.2967
R^2= 0.9874
0
20000
40000
60000
80000
100000
Ec O157:H7 (CFU mL^{-1})
C
0.8
0.6
0.4
0.2
R_{TL}
0
S (10^4)
Ec (10^2)
Ec (10^2) + S (10^4)
Ec (10^4)
Ec (10^4) + S (10^2)
Bacteria (CFU mL^{-1})

Figure 4. A) Overall response of FLFIA to various concentrations of *E. coli* O157:H7 in standard buffer. Ratio of test line intensity (R_{TL})= final intensity/initial intensity of TL. B) Logarithmic response of FLFIA to *E. coli* O157:H7 (Ec O157:H7) concentrations from 50 to 10^5 CFU mL^{-1} in standard buffer. C) Specificity test against higher and lower concentrations of non-specific pathogen (*Salmonella* Typhimurium, S) either alone or in presence of the target pathogen, *E. coli* O157:H7 (Ec) were investigated. The dashed red lines (A and C) represent the limit of detection of *E. coli* O157:H7 in standard buffer by FLFIA (≈57 CFU/ mL), which was estimated as the mean value of blank buffer R_{TL} plus three times its SD. The error bars represent the standard deviation of at least 3 replicates.


A
Minced beef
River water
R_{TL}
0.8
0.6
0.4
0.2
0
50
10^2
10^3
10^4
10^5
10^6
Ec O157:H7 (CFU mL^{-1})


B
R_{TL}
0.7
0.6
0.5
0.4
Y= 0.026 ln(x) + 0.3444
R^2= 0.9592
0
20000
40000
60000
80000
100000
Ec O157:H7 (CFU mL^{-1})


C
R_{TL}
0.7
0.6
0.5
0.4
0.3
Y= 0.0261 ln(x) + 0.2693
R^2= 0.9542
0
20000
40000
60000
80000
100000
Ec O157:H7 (CFU mL^{-1})

693

Figure 5. A) Overall response of FLFIA strips to various concentrations of *E. coli* O157:H7 (Ec O157:H7) in minced beef and river water samples. The dashed lines represent the limit of detection of *E. coli* O157:H7 in minced beef (red) and water (blue) (≈178 and 133 CFU g^{-1} or mL^{-1} respectively), which were calculated as the R_{TL} mean value of blank minced beef or river water plus three times their SD. B) Logarithmic response of FLFIA to *E. coli* O157:H7 concentrations from 50 to 10^5 CFU g^{-1} in minced beef. C) Logarithmic response of FLFIA to *E. coli* O157:H7 concentrations from 50 to 10^5 CFU g^{-1} in river water. The error bars represent the standard deviation of at least 3 replicates.

720 **Tables**

721 Table 1. Spike and recovery experiment in minced beef and river water.

Real samples ($n \geq 3$)	Initial level of *Ec* O157:H7 (CFU mL^{-1} or g^{-1})	Spiked value of *Ec* O157:H7 (CFU mL^{-1} or g^{-1})	R_{TL} in standard buffer	R_{TL} in real samples	Recovery (%)
River water	0.0	10^3	0.419	0.411	97.98
	0.0	10^4	0.559	0.532	95.11
Minced beef	0.0	10^3	0.419	0.398	95.02
	0.0	10^4	0.559	0.501	92.86

722 Where, n, number of replicates. R_{TL}, Ratio of test line intensity= final intensity/initial intensity of

723 test line. *Ec* O157:H7, *E. coli* O157:H7. CFU, colony forming units.

724

725