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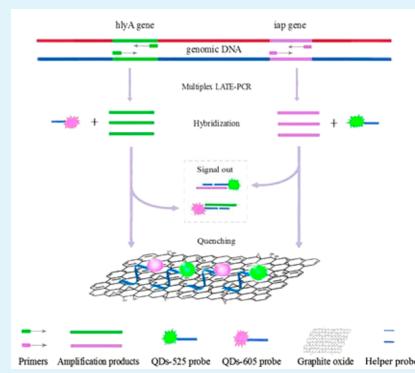
Quantum Dots and Graphene Oxide Fluorescent Switch Based Multivariate Testing Strategy for Reliable Detection of *Listeria monocytogenes*

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ABSTRACT: Graphene oxide (GO) and quantum dots (QDs), as burgeoning types of nanomaterials, have gained tremendous interest in the biosensor field. In this work, we designed a novel multivariate testing strategy that depends on the fluorescence resonance energy transfer (FRET) effect between quantum dots (QDs) and graphene oxide (GO). It integrates the QD–GO FRET principle and QD probes with different emission peaks into a platform, aims at multiplex gene detection of a human infectious and highly pathogenic pathogen, *Listeria monocytogenes* (*L. monocytogenes*). With the development of a multiplex linear-after-the-exponential (LATE) polymerase chain reaction (PCR) system, the single-stranded DNA (ssDNA) products of *hlyA* genes and *iap* genes are obtained by simultaneous amplification of the target genes. Then with the hybridization of ssDNA products and QD probes, simultaneous homogeneous detection of two gene amplification products can be achieved by using GO as a fluorescence switch and monitoring the relevant emissions excited by a single light source. Distinguishable signals corresponding to target genes are obtained. With this developed approach, genomic DNA from *L. monocytogenes* can be detected as low as 100 fg/μL. Moreover, this platform has a good dynamic range from 10² to 10⁶ fg/μL. It is indicated that this platform has potential to be a reliable complement for rapid gene detection technologies and is capable of reducing the false-negative and false-dismissal probabilities in routine tests.

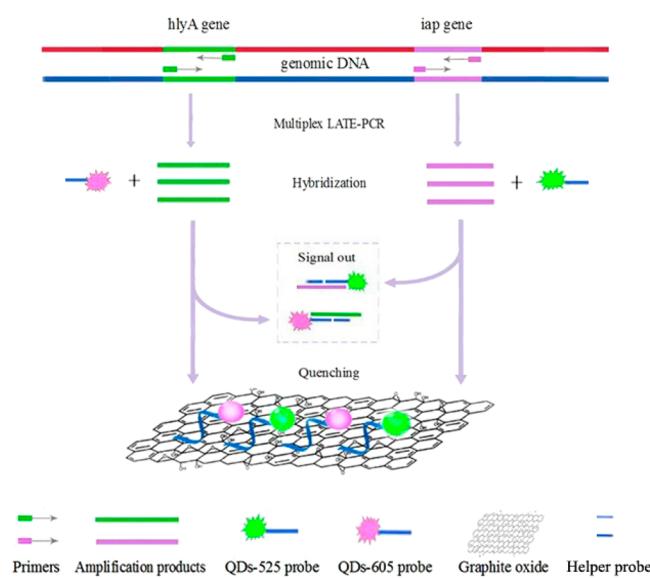
KEYWORDS: multivariate testing strategy, quantum dots, graphene oxide, linear-after-the-exponential PCR, foodborne pathogenic bacteria



1. INTRODUCTION

Graphene oxide (GO) and quantum dots (QDs), as burgeoning types of nanomaterials, have gained tremendous interest in the biosensor field for the design and fabrication of biosensors because of their excellent physical and chemical properties. GO has a hydrophilic surface with carboxyl and phenol hydroxyl groups, exhibiting good dispersion and excellent stability.^{1–8} Owing to the π–π stacking effect between nucleic acid bases and GO, the adsorption of single-stranded nucleic acid by GO is a stable interaction.^{9,10} Conversely, the formation of the double-helix nucleic acid structure hides the nucleic acid bases perfectly, so the adsorption capacity of GO is greatly reduced.¹¹ In addition, one outstanding property of GO is that it can be used as a fluorescence quenching agent with a broad absorption band through fluorescence resonance energy transfer (FRET).^{12–14} In comparison, QDs have been used as excellent fluorescent labels for biological imaging, sensing, and diagnostics. They have a variety of advantages, such as high fluorescent quantum yield; narrow, symmetric, and stable fluorescence; and size-dependent tunable absorption and emission.^{15–19} Surprisingly, it appears that the FRET between QDs and GO results in an outstanding quenching effect. This effect can be used as a fluorescence switch to obtain a high signal-to-noise ratio (SNR) for biological sensing. Based on this principle, a few QD–GO-based biomolecular sensing platforms have been developed in recent years.^{20,21}

Scheme 1. Principle of the QD–GO Multiplex Gene Monitoring Platform for *Listeria monocytogenes*



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Table 1. Sequences of Primers

target gene	limiting primer's sequence (5'-3') and theoretical annealing temperature at 1000 nM		excess primer's sequence (5'-3') and theoretical annealing temperature at 1000 nM	
<i>hlyA</i>	GCTGCCGTAAGTGGGAATCTGTCTCAG	70.4 °C	ATGATTGAACTTCATCTTTGC	59.9 °C
<i>iap</i>	CTACTCAACAAGCTGCACCTGCTGCAGA	72.1 °C	GACAGCGTGTGTAGTAGCAT	61.1 °C

Table 2. Sequences of 5'-Biotin-Labeled Probes

target gene	sequence (5'-3') of 5'-biotin-labeled probes
<i>hlyA</i>	GCTGCCGTAAGTGGGAATCTGTCTCAGGTGATGTAGAAT
<i>iap</i>	CTACTCAACAAGCTGCACCTGCTGCAGAAACAAAAACTGA

Table 3. Sequences of Helper Probes

target gene	sequence (5'-3') of helper probe
<i>hlyA</i>	TAACAAATATCATCAAAATTCTTCCTTCAAAGCCGTAAT
<i>iap</i>	AGTAAAACAAACTACACAAGCAACTACACCTGCACCTAAA

Inspired by the excellent performance of the QD-GO FRET platform and size-dependent tunable emission of QDs, a novel multivariate testing strategy was designed. In this strategy, two kinds of quantum dots with differentiable emission wavelengths

are employed as the probes corresponding to two separate target genes. By integrating the QD-GO FRET principle and two quantum dot probes with different emission peaks into a platform, one can obtain distinguishable signals corresponding to target genes. Based on the principles of probability statistics, the probability of independent random events occurring at the same time is far less than that of a single event occurring independently. Thus, multiplex gene detection has the potential to provide a more reliable approach than single-gene testing. Combined with severe food-security problems, we migrated the

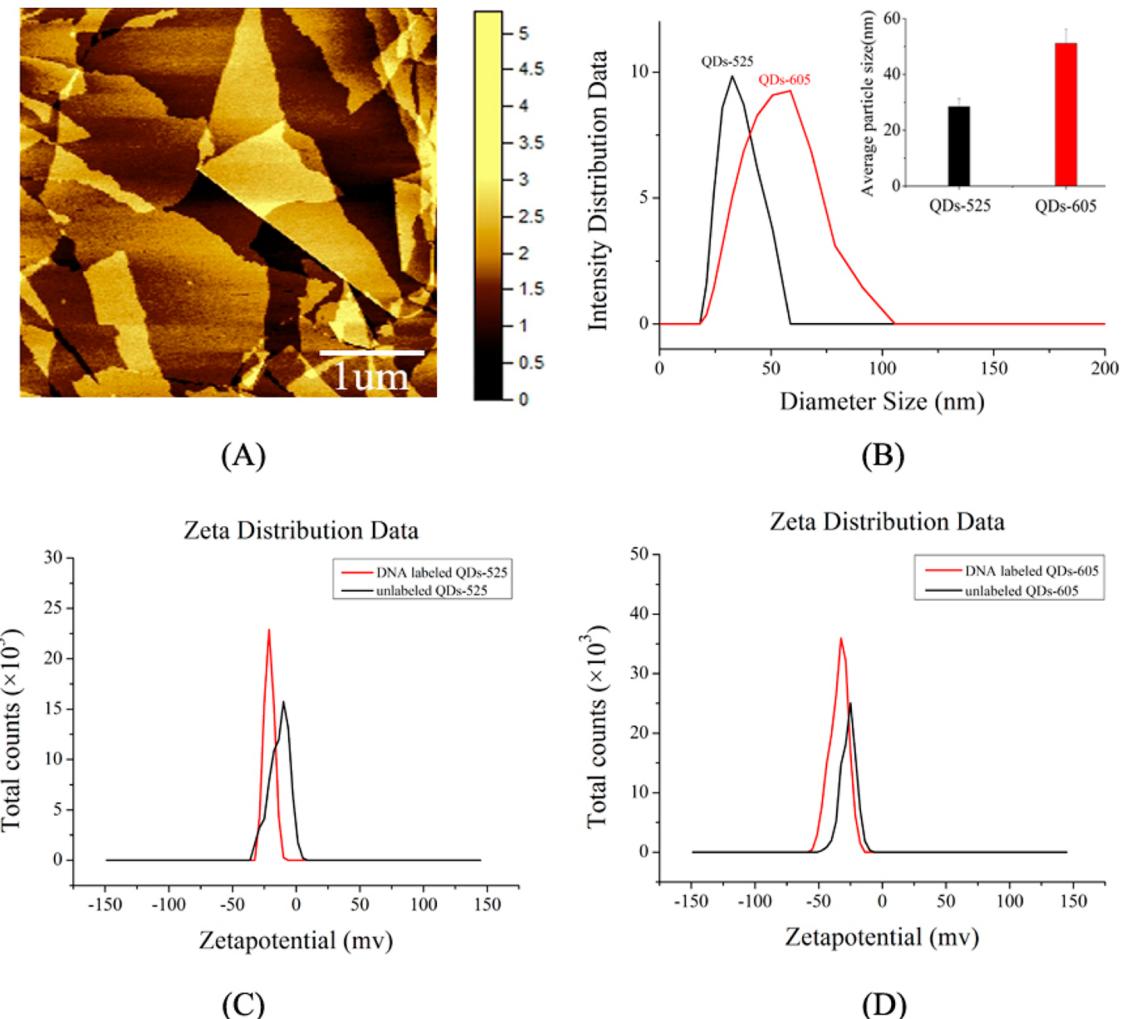


Figure 1. Characterization of QD probe formation and GO. (A) AFM image of GO. (B) Particle sizes of QDs. (C,D) Potential variations between labeled and unlabeled (C) QDs-525 and (D) QDs-605.

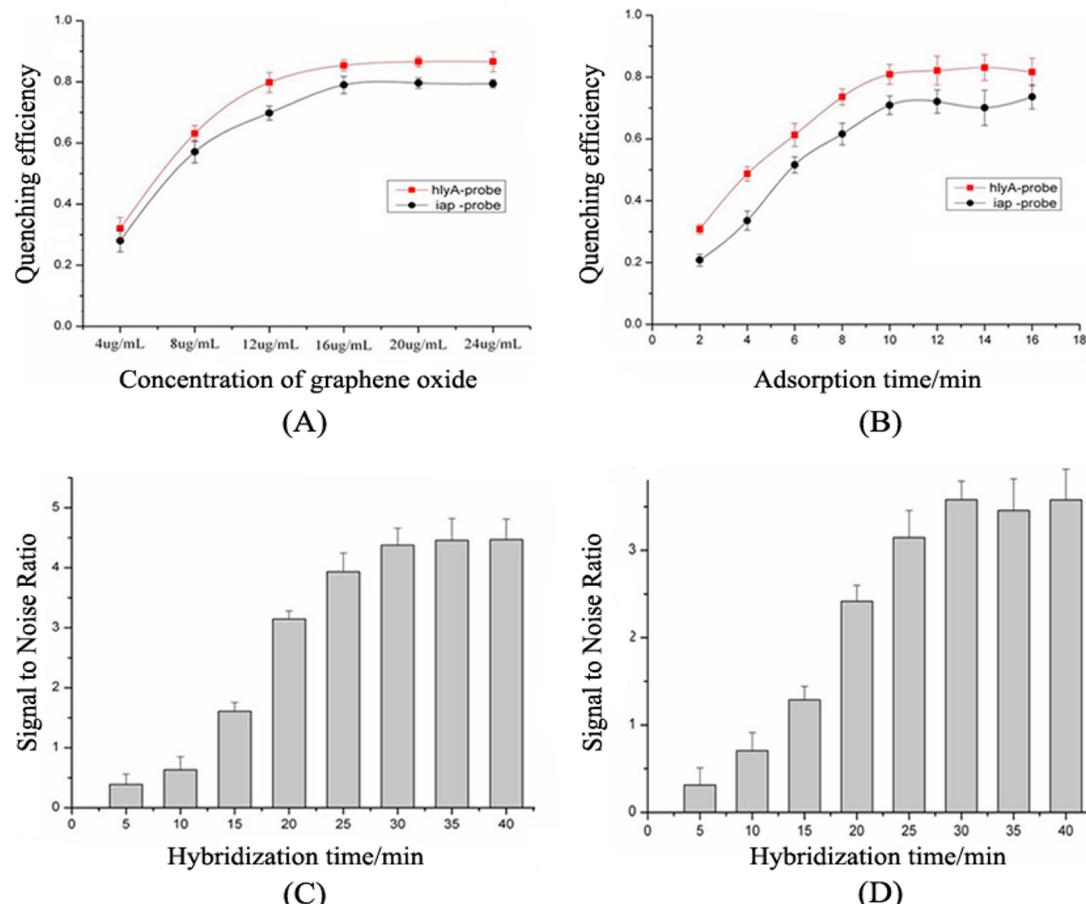


Figure 2. Parameter optimization for the QD–GO multiplex gene monitoring platform. (A) Evaluation of the effect of GO concentration on the quenching ability of QDs. (B) Time dynamics of QD probe adsorption. (C,D) Hybridization time optimizations for (C) *iap* and (D) *hlyA* probes.

multivariate testing strategy to detect the multiplex gene of *Listeria monocytogenes* (*L. monocytogenes*, a human infectious and highly pathogenic pathogen) for the reduction of the probabilities of false negatives and false dismissals in routine tests.

L. monocytogenes is an etiologic microorganism that is transmitted through the food chain and leads to listeriosis.²² In recent years, foodborne pathogenic bacteria have caused huge economic losses to society, posing a serious challenge to human health and food security. Therefore, analyses of the presence of pathogenic bacteria has become standard practice for ensuring food security and quality.²³ Existing technologies for the monitoring of foodborne pathogenic bacteria are mainly made up of conventional monitoring methods and emerging rapid gene detection technologies. Conventional methods^{24–26} rely on specific separation and enrichment to isolate viable bacterial cells in foods and are laborious and time-intensive, requiring several days to produce results. Meanwhile, the existing rapid gene detection technologies^{27–29} are mostly focused on single-gene testing. They are not reliable enough for infectious, widespread, and highly pathogenic organisms in routine tests. Furthermore, the repeated occurrence of food safety incidents compelled us to reexamine the securities of existing technologies for the monitoring of foodborne pathogenic bacteria. Thus, considerable interest has been evoked in concepts of multiplex gene detection.^{30–32}

However, multiplex gene detection methods face the problem of multiplex feature signals breaking down at one-step operation. Thus, similar absorption peaks and distinguishable

emission peaks must be provided simultaneously, which greatly limits the practical application of such techniques. In the actual process, electrophoresis^{33,34} and real-time quantitative methods^{35,36} are employed as the signal producing components for multiplex gene detection. Electrophoresis could provide feature signals of the target gene according to the fragment size, but it is hindered by low sensitivity. Furthermore, real-time quantitative methods often employ SYBR green I as the signal amplifier. This means that these methods can use only superposition of multiplex gene detection signals and cannot obtain the feature signals corresponding to the target gene separately. Fortunately, nanomaterials fill the deficiency of multiplex gene detection techniques, by enabling the production of fluorophores with different emission wavelengths excited by a single light source.

In this work, we constructed a novel multiplex gene detection assay that depends on a QD and GO nanoplatform. It aims at multiplex gene monitoring of *L. monocytogenes* that can overcome false negatives and reduce the false-dismissal probability in routine tests. In this approach, the linear-after-the-exponential polymerase chain reaction (LATE-PCR),³⁷ an outstanding method for generating single-stranded DNA (ssDNA) amplification products, is employed as the amplification system, and the *iap* and *hlyA* genes of *L. monocytogenes* are used as the target genes. The overall concept of the strategy is shown in Scheme 1. This platform consists of three parts: multiplex LATE-PCR amplification, DNA hybridization, and GO–QD signal detection. When the genome of *L. monocytogenes* exists, the multiplex LATE-PCR amplification system can obtain ssDNA

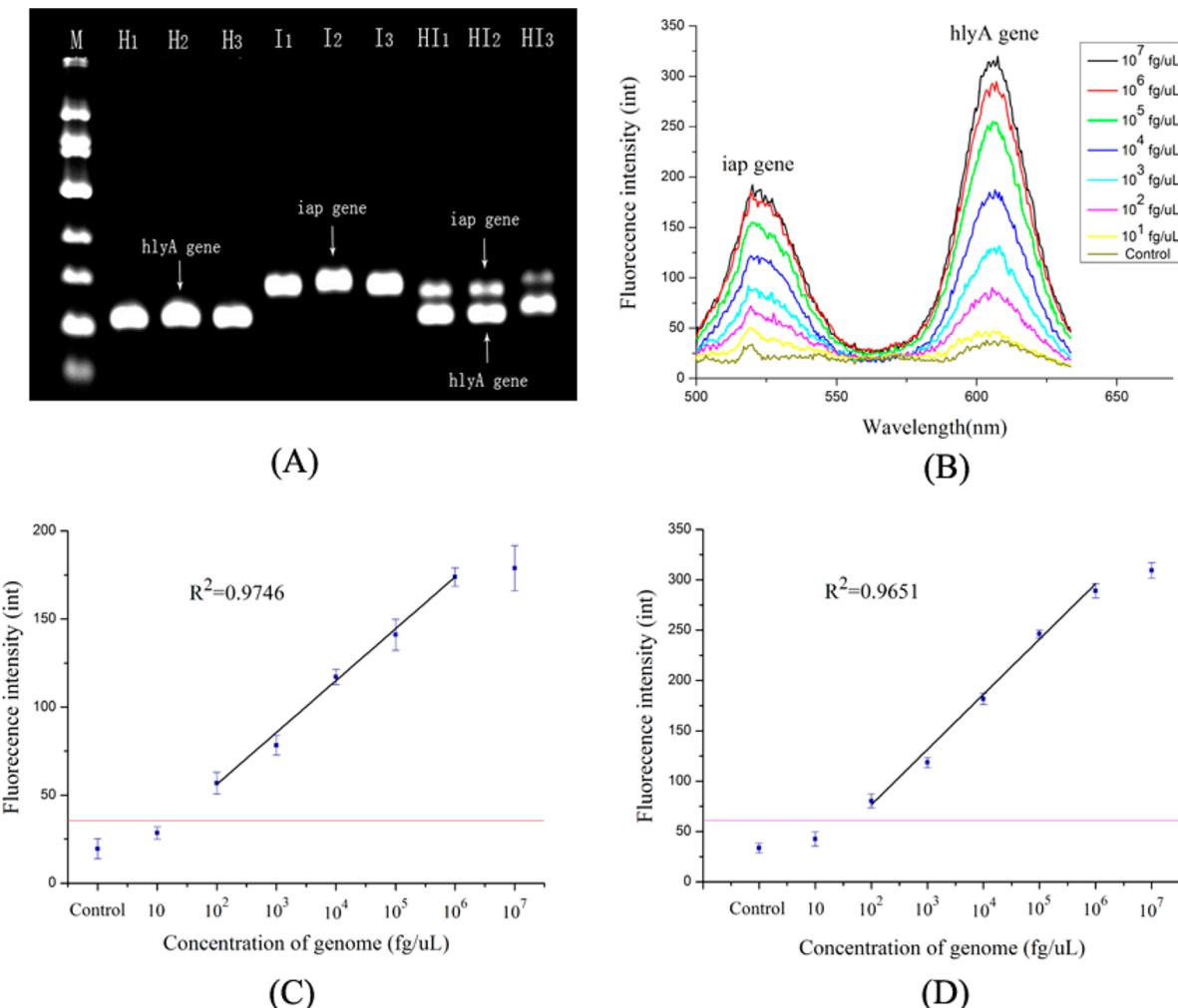


Figure 3. Sensitivity results and data analysis. (A) Electrophoresis identification of the multiplex LATE-PCR amplification. Lanes H₁–H₃ are the LATE-PCR amplification products of the *hlyA* gene, and lanes I₁–I₃ are those of the *iap* gene. Lanes HI₁–HI₃ represent three parallel experiments of multiplex LATE-PCR. (B) Fluorescence spectroscopy experiments for multiplex gene monitoring of *Listeria monocytogenes* derived from different concentrations of genomic DNA. (C,D) Linear regression analyses of monitoring on the (C) *hlyA* and (D) *iap* genes.

amplification products of the *iap* and *hlyA* genes at an efficiency between linear amplification and exponential amplification. Then, QD probes are added and incubated with the ssDNA amplification products to achieve sufficient hybridization of the ssDNA amplification products and QD probes. At the same time, helper probes are imported to constitute a longer double-chain structure. Then, the single-stranded QD probes are quenched by GO. However, partial formation [40 base pairs (bp)] of double-stranded DNA (dsDNA) through the hybridization of QD probes and amplification products leads to sufficient resistance to the quenching effect induced by GO. Therefore, the nanoplatform can achieve characteristic signals corresponding to the target genes. In addition, benefiting from the size-dependent tunable absorption and emission of QDs, more characteristic genes of *L. monocytogenes* could be accessed by this platform as a molecular target, according to the actual demands. Thus, it is demonstrated that the QD–GO nanoplatform has the potential to be a versatile tool for multiplex gene monitoring.

2. MATERIALS AND METHODS

2.1. Reagents. *Listeria monocytogenes* (CMCC54007), *Salmonella enterica* (CMCC50040), and *Escherichia coli* O157:H7 (GW1.2020)

were obtained from Guangzhou Institute of Microbiology, Guangzhou, China. Quantum dots labeled with streptavidin were synthesized by Wuhan Jiayuan Quantum Dots Co., Ltd. (Wuhan, China). QDs-525, coated with 2–4 streptavidin, has a narrow symmetrical emission peak at 525 nm, whereas the emission peak of QDs-605, coated with 4–8 streptavidin, is at 605 nm. Both QDs-525 and QDs-605 have high excitation efficiencies in the range of 200–300 nm. Graphene oxide (1 mg/mL) was purchased from Xianfeng Nanotechnologies Co., Ltd. (Nanjing, China). Taq DNA polymerase and deoxyribonucleoside triphosphate (dNTP) mix were the products of Takara Biotechnology Co., Ltd. (Dalian, China). All oligonucleotides and probes used in our research were synthesized and purified by high-performance liquid chromatography (HPLC) at Invitrogen. SYBR I and SYBR II dyes were purchased from Invitrogen. The regents related to electrophoresis were purchased from Bio-Rad Laboratories (Hercules, CA). Phosphate-buffered saline (PBS) buffer (20×) was purchased from Shanghai Sangon Biotechnology Co. Ltd. (Shanghai, China).

2.2. Extraction of Genomic DNA. The genomic DNA of *L. monocytogenes* (CMCC54007), *Salmonella enterica* (CMCC50040), and *Escherichia coli* O157:H7 (GW1.2020) was extracted according to the manufacturer's protocol from the TIANamp Bacteria DNA Kit (Tiangen Biotech, Beijing, China). The extractions were quantified by measuring the optical density at 260 nm with a spectrophotometer (Eppendorf BioPhotometer, Eppendorf AG, Hamburg, Germany).

2.3. Multiplex LATE-PCR System. The multiplex LATE-PCR system contained dNTP mix (0.2 mM), taq DNA polymerase

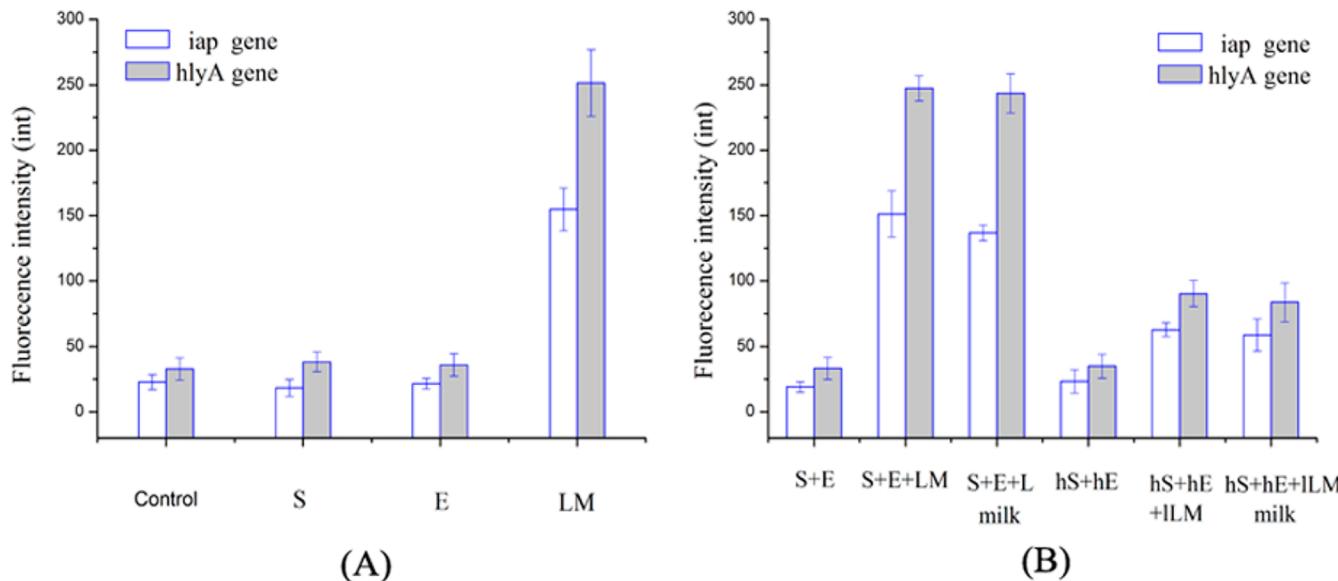


Figure 4. Specificity of the QD-GO multiplex gene monitoring platform. (A) Specificity for equivalent concentrations of food-borne pathogenic bacteria. (B) Specificity for discriminative concentration. S, E, and LM represent *Salmonella enterica*, *Escherichia coli*, and *L. monocytogenes*, respectively (at a concentration of 10^5 fg/ μ L). hS and hE correspond to *Salmonella enterica* and *Escherichia coli*, respectively, at a high concentration of 10^7 fg/ μ L. ILM is *L. monocytogenes* with a lower concentration of 10^3 fg/ μ L.

(0.025U/ μ L), the corresponding buffer (1 \times), limiting primers, and excess primers. The limiting primers and excess primers were designed according to the principle of LATE-PCR³⁷ and reached a molar ratio of 1:40. The sequences of primers corresponding to the *hlyA* (GenBank accession number AF253320) and *iap* (GenBank accession number AY174670) genes are listed in Table 1, yielding a 116-nucleotide (-nt) fragment from the *hlyA* gene and a 137-nt fragment from the *iap* gene.

2.4. Polyacrylamide Gel Electrophoresis. To verify the amplification feasibility of the multiplex LATE-PCR system, the products of amplification were analyzed on a Bio-Rad slab electrophoresis system (Bio-Rad Laboratories, Hercules, CA). A 10% native polyacrylamide gel (29:1 acrylamide/bisacrylamide) loaded with 10 μ L samples was run at room temperature for 45 min at 120 V in Tris-borate-EDTA (TBE, 1 \times) buffer, after which coloring with SYBR I and SYBR II and photographing with a Bio-Rad digital imaging system were performed.

2.5. Preparation of Probes of Quantum Dots. To establish a multiplex gene detection platform, two kinds of quantum dots labeled with streptavidin, QDs-525 and QDs-605 (Wuhan Jiayuan Quantum Dots Co., Ltd., Wuhan, China) were employed as fluorophores of fluorescent probes. QDs-525 targeted the *iap* gene, and QDs-605 corresponded to the *hlyA* gene. Aiming at the target sequences, we designed two ssDNA probes labeled with biotin at the 5' terminus, constituting *iap* and *hlyA* probes with QDs-525 and QDs-605, respectively. The sequences of the ssDNA probes, labeled with biotin at the 5' terminus, are listed in Table 2.

For the preparation of QD probes, the ssDNA probes and QDs (1 μ M) were mixed at a molar ratio of 30:1 and then subjected to thermostatic reactions at 37 °C for 30 min. The disconnected ssDNA probes were filtered on a Nanosep 100K OMEGA tubular ultrafiltration membrane (Pall Corporation, Port Washington, NY). After twice being spun and washed with PBS buffer (1 \times), the final products were redissolved in PBS buffer (1 \times) and stored at 4 °C.

2.6. Construction of the QD-GO Multiplex Gene Monitoring Platform. The construction of the QD-GO platform consisted of the following steps: The ssDNA products from the multiplex LATE-PCR amplification system were mixed with a certain amount of QD probe. The final concentration of *hlyA* probes was fixed at 15 nM. At this concentration, QDs-605 gains adequate fluorescence intensity for the strategy of this platform. To make up for the lower quantum yield of QDs-525 and to equalize the lower labeling rate of streptavidin, we set the concentration of *iap* probe as 30 nM, or twice that of the *hlyA*

probe. The mixture of amplification products and probes was incubated for 30 min at 37 °C. In this process, QD probes hybridize with the amplification products and helper probes (the sequences of which are listed in Table 3). Along with the partial formation (40 bp) of double-stranded DNA (dsDNA), that led to sufficient resistance to the quenching effect induced by GO. Then, the fluorescence was measured on a Perkin-Elmer LS55 luminescence spectrometer (Wellesley, MA) after 10 min of incubation.

3. RESULTS AND DISCUSSION

3.1. Characterization of GO and QD Probe Formation.

The GO and QD probe formation were characterized. We first observed the surface morphology of a GO nanosheet by atomic force microscopy. The homogeneous sheet is shown in Figure 1A. The results show that the size of a single GO sheet was about 1 μ m. Furthermore, the particle sizes of the QDs were measured by particle size analyzer, as shown in Figure 1B. The average diameter of streptavidin-labeled QDs-525 was 28.42 ± 2.83 nm, and that of streptavidin-labeled QDs-605 was 51.11 ± 5.11 nm. These diameters include the hydration layer on the surface of the QDs. Then, we studied the initial surface charge of the QDs, which was negative. The value of zeta potential was -9.94 ± 0.75 and -17.45 ± 2.13 mV for QDs-525 and QDs-605, respectively. With the DNA label on the QDs, the surface charges of the QDs showed an obvious change (see Figure 1C,D). The charges of DNA-labeled QDs exhibited a negative enhancement as a result of the negative charge of the DNA probes.

3.2. Parameters of Optimization for the QD-GO Multiplex Gene Monitoring Platform. The concentration of GO has a strong effect on the performance of this nano-platform. At a high concentration of GO, the amplification products could be adsorbed onto the GO surface. This led to a dramatic decrease in the sensitivity. Nevertheless, a low concentration of GO could give rise to a background fluorescence signal that caused a decrease of the signal-to-noise ratio (SNR). To determine the optimized concentration of GO, the effect of GO concentration on the fluorescence quenching ability was

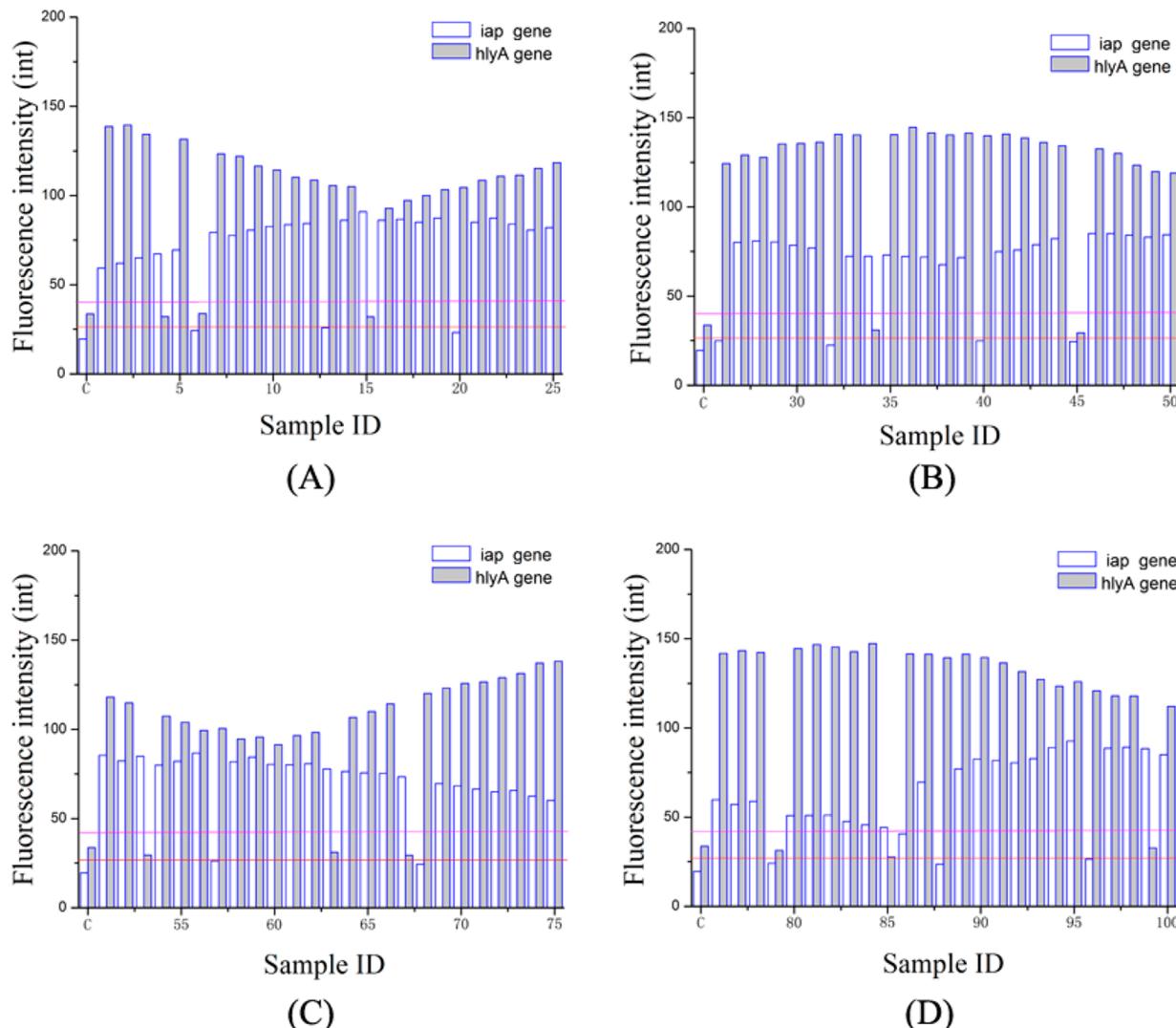


Figure 5. Detections of 100 medium cultured *L. monocytogenes* samples by the QD–GO multiplex gene monitoring platform. Detection signals of the cultured *L. monocytogenes*: sample IDs (A) 1–25, (B) 26–50, (C) 51–75, and (D) 76–100.

evaluated using GO concentrations of 4, 8, 12, 16, 20, and 24 $\mu\text{g}/\text{mL}$. The concentration of QDs-525 probe was 30 nM, reaching a molar ratio to QDs-605 of 2:1. As shown in Figure 2A, with increasing GO concentration, the quenching efficiency also increased. When the GO concentration was 16 $\mu\text{g}/\text{mL}$, the quenching efficiency remained at a stable level. Further addition of GO did not produce a significant increase in quenching efficiency. Therefore, we employed 16 $\mu\text{g}/\text{mL}$ GO as the optimum concentration for the platform.

On the other hand, the time dynamics of adsorption is also an important parameter for the platform. Using a short adsorption time, the QD probes could not be effectively adsorbed onto the GO. However, a long incubation time could result in random adsorption of hybrid products onto the GO. Therefore, to improve the efficiency of the nanodetection platform, GO adsorption time optimization experiments were executed. The *iap* and *hlyA* probes were fixed at 30 and 15 nM, respectively, with a GO concentration of 16 $\mu\text{g}/\text{mL}$. Then, at different time points, the adsorption efficiency was recorded, as shown in Figure 2B. As can be seen, with increasing adsorption time, the adsorption efficiency gradually increased. When the adsorption time was 10 min, the adsorption efficiency was stable.

Therefore, 10 min was chosen as the optimal adsorption time in subsequent experiments.

In addition, the hybridization time of the amplification products and QD probes has a direct bearing on the SNR. For the optimal hybridization time, 1 ng/ μL genomic DNA was added to the nanoplatform, and optimization experiments were performed using different hybridization times. As shown in Figure 2C,D, the SNR increased with the extension of hybridization time and reached a stable level after 30 min. Therefore, 30 min was employed as the optimal hybridization time.

3.3. Sensitivity Results of QD–GO Multiplex Gene Monitoring Platform and Data Analysis. For verification of the amplification system, 1 ng of genomic DNA of *L. monocytogenes* was amplified by the multiplex LATE-PCR amplification system. The amplification products were detected by 10% polyacrylamide gel electrophoresis. As shown in Figure 3A, the products of multiplex LATE-PCR amplification system were stably produced from three parallel amplification experiments. In addition, we evaluated the sensitivity of the QD–GO multiplex gene monitoring platform. In this design, the concentration of genomic DNA was varied from 10 to 10^7 fg/ μL . After amplification by the multiplex LATE-PCR

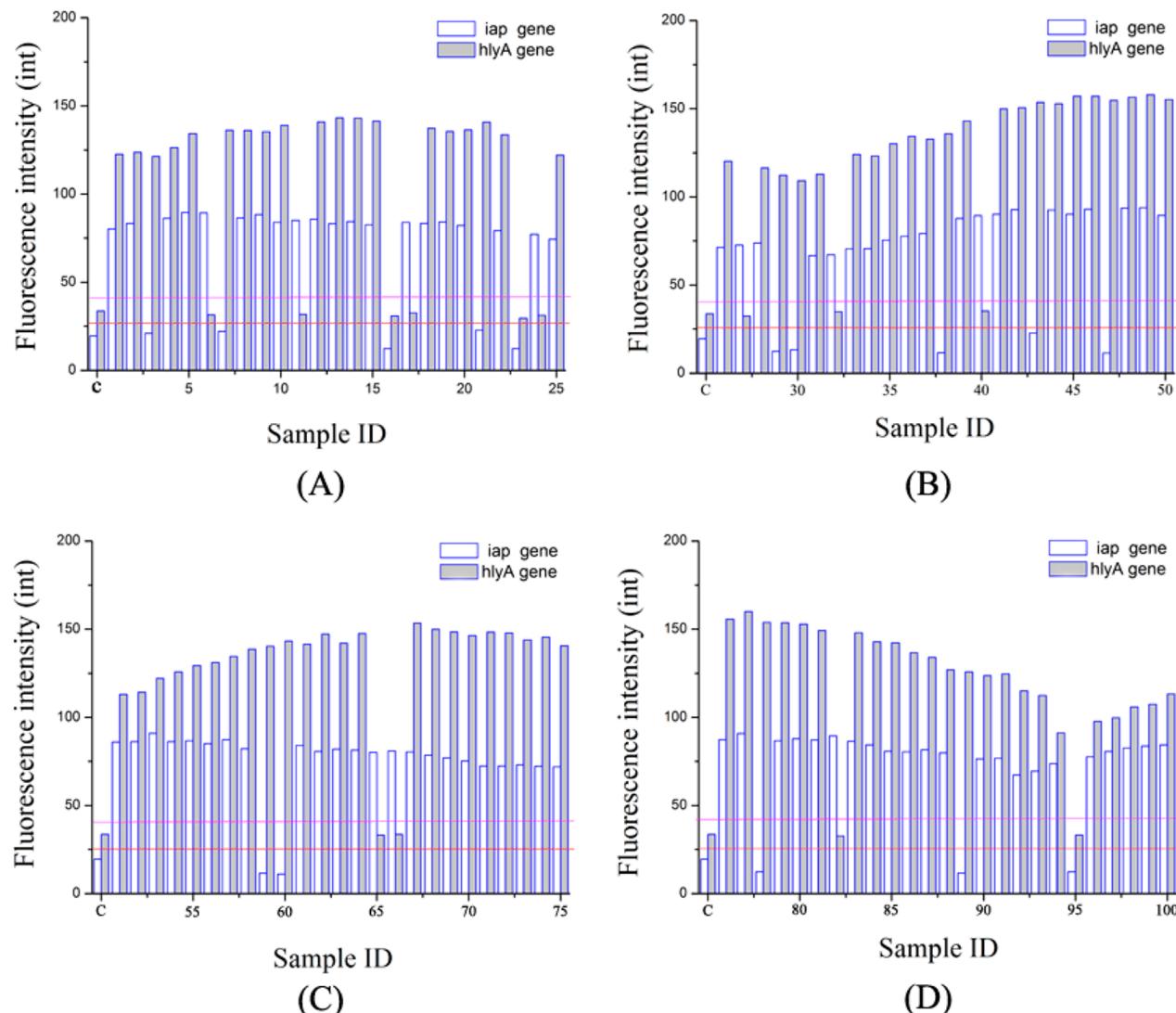


Figure 6. Detections of 100 spiked milk samples by the QD–GO multiplex gene monitoring platform. Detection signals of milk-cultured *L. monocytogenes* for sample IDs (A) 1–25, (B) 26–50, (C) 51–75, and (D) 76–100.

amplification system, the amplification products of target genes were mixed with preprepared QD probes and helper probes. The mixture was incubated at 37 °C for 30 min. Then, GO was added to the system, reaching a final concentration of 16 μg/mL and leading to a quenching effect on the excess QD probes within 10 min. Finally, changes in the fluorescence intensity of the final mixture were observed and recorded (see Figure 3B). The fluorescence intensity synchronously decreased with the reduction of genomic DNA. When the concentration of genomic DNA decreased to 10 fg/μL, the fluorescence intensity tended to be coincident with that of the control group. Nevertheless, the group with 100 fg/μL genomic DNA had an obvious enhancement compared with the control group. It was demonstrated that the QD–GO multiplex gene monitoring platform achieved a sensitivity of 100 fg/μL. We performed linear regression analysis of the fluorescence intensities with different genomic DNA concentrations for verification of the reliability and feasibility of this platform. Figure 3C shows the linear regression analysis of monitoring of the *iap* gene. It was found that the results of sensitivity had a good linear relation, and an R^2 value of 0.9746 was obtained. Meanwhile, the linear regression analysis of monitoring of the *hlyA* gene is shown in

Figure 3D, achieving an R^2 value of 0.9651. This indicates the feasibility and reliability of the platform used for multiplex gene monitoring of *L. monocytogenes*. Moreover, this platform exhibited a good dynamic range from 10^2 to 10^6 fg/μL.

3.4. Specificity of the QD–GO Multiplex Gene Monitoring Platform. Evaluation of specificity is an important quality index in biological monitoring methodology. To further evaluate the performance of the platform, samples of the genomic DNA of *Salmonella enterica*, *E. coli* O157:H7, and *L. monocytogenes* with an isometric concentration of 10^6 fg/μL were also analyzed using this platform. As shown in Figure 4A, the fluorescence intensities of *Salmonella enterica* and *E. coli* O157:H7 exhibited no significant enhancements, whereas *L. monocytogenes* gave a dramatic response, which confirms that this platform has highly responsive specificity to *L. monocytogenes*.

For discrimination of the *L. monocytogenes* response from that of nontarget species, we mixed *L. monocytogenes* with the common food-borne pathogens *Salmonella enterica* and *E. coli* O157:H7 and then applied the standard program of this platform. The results show that this strategy can discriminate *L. monocytogenes* from the mixed system (see Figure 4B).

The milk samples also gave obviously characteristic signals. Thus, this method exhibits preferable discrimination performance. In addition, we detected the low-level response of the target *L. monocytogenes* against a high level of nontarget species (see Figure 4B), which also demonstrates the discrimination capacity of this platform.

3.5. Data Supporting Reductions of False-Negative and False-Dismissal Probabilities. The original intention for constructing this platform was to reduce the false-negative and false-dismissal probabilities. For data supporting the reduction of the false-negative and false-dismissal probabilities, we tested 100 cultured *L. monocytogenes* samples with this platform. The results are displayed in Figure 5, which shows that the single-gene testing of *iap* and *hlyA* genes had false-negative probabilities of 11% and 12%, respectively. In contrast, the multivariate testing strategy achieved a false-negative probability of 3%. In addition, 100 spiked milk samples were also tested (see Figure 6). The results again demonstrated a reduction of the false-negative and false-dismissal probabilities and provided a better performance. We also found that the false-negative and false-dismissal probabilities increased with the complexity level of the substrate in which *L. monocytogenes* was grown. The milk samples reached higher false-negative and false-dismissal probabilities with single-gene tests (*iap*, 15%; *hlyA* 13%) than the conventional culture medium. Thus, this platform could significantly reduce the probabilities of false negatives and false dismissals.

4. CONCLUSIONS

In summary, we have demonstrated that a multiplex gene monitoring platform can be a rapid, sensitive, and reliable method for the detection of *L. monocytogenes*. To the best of our knowledge, this is the first reported example of a multiplex gene monitoring platform for foodborne pathogen detection, based on the QD-GO nanoplateform. The proposed method consists of multiplex LATE-PCR amplification, DNA hybridization, and QD-GO signal detection system. Benefiting from the high amplification efficiency of LATE-PCR, excellent quenching ability of GO, and high quantum yield of QDs, three remarkable advantages can be achieved with this platform, compared to conventional assays. First, the whole detection process is rapid, being accomplished in 1.5 h, so this platform meets the requirements for rapid detection exactly. Second, this platform achieved a high sensitivity level of 100 fg/μL that ensured its feasibility for routine inspection and observation. Third, this platform was aimed at multiplex gene monitoring. The emergence of one or more characteristic fluorescence peaks corresponding to the target genes can be regarded as a positive result that significantly reduces the probabilities of false negatives and false dismissals in routine tests. Taken as a whole, this platform has the potential to be a versatile tool for the monitoring of infectious, widespread, and highly pathogenic foodborne pathogens.

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Notes

The authors declare no competing financial interest.

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