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Microarray detection method for pathogen genes by on-chip signal amplification using terminal deoxynucleotidyl transferase

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Abstract

A microarray detection method based on on-chip signal amplification using terminal deoxynucleotidyl transferase (TdT) was developed to visualize pathogenic genes. Cyclic olefin copolymer (COC) substrate for microarrays was treated with oxygen plasma to induce hydrophilic surface properties. The capture probe DNA was immobilized on the COC surface by UV irradiation. The 3′ end of the capture probe DNA immobilized on the COC surface was modified with a phosphate group to provide resistance against the TdT reaction. Therefore, the TdT reaction was triggered only when the capture probe DNA acquired the target gene, and biotin-11-deoxyuridine triphosphate (b-dUTP) was continuously added to the 3′ end of the target gene. Thereafter, streptavidin-conjugated gold nanoparticles (s-AuNPs) tagged the poly uridine tails by the biotin–streptavidin interaction. The visual signal was amplified by silver enhancement in the presence of the s-AuNPs. The usefulness of this detection method was confirmed by analyzing four pathogens and allowing their visual identification.

Keywords: DNA microarray, Terminal deoxynucleotidyl transferase, Cyclic olefin copolymer, On-chip, Pathogenic gene

Introduction

Food poisoning caused by ingesting contaminated water or food is a ubiquitous problem worldwide [1]. Pathogenic microorganisms cause high proportion of food poisoning cases, and symptoms such as diarrhea, vomiting, high fever, or even death can occur in severe cases [2]. Therefore, it is essential to accurately and quickly detect pathogenic microorganisms to improve public health. Traditional culture-based methods for detecting microorganisms are labor-intensive and time-consuming [3]. Although various molecular biological detection methods, such as enzyme-linked immunosorbent assays, polymerase chain reaction (PCR), and in situ hybridization assays, have been developed, the simultaneous diagnosis of microorganisms remains challenging [4]. To overcome this limitation, developing a detection method that can quickly, sensitively, and simultaneously analyze multiple microorganisms is necessary.

A DNA microarray is a collection of microscopic dots in which DNA is arranged and attached to a solid surface or membrane. This technique is widely applied in gene expression analysis, genotyping, clinical diagnoses, environmental monitoring, and food safety testing for pathogen detection because of its high throughput and economic advantages [5]. Pathogenic genes in test samples can be captured on array spots through hybridization reactions, and this binding event can be converted into fluorescence, luminescence, or colorimetric signals [6]. In particular, because colorimetric sensor arrays do not require expensive analysis equipment for signal detection, they have the advantage of being simpler and more economical than fluorescent sensor arrays

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[7]. Small-sized portable microdevices based on various materials, such as glass [8], paper [9], silicon [10], polydimethylsiloxane [11, 12], and cyclic olefin copolymers (COCs) [13, 14], have been investigated for simplified on-site detection. COCs are suitable substrates for microarray chips because of their high chemical resistance, low water absorption, and high optical transparency [15–18]. They are a good alternative to conventional materials, such as glass, because fabricating microsystems, such as the reaction chamber and microfluidic channel, is easy and economical. The immobilization method of poly(thymine)10-poly(cytosine)10 tagged (TC tag) DNA oligonucleotides through UV irradiation on unmodified COC substrates enabled the simple fabrication of a low-cost DNA microarray chip [19]. Although a more specific principle of the binding mechanism between the COC substrate and the DNA oligonucleotide has not been elucidated, the TC tag DNA oligonucleotides immobilized on the microarray chip showed stable properties even at high-temperature conditions (100 °C, 20 min), and its function as a capture probe for microarray analysis was confirmed [19, 20]. Terminal deoxynucleotidyl transferase (TdT) is an enzyme that catalyzes the addition of nucleotides to the 3′ terminus of single-stranded or double-stranded DNA in a template-independent manner [21]. The sequence of the TdT reaction products depends on the components of the deoxynucleotide triphosphate pool added to the reaction. The long single-stranded DNA structures produced make TdT a powerful tool for biological analyses [22–24].

In this study, we fabricated a DNA microarray chip using a COC substrate through a simple process involving plasma and UV treatment. We designed a visual identification method using on-chip signal amplification technology and gold nanoparticles to check for pathogenic genes. Optimal conditions for on-chip signal amplification, colorimetric signal formation, and DNA microarray fabrication were investigated. In addition, four actual pathogens were used as detection targets to demonstrate the usefulness of this detection method.

Materials and methods

Chemicals and reagents

All synthetic DNA oligomers used in this study were purchased from Bioneer (Daejeon, Korea), and sequence information for DNA oligomers is listed in Additional file 1: Table S1. The COC chip (length: 75 mm × width: 25 mm × height: 1.7 mm) was custom-made by MNTEK, Inc. (Suwon, Korea). The attachable HybriWell chambers (6 well, length: 9.8 mm × width: 20 mm × depth: 0.25 mm) were purchased from Grace Bio-Labs (OR, USA). TdT and biotin-11-deoxyuridine triphosphate (b-dUTP) were purchased from Thermo Fisher Scientific (Vilnius, Lithuania). A multiplex PCR kit was purchased from Qiagen (Hilden, Germany). Lambda exonuclease was purchased from New England Biolabs (MA, USA). The Streptavidin-conjugated gold nanoparticle (s-AuNP; 10 nm) solution was purchased from Cytodiagnostics (Burlington, ON, Canada). A silver enhancement kit and isopropyl alcohol were purchased from Sigma-Aldrich (MO, USA). Absolute ethanol (HPLC grade) was purchased from Fisher Scientific (NJ, USA). Micro Spotting Solution Plus (2×) was purchased from Arrayit Corporation (Sunnyvale, USA). Saline-sodium citrate buffer (20×, pH 7.0) was purchased from Rockland (ME, USA).

Bacterial strains and culture conditions

Escherichia coli O157:H7 (NCCP 11091) was obtained from the National Culture Collection for Pathogens (NCCP, Korea). *Listeria monocytogenes* (KCTC 3569) was obtained from the Korean Collection for Type Cultures (KCTC, Korea). *Bacillus cereus* (ATCC 14579) and *Salmonella enterica Typhimurium* (ATCC 14028) were obtained from the American Type Culture Collection (ATCC, MD, USA). The four pathogens (*E. coli O157:H7*, *Listeria monocytogenes*, *Bacillus cereus*, and *Salmonella enterica Typhimurium*) were cultured in a brain heart infusion liquid medium at 37 °C with shaking for 12–16 h. The concentrations of the cultured cells were determined by optical density at 600 nm using a UV-Vis spectrometer (Thermo Fisher Scientific, MA, USA). The cells were then diluted in sterile water to a concentration of 10^8 cells/mL and stored at − 20 °C until use.

Fabrication of DNA microarray chip

The custom-made COC chip was washed sequentially in distilled water (DW), ethanol, and isopropyl alcohol for 5 min each before use and then thoroughly dried using an air gun. Subsequently, for the hydrophilization of the COC chip surface, the COC chip was exposed to oxygen plasma (CUTE, Femto Science Inc., South Korea) at 30 W for 10 s with a flow rate of 20 sccm. Optimal spotting buffer conditions were investigated to obtain uniform microarray spots on the COC chip. For this, the fluorescent probe DNA had the 5′ end modified by cy3 and the 3′ TC tag (poly thymine 10-poly cytosine 10) diluted in spotting solution A (150 mM sodium phosphate buffer with 0.01% tween 20, pH 8.5) or B (1× micro spotting solution plus, Arrayit, USA) to a final concentration of 0.5 μM. Spotting was performed using an XactII microarrayer (LabNEXT, USA) with an Xtend microarray pin with a diameter of 500 μm and then dried under 70% humid conditions for 10 min. The array spots were exposed to UV light at 254 nm with a power of 3 mW/cm^2 for 30 min using an XL-1000 UV crosslinker (Spectronics Corporation, NY, USA). Subsequently, to remove

unbonded DNA molecules, the COC chip was washed using a washing buffer (0.1× SSC buffer with 0.01% SDS, pH 7) for 10 min and rinsed with DW. The prepared microarray chip was stored until use in a chamber where humidity (52%) and temperature (25 °C) were maintained and was usually used for subsequent experiments immediately. Fluorescence images of the array spots were obtained with a GenePix 4100 microarray scanner (Molecular Devices, CA, USA).

Optimization of TdT reaction parameters and s-AuNP concentration

The DNA microarray chip was prepared by fixing 100 μM of positive probe DNA with the 5′ end modified with a TC tag on the COC chip in the same manner described in Fabrication of DNA microarray chip section. To investigate the optimal TdT reaction time, 25 μL of TdT reaction solution (1× TdT reaction buffer, 200 μM of b-dUTP, and 60 units of TdT) in a reaction chamber on a COC chip was incubated at 37 °C for various durations (0–4 h), and then inactivated at 70 °C for 20 min. Then, 25 μL of the s-AuNP solution at an optical density (OD) of 0.3 was loaded onto the array spots and hybridized at 37 °C for 1 h. The COC chip was washed with the washing buffer (0.1× SSC buffer with 0.01% SDS, pH 7) for 10 min before and after the hybridization reaction. The silver enhancement reaction for augmenting the colorimetric signal of the array spots was performed using a cover slide for 4 min at 25 °C and imaged using a FastGene® GelPic LED Box (Nippon Genetics) after rinsing with DW. To measure the colorimetric signal of the array spots, the COC chip images were transformed into black and white inversion images corrected brightness and sharpness using Image J software. And then, the intensity of 16 pixels at the array spot center was expressed as "grey value" representing the brightness of pixels by Image J software. The "mean grey value" represented the average of the grey value obtained from each array spot. Various concentrations of b-dUTP (0–200 μM) were investigated for a TdT reaction of 1 h to determine optimal conditions. In addition, the different concentrations of s-AuNP (0–0.5 OD) were analyzed in the same manner as in the previous experiment, with a TdT reaction of 1 h and a b-dUTP concentration of 120 μM.

Qualitative analysis of real samples

Four pairs of primers capable of specifically hybridizing with each pathogenic gene were used for PCR (Additional file 1: Table S1). PCR amplifications were conducted in a 50 μL mixture containing 0.2 μM of each primer pair, 10^5 cells of the target pathogen, and 1× PCR master mix (Qiagen, Hilden, Germany) using a T100 Thermal Cycler (Bio-Rad, CA, USA). The following PCR program was used: 95 °C for 15 min, 35 cycles at 95 °C for 30 s, 56 °C for 90 s, and 72 °C for 1 min. For exonucleolytic digestion, 50 μL of mixture solution containing the PCR product with a final 1× lambda exonuclease reaction buffer and 25 units of lambda exonuclease was incubated at 37 °C for 15 min and inactivated at 75 °C for 10 min. This solution was loaded onto a COC chip upon which each capture probe DNA was immobilized in the same manner as described in Fabrication of DNA microarray chip section and incubated at 37 °C for 2 h in the reaction chamber to induce the hybridization of the target gene with the capture probe DNA. The TdT reaction solution (25 μL; 1× TdT reaction buffer, 200 μM b-dUTP, 60 units of TdT) in a reaction chamber on a COC chip was incubated at 37 °C for 2 h and then inactivated at 70 °C for 20 min. Next, 25 μL of s-AuNP solution (0.3 OD) was loaded onto the array spots using a reaction chamber and hybridized at 37 °C for 1 h, and a silver enhancement reaction using a cover slide was conducted at 25 °C for 4 min. The COC chip was washed with the washing buffer (0.1× SSC buffer with 0.01% SDS, pH 7) for 10 min before and after the TdT reaction and hybridization reaction of s-AuNP. Finally, the COC chip was imaged using a FastGene® GelPic LED Box (Nippon Genetics) after rinsing with DW and drying with an air gun.

Results and discussion

Microarray-based assay design for visual identification of pathogenic genes

A microarray-based detection method using terminal nucleotide addition reaction for visual identification of pathogenic genes was developed through the fabrication of an unmodified COC-based microarray chip and optimization of colorimetric signal formation. As shown in Fig. 1, the capture probe DNA capable of partially complementarily binding to the target gene was consistently arranged by a contact microarray spotter and fixed on the surface of the COC chip. The target gene loaded onto the microarray chip was hybridized with the capture probe DNA that had sequence complementarity and b-dUTP was continuously added to the 3′ end with the TdT reaction. The Poly b-dUTP synthesized selectively forms a complex with s-AuNPs through a biotin-streptavidin interaction, causing the formation of black spots that can be observed with the naked eye through an additional silver enhancement reaction for 4 min. However, in the case the target gene was absent, the synthesis of poly b-dUTP in the array spot was inhibited because TdT could not use a phosphate-protected capture probe at the 3′ as a reaction substrate. This microarray-based detection method saves time, cost, and labor required for multi-target or multiple samples analysis as it can analyze numerous targets simultaneously compared to the general real-time

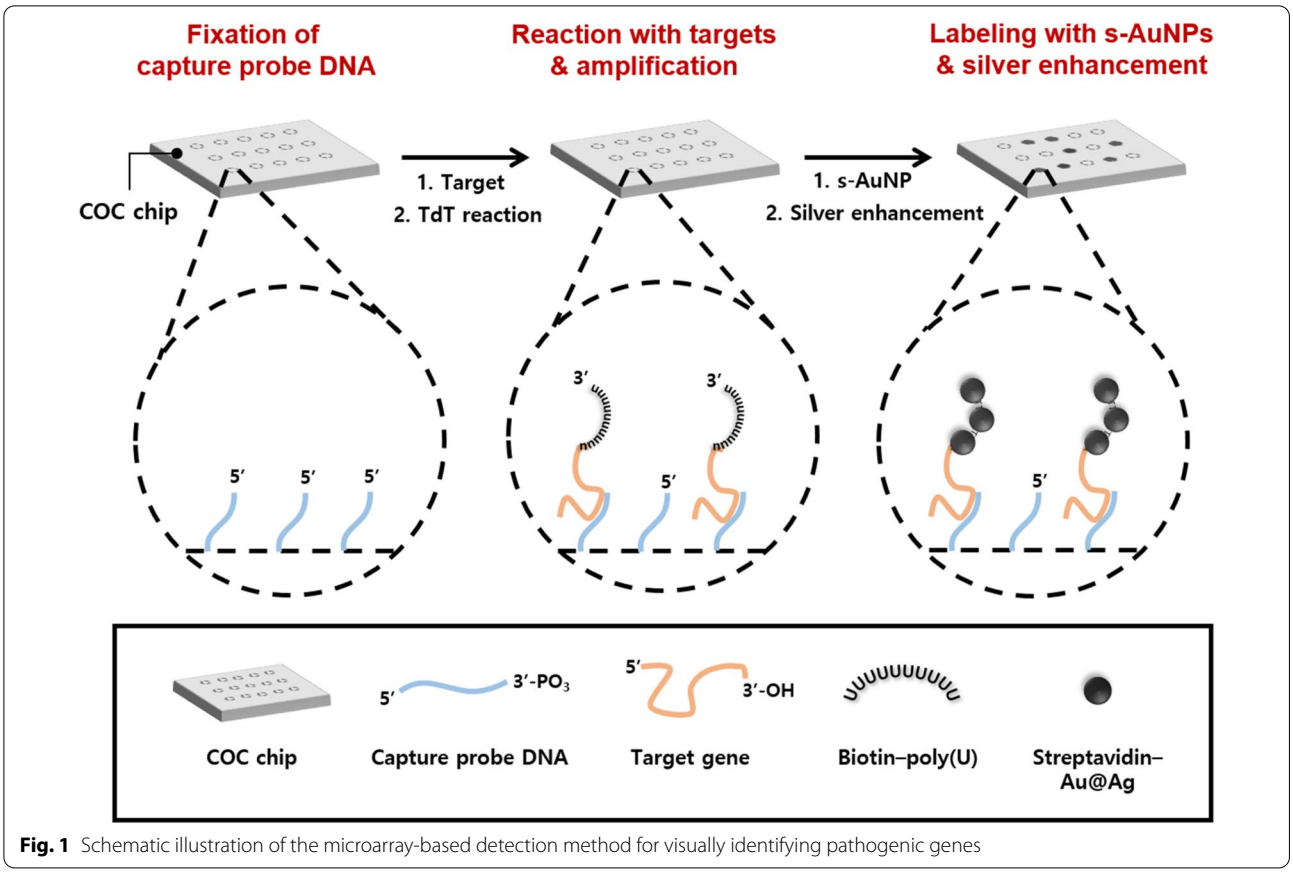

Fig. 1 Schematic illustration of the microarray-based detection method for visually identifying pathogenic genes

polymerase chain reaction that uses fluorescent probes within the range where their absorption wavelengths do not overlap each other. In addition, since the colorimetric signal formed in the microarray spot minimizes the use of complex and expensive equipment for signal analysis, intuitive result analysis is possible, and is cost-effective compared to the fluorescence signal-based microarray detection method. In the past, for the detection of a target gene, a sandwich assay using simple and direct binding between target DNA and AuNP probes was usually performed on a colorimetric microarray chip [25, 26]. On the other hand, in our method, the generation of the b-dUTP tail from one target gene by using the TdT reaction has the advantage of binding a larger amount of AuNPs to the target gene. These characteristics act as factors that amplify the colorimetric signal.

Optimization of spotting conditions for DNA microarray chip fabrication

To ensure uniform microarray spot formation on the non-modified COC chip surface, it was sequentially washed with water, ethanol, and isopropanol before use and treated with oxygen plasma to induce hydrophilicity of the COC surface. To produce high-quality DNA array spots using a contact microarray system, the pickup height and speed of the microarray pins were determined to be 1.5 mm and 3 s, respectively (data not shown). And to prevent cross-contamination between samples, 5 cycles of DW washing for 30 s and air drying for 30 s were determined as the optimal washing conditions (data not shown). Optimal spotting buffer conditions were also investigated, as shown in Fig. 2. Two types of spotting solutions were compared. For buffer A (150 mM sodium phosphate buffer with 0.01% tween 20) [19], the fluorescent probe DNA was intensively fixed at the edge of the spot, while commercial buffer B (1× micro spotting solution plus produced by the Arrayit Corporation) tended to be non-uniformly fixed at the center of the spot. Because the uniform distribution of the capture probe DNAs on the microarray spot was essential for the efficiency of subsequent hybridization and enzymatic reactions, we further tested the mixing conditions of buffers A and B. We found that uniformly fixed spots formed when the two buffers were mixed at a volume ratio of 1:1 (Fig. 2C). This phenomenon may be influenced by the concentration of surfactant present in the spotting buffer. In previous studies, it has been reported that when the amount of surfactant in the spotting buffer is small, the fluorescence

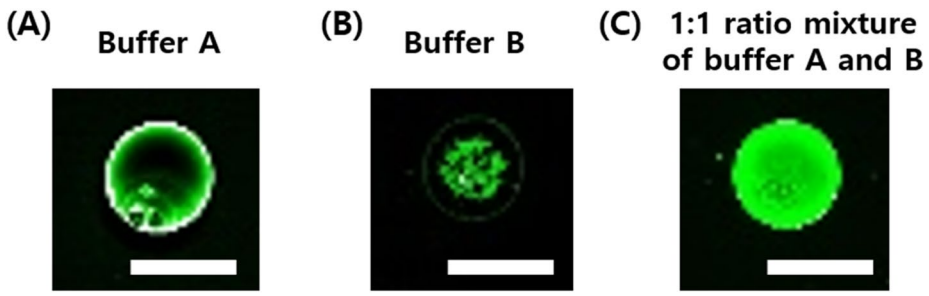

Fig. 2 Fluorescence scan image of fluorescent probe DNA immobilized on a COC chip to determine the optimal spotting buffer conditions. The scale bar is 500 μm

signal of the microarray is concentrated in the center of the spot, and when the amount is large, a coffee ring pattern occurs [19, 27]. However, in determining the quality of microarray spots, not only the concentration of the surfactant but also other factors such as the surface properties of the solid substrate, the viscosity of the solution, drying conditions, and microarray type were thought to act in a complex way.

Optimization of experimental parameters

To optimize the TdT reaction conditions, the optimal reaction time and concentration of b-dUTP were determined. Regarding the TdT reaction time, the mean grey values of spots according to the TdT reaction time (0, 0.5, 1, 2, and 4 h) were 48.37 ± 28.16, 101.49 ± 26.33, 164.35 ± 34.42, 183.06 ± 26.88, and 177.3 ± 26.32, respectively. Two hours was determined to be the optimal TdT reaction time. The grey value of the microarray spot increased with time and showed a tendency to become saturated at 2 h (Fig. 3A). The mean grey values of spots according to b-dUTP concentration (0, 40, 80, 120, 160, and 200 μM) were 20.91 ± 1.17, 35.16 ± 9.28, 61.72 ± 17.82, 121.34 ± 8.46, 93.99 ± 25.53, and 110.19 ± 30.29, respectively. Therefore, a concentration of 120 μM led to the highest grey value and was determined to be optimal (Fig. 3B). In addition, the optimal concentration of the s-AuNP solution necessary to produce a colorimetric signal was also investigated, and 0.3 OD was determined as the optimal concentration, as shown in Fig. 3C. The mean grey values of spots according to s-AuNP concentration (0, 0.1, 0.2, 0.3, 0.4, and 0.5 OD) were 22.78 ± 0.57, 95.12 ± 10.8, 139.92 ± 10.12, 193.92 ± 22.74, 168.05 ± 28.67, and 137.55 ± 28.01, respectively. One reason the grey value decreased as the concentration of s-AuNP increased in this result could be due to electrical repulsion between s-AuNPs. In our previous study [28], the surface charge of s-AuNP was confirmed to be – 24.8 mV, and the biotin-labeled poly uridine tail generated from the array spot will also be negatively charged by the sugar-phosphate backbone. Therefore, excessive s-AuNP concentration conditions can inhibit the s-AuNP binding efficiency to the array spot due to the increased electrical repulsion between particles. To form the highest colorimetric signal at the array spot, the optimal condition was briefly set to a TdT reaction time of 2 h, b-dUTP concentration of 120 μM, and s-AuNP concentration of 0.3 OD. Captured images of the array spots formed on the COC chip was

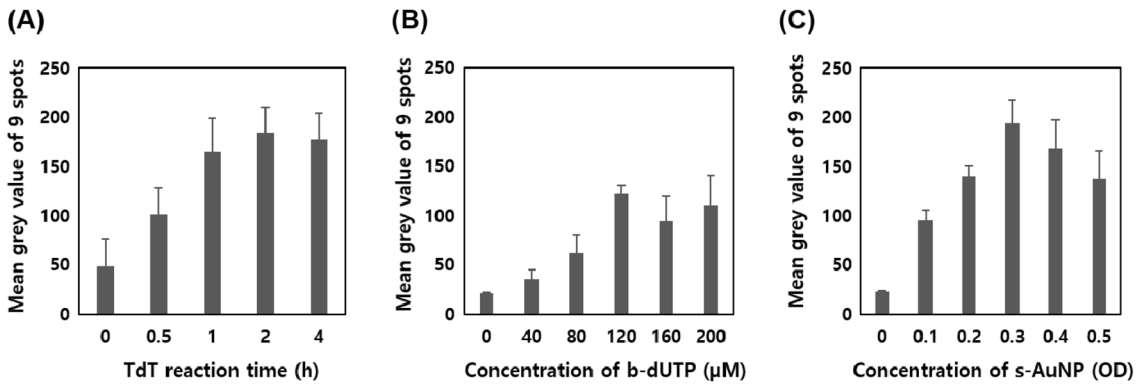

Fig. 3 Optimization results for TdT and s-AuNP hybridization reactions

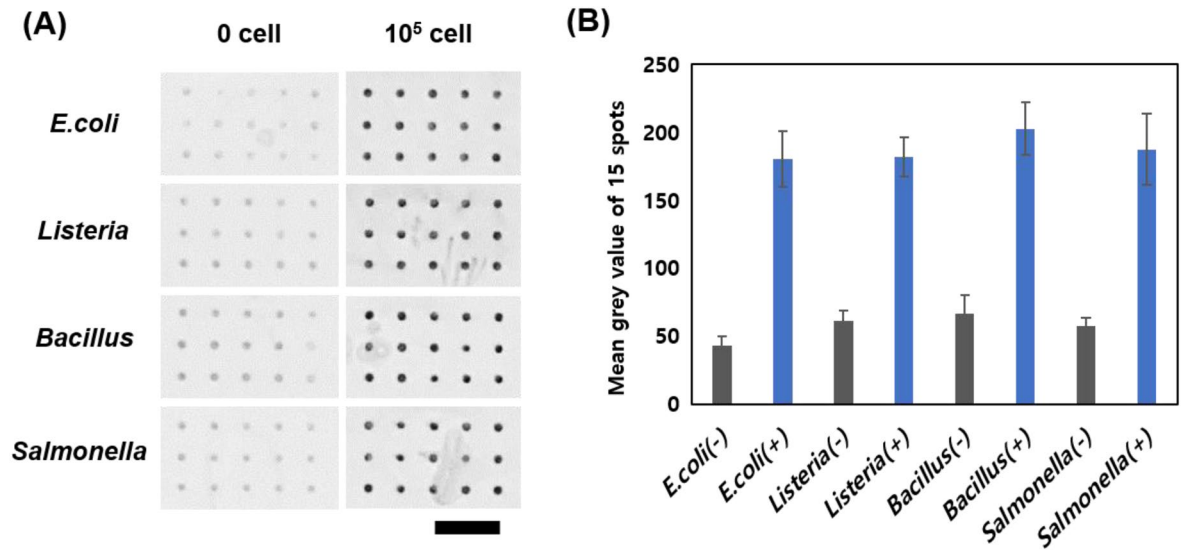

Fig. 4 **A** Grey capture images of array spots formed on the COC chip to detect four pathogenic genes. Scale bar is 4 mm. **B** Bar graph of the grey values of (**A**) images analyzed using Image J software. The symbol (− or +) indicates the amount of each pathogen (0 or 10^5 cells)

used for the characterization in Fig. 3 (Additional file 1: Fig. S2).

Usability of the proposed method

The applicability of the proposed method for actual pathogenic gene analysis was evaluated. First, to produce target dsDNAs from four kinds of microbial culture mediums, a PCR was performed using the four types of target-specific forward primers and reverse primers modified with a 5′ phosphate group. Subsequently, PCR products were treated with lambda exonuclease such that only ssDNAs capable of hybridizing with each capture probe DNA remained (Additional file 1: Fig. S3). These test samples were individually prepared in the presence or absence of each pathogen and analyzed on a COC chip to which each target-specific capture probe DNA was immobilized. As shown in Fig. 4A, it was visually apparent that the colorimetric signal of the microarray spots enhanced remarkably in the presence of each target ssDNA for all pathogens. In addition, the average grey values of spots obtained by analyzing Fig. 4A through Image J software are 42.74 ± 6.98 for *E. coli* (0 cell), 180.31 ± 20.38 for *E. coli* (10^5 cell), 60.97 ± 7.66 for *Listeria* (0 cell), 182.04 ± 14.5 for *Listeria* (10^5 cell), 66.28 ± 14.16 for *Bacillus* (0 cell), 202.8 ± 19.26 for *Bacillus* (10^5 cell), 57.42 ± 6.31 for *Salmonella* (0 cell), and 187.44 ± 26.29 for *Salmonella* (10^5 cell) (Fig. 4B). These results suggest that the present detection method could easily help identify the presence or absence of each pathogenic gene from several pathogen samples with the naked eye.

Conclusion

To summarize, we fabricated a DNA microarray chip made of a COC substrate and developed a detection method for visually identifying pathogenic genes. The DNA microarray chip was easily fabricated through oxygen plasma and UV treatment, and optimal DNA spotting buffer conditions were investigated. In addition, the optimal conditions for the signal amplification and colorimetric reactions occurring on the surface of the array spots were successfully established. Four kinds of pathogen samples were analyzed to confirm the usefulness of this detection method, and the presence of pathogens could be visually confirmed without complex analysis equipment. As a result, a system capable of the simultaneous detection of multiple genes was realized through this study, and its use in detecting precise pathogen was confirmed.

Abbreviations

b-dUTP: Biotinylated deoxyuridine triphosphate; COC: Cyclic olefin copolymer; OD: Optical density; PCR: Polymerase chain reaction; s-AuNP: Streptavidin-conjugated gold nanoparticle; SDS: Sodium dodecyl sulfate; SSC: Saline-sodium citrate; TdT: Terminal deoxynucleotidyl transferase.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40486-022-00153-8.

Additional file 1: Table S1. Oligonucleotide information used in this study. **Figure S1.** Images of (A) a COC chip and (B) attachable reaction chambers. (C) An image of 25 µL of green dye solution injected into the reaction chambers attached to the COC chip. Scale bar is 25 mm. **Figure S2.** Capture images of array spots formed on the COC chip for

optimization of experimental parameters. Scale bar is 4 mm. **Figure S3.** Agarose TBE gel (2.5%) electrophoresis results of the PCR or PCR and lambda exonuclease reaction product for the four pathogens. Lane M: GeneRuler 50 bp DNA ladder (Thermo Scientific) (1.5 µL per lane), lanes 1, 3, 5, and 7: PCR product of E. coli O157:H7, *Listeria monocytogenes*, *Bacillus cereus*, and *Salmonella enterica Typhimurium*, respectively. Lanes 2, 4, 6, and 8: lambda exonuclease reaction product after PCR of E. coli O157, *Listeria monocytogenes*, *Bacillus cereus*, and *Salmonella enterica Typhimurium*, respectively. Gel electrophoresis was conducted at 45 V for 90 min and gel staining was conducted with 1 × SYBR Green I and II for 20 min.

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Author contributions
TWK contributed to the design of experimental methods and the acquisition of most data. MCL and JAL collected and analyzed the data. SWC contributed to the interpretation of the experimental results. MAW developed the research concept and critically revised and approved the manuscript. All authors have read and approved the final manuscript.

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Data availability
All data generated or analyzed during this study are included in this published article and its Additional files.

Declarations

Ethics approval and consent to participate
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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