



An integrated smartphone-based lateral flow assay utilizing enzyme-loaded nanoprobe for rapid and ultrasensitive on-site detection of microcystin-LR

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ABSTRACT

The increasing prevalence of harmful cyanobacterial blooms has intensified concerns over microcystin-LR (MC-LR) contamination in freshwater, creating an urgent demand for rapid and decentralized water quality monitoring. Although liquid chromatography–tandem mass spectrometry (LC–MS/MS) and enzyme-linked immunosorbent assay (ELISA) provide laboratory-grade sensitivity, these methods require complex instrumentation and trained personnel, which limits their applicability in field settings. Conventional lateral flow assays (LFAs) offer portability and ease of use but generally lack the sensitivity required for reliable detection of MC-LR at sub-regulatory concentrations. In this study, we present a user-friendly semiautomated paper-based lateral flow immunosensor engineered as a point-of-care testing (POCT) platform for on-site MC-LR screening. To address intrinsic sensitivity limitations, enzyme-loaded signal-amplifying nanoprobe (ELNPs) were engineered to enable catalytic signal amplification through an on-chip TMB reaction. The multilayer paper-based microfluidic device supported capillary-driven fluid flow and incorporated dedicated washing and substrate inlets, which allowed controlled enzymatic reactions while minimizing background interference. A custom 3D-printed smartphone reader was also constructed to enable standardized imaging and RGB-based quantitative analysis of the device output. The resulting platform detected MC-LR across a range of 85 to 400 pg/mL with a limit of detection of 85 pg/mL within 15 min, which is significantly lower than the guideline value established by the World Health Organization (WHO) for drinking water. Moreover, the system demonstrated high selectivity and strong agreement with LC–MS/MS when applied to real freshwater samples. Overall, this integrated platform provides a practical and portable POCT solution for rapid on-site water quality monitoring.

1. Introduction

The increasing global concern over the rise of harmful cyanobacterial blooms, driven by anthropogenic factors such as global warming and nutrient-rich water pollution, represents a critical environmental challenge [1,2]. These blooms often lead to the production of potent cyanotoxins, particularly microcystin-LR (MC-LR), a cyclic heptapeptide recognized as a highly potent hepatotoxin. The presence of MC-LR in freshwater environments poses significant risks not only to aquatic life but also directly to human health through contaminated drinking water and the consumption of contaminated aquatic products [3]. Given this substantial threat, the World Health Organization (WHO) has established a maximum allowable concentration of 1 ng/mL for MC-LR in drinking water, underscoring the urgent need for robust, sensitive, and

rapid detection technologies [4]. The accelerating pace of climate change and continued water quality degradation necessitate the development of on-site, real-time detection methods for effective environmental monitoring and public health protection [5].

Currently, the quantification and identification of MC-LR in environmental matrices predominantly rely on established instrumental techniques such as high-performance liquid chromatography (HPLC) and liquid chromatography–tandem mass spectrometry (LC–MS/MS). These methods are valued for their exceptional sensitivity and their ability to provide structural confirmation of toxin variants. To overcome the limitations of these conventional chromatographic methods, a range of screening tools and advanced sensing platforms have been extensively developed for environmental monitoring. For instance, the enzyme-linked immunosorbent assay (ELISA) has been widely adopted as a

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primary screening tool owing to its high-throughput capacity [6–8]. Complementing these methods, advanced sensing platforms, such as electrochemical (EC), fluorescence (FL), and chemiluminescence (CL) sensors, have been developed for highly sensitive detection [9–14]. Building upon these modalities, several sensors have been specifically developed for the precise detection of MC-LR. For example, recent EC approaches have utilized laser-induced graphene and tetrahedral DNA-based aptasensors to facilitate the on-site electrochemical detection of MC-LR [15,16]. FL-based methods have introduced unique fluorescent probes designed to effectively exclude interference, enabling accurate MC-LR monitoring in natural lakes and biological cells [17,18]. Furthermore, CL and ECL platforms have achieved ultra-sensitive detection of MC-LR by employing multivalent bifunctional nanobodies or synergistic trimetallic (PdMoCu) metallene structures for enhanced signal transduction [19,20]. Despite their exceptional sensitivity, these advanced methodologies inherently share critical drawbacks for real-time field surveillance. ELISA involves multistep workflows with repeated washing and prolonged incubations, while EC, FL, and CL sensors often require specialized benchtop equipment and complex electrode fabrication. These logistical constraints hinder deployment in remote or resource-limited settings [21].

To bridge the gap between analytical reliability and the practical demands of rapid on-site monitoring, lateral flow assay (LFA) technology has emerged as a promising alternative for the fast and simple detection of MC-LR. By utilizing capillary action, LFA platforms eliminate the need for external power sources while providing rapid results (15–30 min), cost-effectiveness, and high portability [22]. Specifically, LFA-based sensors for MC-LR detection leverage the high specificity of antibody–antigen interactions, providing a user-friendly and selective diagnostic tool [23]. Despite the significant advantages in speed and simplicity offered by conventional gold nanoparticle-based LFAs, these systems often exhibit limited sensitivity for quantifying trace amounts of MC-LR in real environmental samples. To address this limitation, various signal amplification strategies have been explored, including aptamer probes [24], scFv-AP fusion proteins [25], gold nanostars [26], Au@Pt nanozymes [27], fluorescent microspheres (FMs) [28], and quantum dots (QDs) [29]. However, many of these approaches struggle to consistently achieve reliable quantification below the WHO drinking-water guideline of 1 ng/mL without sacrificing the inherent simplicity of the assay. Therefore, effective field monitoring requires an advanced signal amplification system capable of reliably detecting MC-LR concentrations well below regulatory thresholds while preserving the inherent convenience of LFA platforms. While enzyme-mediated signal amplification is highly effective for enhancing sensitivity, its application in conventional LFAs is severely limited by the necessity of multi-step processes. Sequential washing and substrate addition steps typically require complex manual handling, external pumps, or bulky equipment, which constrains the point-of-care (POC) applicability of the sensor.

To address the sensitivity limitations of conventional LFAs for on-site MC-LR detection, this study introduces a structurally engineered, semi-automated microfluidic platform. Building on the robust signal amplification capability of our previously established enzyme-loaded signal-amplifying nanoprobe (ELNPs), this work focuses on advanced fluidic control and systemic integration. First, to overcome the limitations associated with the multi-step procedures of enzymatic LFAs, we introduced a fluidic control mechanism. We designed a three-dimensional (3D) multilayer paper-based device equipped with a top PET microfluidic channel and sequentially operable inlets. This structural design spatiotemporally separates the competitive binding phase from the signal generation phase. This precise fluidic control enables capillary-driven management of washing steps and on-chip enzymatic colorimetric reactions between horseradish peroxidase (HRP) and TMB substrate, removing the need for external fluidic control equipment or complex manual washing.

Second, to overcome the reliance on benchtop instruments and specialized personnel, we integrated a smart, image-assisted readout

mechanism. Recently, the utilization of digital image processing for on-site optical readout has emerged as a useful strategy in environmental analysis, effectively translating visual color changes into quantitative data, as demonstrated in recent portable sensors [30,31]. In line with this trend toward smart, image-assisted monitoring, we successfully combined the miniaturized paper chip with a custom 3D-printed smartphone reader capable of inverse RGB data processing. This integration addresses a common trade-off in biosensor development between high sensitivity and field applicability. By unifying our ELNPs with automated fluidic hardware and smart optical readout, we established a practical and user-friendly quantitative screening system. The integrated platform provides a fast, simple, and sensitive route for the on-site monitoring of MC-LR in freshwater, balancing laboratory-grade analytical rigor with the logistical requirements of practical field deployment.

2. Materials and methods

2.1. Chemicals and reagents

Colloidal gold nanoparticles (AuNPs, 30 nm in diameter) were purchased from BBI Solutions (UK). Distilled water was purified using the Human Power I+ water purification system (Human Science, Korea). HRP-conjugated anti-mouse IgG, bovine serum albumin (BSA), MC-LR standard solution, BSA-conjugated MC-LR, saxitoxin (STX) dihydrochloride solution, trifluoroacetic acid (TFA), ammonium acetate, formic acid, methanol, Whatman GF/C glass microfiber filter (1.2 μ m pore size), C18 solid-phase extraction (SPE) cartridges (Discovery DSC-18), 3,3',5,5'-tetramethylbenzidine (TMB), 0.01 M phosphate-buffered saline (PBS, pH 7.4), and Tween 20 were purchased from Sigma-Aldrich (USA). Acetonitrile was purchased from Daejung Chemicals (Korea). HRP, HRP-conjugated streptavidin, goat anti-HRP IgG, and Zeba spin desalting columns (7 K MWCO) were purchased from Thermo Fisher Scientific (USA). Gramicidin S was purchased from MedChemExpress (USA). Nitrocellulose membranes (CNPC-SS12, 10 μ m), glass fiber membranes (PT-R1), and absorbent pads (AP080) were purchased from MDI (India). A4-size photo paper (Formtec, IH4021), A4-size double-sided adhesive tape (Formtec, BS-210), adhesive polyethylene terephthalate (PET) film (Printec, G2140), and overhead projector (OHP) transparency film (Printec, V2300) were purchased from a local supplier. Anti-MC-LR IgG (MC10E7) and anatoxin-a (ATX) were purchased from Enzo Life Sciences (USA). A commercial MC-LR ELISA kit (KA1496) was purchased from Abnova (Taiwan). Transmission electron microscopy (TEM) grids (carbon type A, 300 mesh, Cu) were purchased from Ted Pella (USA). All other chemicals used in this study were commercially obtained at reagent grade.

2.2. Apparatus and instruments

The optical and structural characteristics of the synthesized ELNPs were analyzed using a UV–Vis spectrophotometer (NanoDrop 2000, Thermo Scientific, USA) and a transmission electron microscope (TEM, JEOL1010, Japan). The surface charge of the nanoparticles was measured using a zeta potential analyzer (SZ-100, HORIBA, Japan). Structural components for the LFA device were precisely patterned using a laser cutter (L3020, REXBOT, Korea). The portable dark-box reader was custom-designed and fabricated via a commercial 3D printing service. Image capture and RGB data processing for colorimetric analysis were performed using a smartphone (Galaxy S23 Ultra, Samsung, Korea). The absorbance of the colorimetric reactions was measured using a microplate reader (LTEK INNO, Korea). Additionally, for the validation of real environmental samples, target concentrations were confirmed using an LC–MS/MS system (TSQ Altis, Thermo Scientific, USA).

2.3. Synthesis of ELNPs

The ELNPs were synthesized following a previously reported method [32]. The synthesis involved a two-step process consisting of AuNP coating with streptavidin–HRP followed by conjugation with biotinylated antibodies. For AuNP coating, 6.4 μL of streptavidin–HRP (1.25 mg/mL) was added to 1 mL of colloidal AuNP solution (30 nm, 2×10^{11} particles/mL), and the mixture was stirred at 700 rpm for 1 h at room temperature ($\sim 25^\circ\text{C}$). Next, anti-MC-LR IgG was biotinylated to facilitate coupling through streptavidin–biotin interactions. Sulfo-NHS-biotin reagent (2.66 μL , 2.5 mM) was mixed with 50 μL of anti-MC-LR IgG solution (1 mg/mL) and incubated at 850 rpm for 1 h at 25°C . Unreacted biotin reagents were removed by purification using a PBS buffer-exchange desalting column. Finally, 10 μL of the biotinylated anti-MC-LR IgG (1 mg/mL) was added to the streptavidin–HRP-coated AuNP solution. Excess antibodies were removed by centrifugation at 8000 rpm for 15 min at 25°C . The pellet was resuspended in a storage buffer consisting of 2 μL of 3% BSA and 198 μL of 1% PBST (PBS + Tween 20; pH 7.4). The resulting ELNPs were stored at 4°C in the dark. The optical and structural characteristics of the ELNPs were analyzed using a UV–Vis spectrophotometer and TEM, respectively.

2.4. Preparation of the LFA membrane strip

The test line (TL) and control line (CL) were defined on a nitrocellulose membrane by immobilizing specific capture probes. BSA-conjugated MC-LR (1.7 mg/mL) and goat anti-HRP antibody (2.4 mg/mL) served as capture reagents for the TL and CL, respectively. The reagents were applied using a manual contact-printing method with the edge of a glass slide (contact area: 1 mm $\times$ 5 mm) to deliver 0.4 μL per line [33]. Following immobilization, the membrane was dried overnight at 4°C to ensure stable protein fixation.

2.5. Assembly of the paper-based LFA device

The final paper-based device was constructed as a multilayer structure comprising a photo-paper base, adhesive PET films, and an OHP film cover, based on the design in our previous work [34], with minor modifications. First, all structural components, including the photo paper, adhesive films, and functional pads, were precisely patterned using a laser cutter. To assemble the device, a patterned single-sided adhesive PET film was first attached to the photo-paper base substrate. Subsequently, a patterned double-sided adhesive film was layered on top to form the fluidic channel. The sample pad, nitrocellulose membrane (with immobilized probes forming the TL and CL), and absorbent pad were then sequentially positioned in the designated alignment areas on the PET film. Finally, the device was completed by covering the structure with a transparent OHP film placed over the double-sided adhesive layer. This multilayer configuration provided structural stability and enabled consistent, capillary-driven fluid flow within the device.

2.6. Analytical procedure for MC-LR detection

MC-LR detection was performed using the ELNPs in combination with the assembled paper-based LFA device. All procedures were carried out at room temperature. First, 20 μL of diluted ELNP solution (5×10^9 particles/mL) was mixed with 20 μL of MC-LR standard solution at various concentrations and incubated for 5 min to allow competitive binding. The reaction mixture was then loaded onto the sample pad of the device and allowed to flow through the membrane for 4 min. To minimize background noise caused by unbound materials, a washing step was performed by sequentially injecting 10 μL of 0.5% PBST three times over a total washing period of 5 min. Subsequently, 80 μL of TMB solution was introduced through the substrate inlet. The device was then inserted into the custom-designed holder of the portable reader, where

the TMB reaction proceeded for 1 min. Finally, images of the colorimetric detection zones were captured using a smartphone coupled with the reader, and the resulting color intensities were quantitatively analyzed using RGB-based image-processing software. For high-concentration detection, a conventional LFA strip without the microfluidic cover (referred to as a bare strip) was used, and the analytical procedure was identical, except that the ELNP solution concentration was adjusted to 2×10^{10} particles/mL to expand the dynamic detection range.

2.7. Preparation and LC–MS/MS analysis of practical samples

To validate the practical reliability of the developed LFA platform, LC–MS/MS analysis was conducted as a standard reference method. Sample pre-treatment was performed in our laboratory strictly following the Algae Alert System guidelines of the National Institute of Environmental Research (NIER, Korea), which are fundamentally aligned with the WHO standards for total microcystin analysis. Briefly, the collected water samples were subjected to ultrasonic cell lysis for 30 min in an ice bath to extract intracellular microcystins. Following complete cell lysis, the mixture was filtered through a GF/C filter (1.2 μm pore size). The filtrate was then purified and concentrated using a C18 SPE cartridge. After washing the cartridge with 20% methanol, the target analytes were eluted using 0.1% TFA in methanol. The eluate was completely dried under a stream of nitrogen gas and reconstituted in methanol. The quantitative analysis of the prepared samples was performed by an external specialized analytical facility using an LC–MS/MS system. Chromatographic separation was performed on a standard C18 column, utilizing a mobile phase consisting of 10 mM ammonium acetate containing 0.1% formic acid and acetonitrile. The mass spectrometer was operated in electrospray ionization (ESI) mode, and multiple reaction monitoring (MRM) was employed for the highly specific and sensitive quantification of MC-LR.

3. Results and discussion

3.1. Design and working principle of the signal-enhanced MC-LR detection platform

To facilitate on-site monitoring of MC-LR in freshwater, we designed an integrated platform consisting of a signal amplification system based on ELNPs, a paper-based LFA device, and a portable signal-readout system. ELNPs were utilized to enhance the sensitivity of MC-LR detection, and Fig. 1a shows their structural design and synthesis process. Meanwhile, Fig. 1b presents the structural configuration of the paper-based LFA device. This simple and miniaturized device featured microfluidic channels constructed from multilayered patterned paper and PET films, incorporating dedicated inlets for washing buffers and substrate solutions to facilitate on-chip enzyme-substrate reactions. The detection mechanism of MC-LR based on a competitive immunoassay is illustrated in Fig. 1c. Initially, an immunoreaction occurred between the target MC-LR and ELNP probes, followed by an immunoreaction with immobilized capture probes through lateral flow within the paper-based LFA device. Subsequently, a washing step was performed, after which the TMB substrate solution was introduced to initiate the enzymatic colorimetric reaction. In this competitive system, the presence of MC-LR competitively inhibited the binding of ELNPs to the BSA-conjugated MC-LR immobilized on the TL. This behavior represents a typical “signal-off” response, where the colorimetric intensity decreased proportionally with increasing target concentration. To streamline the quantitative analysis, the optical signals were converted into a mean gray value $[(R + G + B)/3]$ to quantify the brightness. In this model, pure black and pure white pixels correspond to values of 0 and 255, respectively. Therefore, as the TL color faded with increasing MC-LR concentrations, the measured brightness value increased. Accordingly, this image-processing strategy provided an intuitive positive correlation between

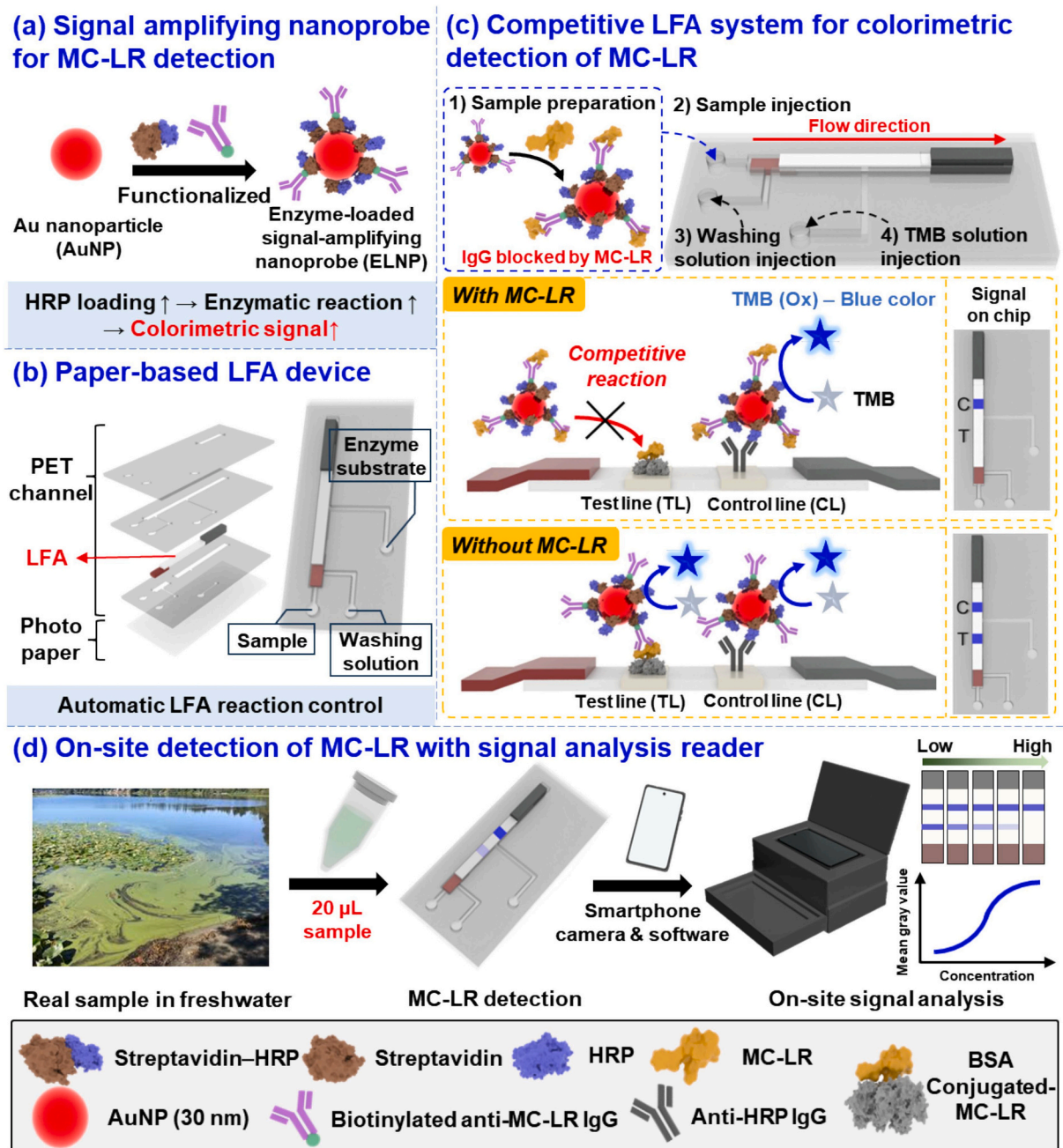


Fig. 1. Schematic illustration of the integrated signal-enhanced platform for on-site MC-LR detection. (a) Preparation of ELNPs for enzymatic signal amplification. (b) Structural schematic of the paper-based LFA device. (c) Mechanism of the competitive immunoassay for MC-LR detection. (d) Overall workflow for on-site monitoring.

the analytical readout and the MC-LR concentration. The complete workflow for on-site MC-LR monitoring using the proposed platform is illustrated in Fig. 1d. The procedure included freshwater sample collection, MC-LR detection using the paper-based LFA device, and instantaneous signal quantification using the fabricated portable reader.

3.2. Synthesis and characterization of ELNPs

To achieve highly sensitive detection of MC-LR within the paper-based LFA system, ELNPs capable of HRP-mediated signal amplification were designed. As shown in Fig. 2a, streptavidin–HRP was first physically adsorbed onto the AuNP surface. Subsequently, biotinylated anti-MC-LR IgG was attached to the remaining streptavidin binding sites through streptavidin–biotin interactions, followed by BSA blocking to

suppress nonspecific surface binding. The successful synthesis of these ELNPs was morphologically confirmed (Fig. 2b). The synthesized ELNPs exhibited a distinct protein layer surrounding an approximately 30 nm AuNP core, resulting in a total particle diameter of approximately 50 nm. Comparative TEM micrographs of the bare AuNPs (left) and ELNPs (right) clearly demonstrated this structural difference. Furthermore, surface optical properties were analyzed using a UV–Vis spectrophotometer to further confirm ELNP synthesis (Fig. 2c). AuNPs exhibited a characteristic optical phenomenon known as localized surface plasmon resonance (LSPR), which resulted from the collective oscillation of electrons at a specific wavelength. This phenomenon produced a strong absorption band in the visible region (500–600 nm). The introduction of functionalized ligands altered the surface morphology and the local refractive index, inducing a shift in this characteristic absorption band.

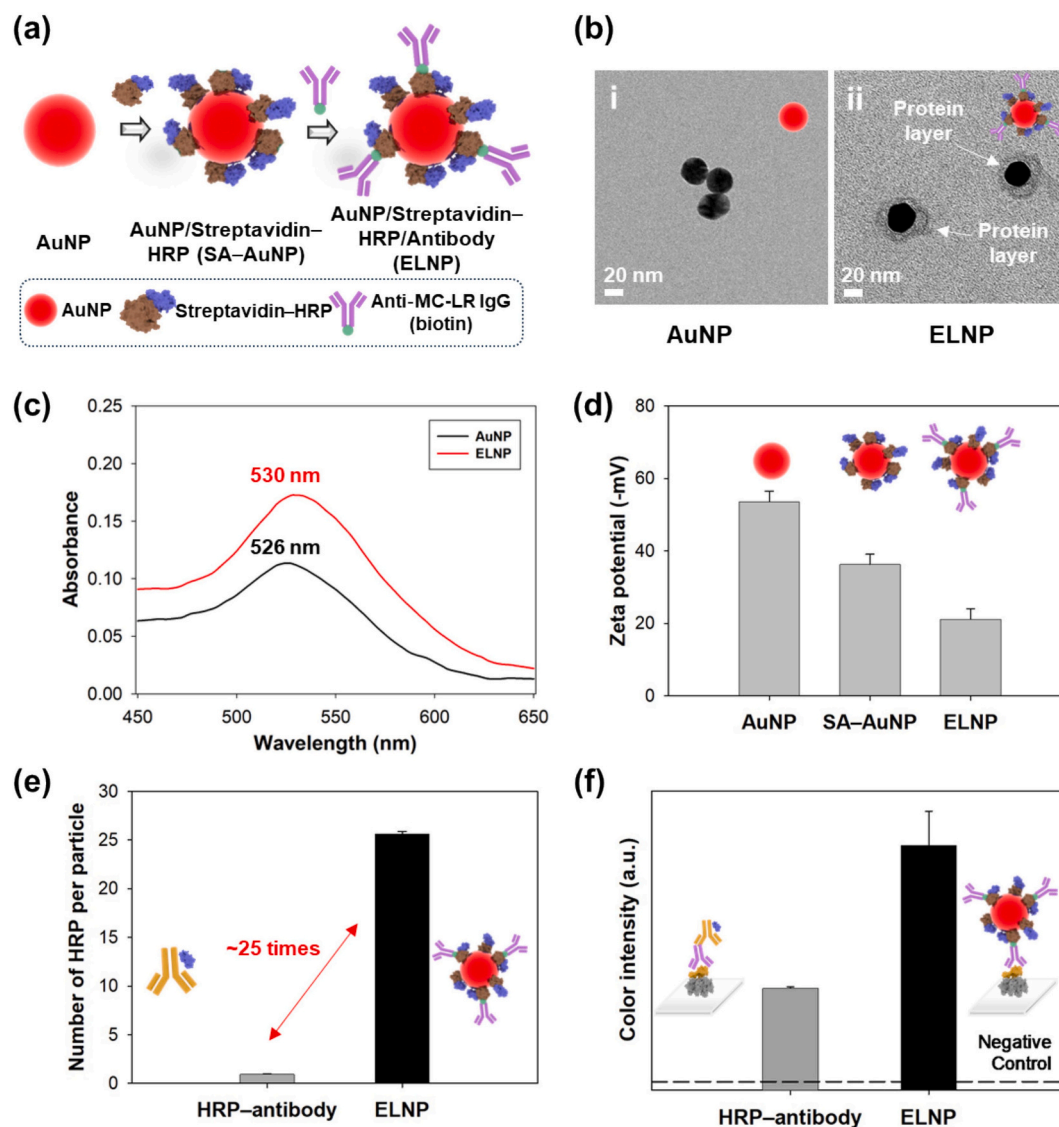


Fig. 2. Synthesis and characterization of ELNPs. (a) Schematic of the ELNPs synthesis process. (b) TEM micrographs of AuNPs (left) and ELNPs (right). (c) UV-Vis spectra confirming surface modification through the redshift of the LSPR peak. (d) Zeta potential values of nanoparticles in each synthesis step. (e) Comparison of enzymatic activity between HRP-conjugated antibodies and ELNPs based on the number of HRP molecules conjugated per nanoparticle. (f) Comparison of color intensity (reciprocal of the mean gray value) between conventional HRP-conjugated antibodies and ELNPs in the LFA platform. All data represent the mean $\pm$ standard deviation of three independent experiments.

Consequently, a distinct red shift of the maximum absorption wavelength from 526 to 530 nm was observed for the prepared ELNPs, confirming successful ligand conjugation on the AuNP surface. To further verify ELNP synthesis, we investigated the impact of ligand conjugation on the nanoparticle surface charge during each functionalization step by measuring zeta potential values. Three types of samples were prepared: bare AuNPs, HRP-conjugated streptavidin-coated AuNPs (SA-AuNPs), and fully functionalized ELNPs. Each sample was diluted in distilled water (pH 6) to evaluate the surface-charge changes in the colloidal dispersed nanoparticles. The results showed zeta potential values of -53.6 ± 2.8 mV for AuNPs, -36.3 ± 2.7 mV for SA-AuNPs, and -21.2 ± 2.8 mV for ELNPs, confirming a progressive decrease in the magnitude of the zeta potential with each functionalization step. Generally, the surface of bare AuNPs was surrounded by citrate molecules, imparting a strong negative charge that provided dispersion stability through interparticle electrostatic repulsion. However, when proteins with an isoelectric point (pI) higher than the solution pH, such as HRP or IgG antibodies, were conjugated to the surface, a protein corona layer formed. This introduced a partial positive charge, thereby decreasing

the absolute value of the zeta potential. Therefore, these zeta potential measurements confirmed that both the HRP-conjugated streptavidin and the biotinylated antibodies were effectively attached to the nanoparticle surface, verifying the successful stepwise synthesis of the ELNPs (Fig. 2d). Meanwhile, the enzymatic performance of the prepared ELNPs was evaluated to investigate their signal-amplification potential. As shown in Fig. 2e, the amount of enzyme per particle was compared between a conventional detection probe (HRP-conjugated secondary antibody) and the synthesized ELNPs. Because the absorbance of the HRP-mediated colorimetric reaction was directly proportional to the number of HRP enzymes, the quantity of HRP immobilized on the ELNPs was quantified using an HRP standard curve (Fig. S1). To determine the absolute number of HRP molecules per single particle, a standard calibration curve was initially established by measuring the absorbance (O. D. at 450 nm) of free HRP at varying concentrations following a 10 min TMB reaction ($R^2 = 0.9996$). By applying identical reaction conditions to the HRP-conjugated anti-mouse IgG and diluted ELNPs, their absorbance values were interpolated into the standard curve to quantify the total number of active HRP molecules in each sample. Dividing this total

value by the known number of nanoparticles yielded the absolute number of HRP molecules per individual particle. Although the potential for reduced specific enzyme activity due to steric hindrance cannot be excluded, the results indicate that each ELNP exhibited an apparent catalytic activity equivalent to approximately 25 free HRP molecules. By contrast, a conventional HRP-conjugated antibody typically possesses only one HRP per molecule. Furthermore, Fig. 2f compares the colorimetric intensity differences, represented by the reciprocal of the mean gray value, on the LFA platform. The TL and CL were immobilized with

0.4 μL of BSA-conjugated MC-LR (0.5 mg/mL) and 0.4 μL of anti-HRP IgG (1 mg/mL), respectively. Equal particle concentrations (3.6×10^8 particles/20 μL) of both the HRP-conjugated anti-mouse IgG and ELNPs were applied, and the resulting grayscale values following the TMB reaction were compared. The application of ELNPs yielded a markedly enhanced colorimetric signal. Consistent with the findings in Fig. 2e, this result demonstrates that unlike conventional secondary antibodies with a low enzyme conjugation ratio (typically 1:1), the synthesized ELNPs could accommodate a high payload of HRP by leveraging the large

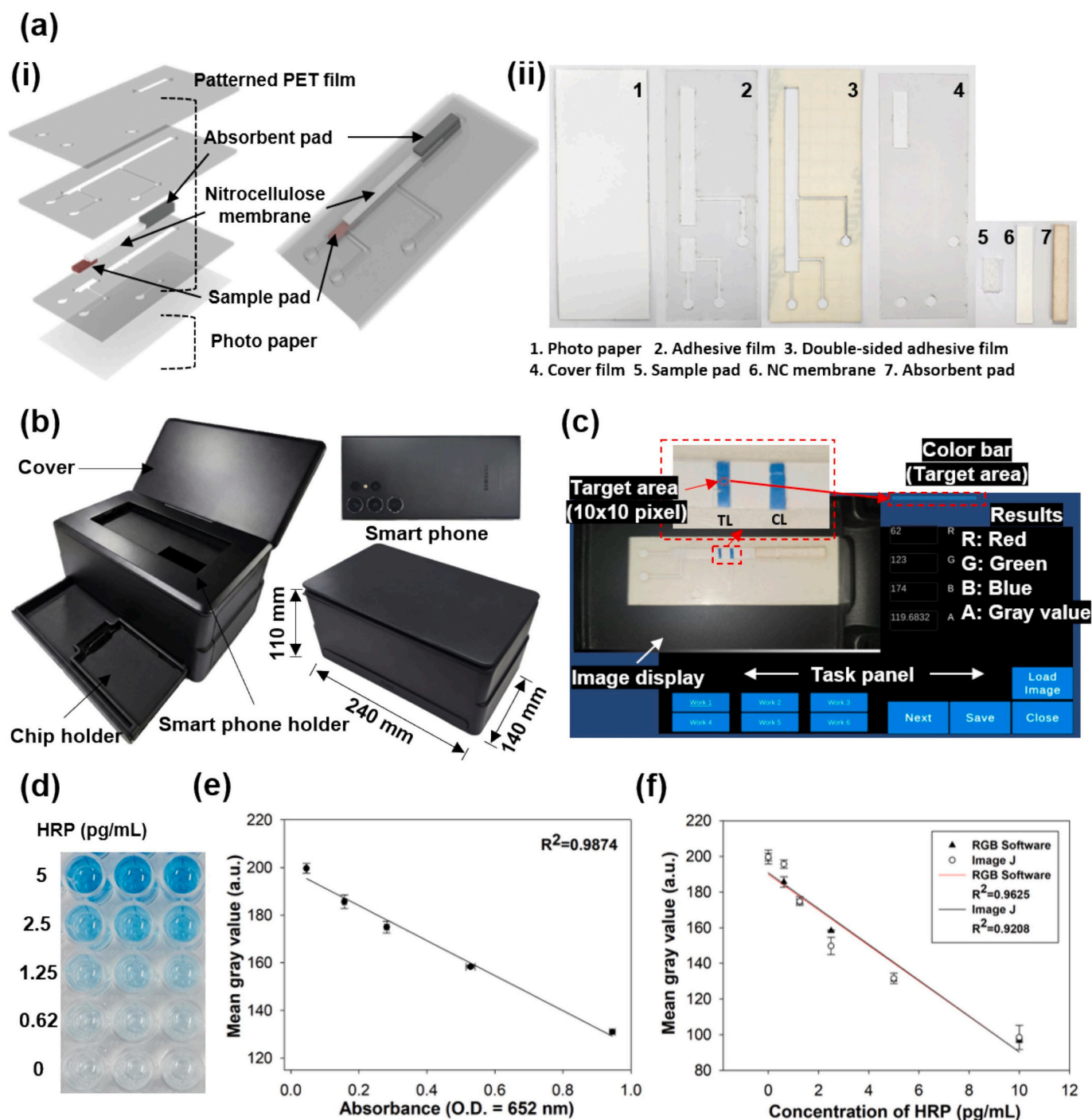


Fig. 3. Overview and analytical validation of the smartphone-based colorimetric detection platform. (a) The paper-based LFA device: (i) schematic and (ii) photograph of individual components. (b) Photograph of the hardware for signal analysis. (c) Photograph of the software for signal analysis. (d) Photograph of the HRP-TMB colorimetric reaction in a microplate with varying concentrations of HRP (0–5 pg/mL). (e) Correlation analysis between the absorbance measured by a microplate reader and the grayscale intensity extracted by the developed RGB software. (f) Comparison of the analytical performance between the developed RGB software and ImageJ. All data represent the mean $\pm$ standard deviation of three independent experiments.

surface area of the AuNPs, thereby significantly amplifying the colorimetric signal. Furthermore, to investigate how this multi-component design affects the visual readout across a range of target concentrations, the LFA strips were observed before and after the TMB reaction (Fig. S2). As illustrated in Fig. S2a, the intrinsic red signal from the AuNP cores prior to TMB addition provided a limited visual response and a narrow dynamic range. Upon TMB addition, however, the enzymatic reaction yielded a distinct blue color that successfully expanded the dynamic range across the tested concentrations (Fig. S2b). This comparison demonstrates that utilizing the AuNP core as a carrier for enzymes offers a more discernible signal and a broader working range than relying solely on the intrinsic optical signal of conventional bare AuNPs.

3.3. Detailed configuration of the detection device and reader system

Fig. 3 illustrates the features of the integrated device developed for the on-site detection of MC-LR. First, Fig. 3a presents (i) a schematic diagram and (ii) photograph of the paper-based LFA device. Because the fabricated device relied on analyzing colorimetric signals generated by an enzyme-substrate reaction, it required a dedicated inlet for the substrate solution and was therefore constructed in a microfluidic-chip format. This microfluidic chip was formed by laminating multiple layers of precisely laser-cut photo paper and PET films to create fluidic channels. Because the photo paper came into direct contact with the solution, its coating level critically influenced the fluid flow. Therefore, the optimal photo paper was selected after evaluating its fluidic behavior. Regarding the PET films, single-sided and double-sided adhesive films were strategically selected depending on the specific assembly stage. The reaction zone, where the LFA occurred, comprised a glass-fiber sample pad, nitrocellulose membrane, and cellulose absorbent pad, which were assembled with overlapping regions to ensure continuous flow. The final dimensions of the fabricated paper chip were 27 mm (W) $\times$ 74 mm (L), with a total weight of approximately 3 g. Fig. 3b shows a photograph of the hardware designed for the signal analysis system. To capture the assay results on the device, the built-in camera and flash of a smartphone were utilized. Specifically, the primary camera sensor features a 200 MP resolution, an F1.7 aperture, a 23 mm focal length, and an 85° field of view. Moreover, to eliminate interference from ambient light during image acquisition and thereby ensure high reproducibility, a dark-box-style portable reader was fabricated via 3D printing. This reader comprised a chip holder, a smartphone mount, and an external cover, with overall dimensions of 240 mm (W) $\times$ 140 mm (L) $\times$ 110 mm (H). Within this custom reader, the smartphone was securely mounted parallel to the LFA device to maintain a fixed orthogonal shooting angle (90°). The vertical distance between the camera lens and the reaction zone was fixed at 80 mm, providing a consistent image capture area of 90 $\times$ 60 mm² under uniform flash illumination. Fig. 3c shows a photograph of the software for signal analysis. This software was developed based on the RGB color model. The interface consisted of a display screen for viewing captured images, a pointer to designate a target area of 10 $\times$ 10-pixel, a color bar indicating the recognized color within the target area, a result window displaying the extracted RGB values, and a control panel for operating the save, load, and capture functions. For quantitative analysis, a user manually positions the pointer over the center of the reaction zone to designate the specific target area. Once selected, the software extracts the R, G, and B values of all pixels within this 10 $\times$ 10-pixel area and computes their arithmetic mean to export the final mean gray value. First, as shown in Fig. 3d, images were acquired after reacting various concentrations (0–5 pg/mL) of HRP with TMB for 10 min. Subsequently, as shown in Fig. 3e, the correlation between the absorbance of these colorimetric reactions measured using a microplate reader and the grayscale values analyzed by the developed software was evaluated. Within the linear dynamic range, the results exhibited a high correlation ($R^2 = 0.9874$), indicating that the developed software possessed reliable analytical performance comparable to that of a standard microplate

reader. Finally, Fig. 3f compares the performance of the developed software with ImageJ, a widely used program for RGB signal analysis. By analyzing the HRP–TMB colorimetric reactions across various concentrations, both programs yielded similar results, cross-validating the excellent performance of the developed software in RGB analysis. Furthermore, the influence of smartphone hardware variations was evaluated using simulated lines drawn with standard commercial inks to exclude intrinsic biochemical assay fluctuations. As shown in Fig. S3, an optical comparison of three smartphone models (iPhone 13, Galaxy S8, and Galaxy S23 Ultra) revealed variations in absolute mean gray values due to distinct camera specifications, such as flash intensity and image processing algorithms. Nevertheless, the relative analytical trends remained highly consistent across all devices. This confirms that while device-specific calibration is recommended for precise quantification, the fundamental diagnostic reliability of the system is independent of smartphone hardware.

3.4. Optimization of assay conditions

To ensure stable microfluidic operation and maximize the colorimetric signal-to-noise (S/N) ratio, the physical and temporal assay conditions were systematically optimized. Because fluid migration in our device is fundamentally governed by the capillary action of porous materials rather than pressure-driven closed microfluidics, the internal PET channels were simply designed to accommodate the underlying nitrocellulose membrane. Consequently, optimizing the injected sample volume, rather than modifying the structural channel dimensions, was the primary physical strategy to establish stable capillary flow without structural failure. To visually trace the fluidic dynamics within the microfluidic channels, varying volumes (20, 40, and 60 μ L) of an aqueous dye were injected (Fig. S4). At a low volume of 20 μ L, the fluid was insufficient to completely wet the nitrocellulose membrane, resulting in a halted flow that failed to reach the absorbent pad. Conversely, an excessive volume of 60 μ L caused fluidic overloading, leading to lateral leakage outside the main channel and severe backflow into the adjacent washing buffer inlet. A volume of 40 μ L provided a stable, uniform capillary flow across the entire reaction zone without any leakage. Thus, the total volume of the initial reaction mixture consisting of the sample and ELNPs was strictly fixed at 40 μ L for all subsequent assays. Following the volumetric optimization, the temporal conditions were optimized, as shown in Fig. 4. First, Fig. 4a shows a schematic of the washing process and images of the corresponding colorimetric reaction results at different washing times. In the colorimetric reaction between HRP and TMB, unreacted enzymes present on the membrane that failed to bind to the TL needed to be removed through a separate washing step to eliminate nonspecific signals. Insufficient washing durations retained some HRP, generating nonspecific signals in the surrounding area. Conversely, extended washing durations reduced nonspecific signals, but exposed ELNPs that specifically bound to the TL to possible erosion. Therefore, identifying the washing time that would yield the highest signal-to-noise (S/N) ratio was crucial. The reaction was conducted by immobilizing BSA-conjugated MC-LR on the TL and reacting it with the prepared ELNPs. Subsequently, 10 μ L of washing solution was injected in three aliquots at regular intervals for each tested washing duration (1, 3, 5, 7, and 9 min), followed by the TMB reaction. As shown in Fig. 4b, unreacted ELNPs were not sufficiently removed with only a 1 min washing duration, resulting in strong noise that impeded TL identification. When the washing time was subsequently increased, the S/N ratio reached a plateau after 5 min. This result indicates that unreacted ELNPs were sufficiently removed with washing durations of at least 5 min; therefore, 5 min was selected as the optimal washing time in this experiment. Fig. 4c also presents a schematic of the TMB reaction alongside images of the corresponding colorimetric results at various TMB reaction times. In the HRP–TMB reaction, insufficient reaction times led to insufficient color development to obtain the desired signal. Conversely, prolonged

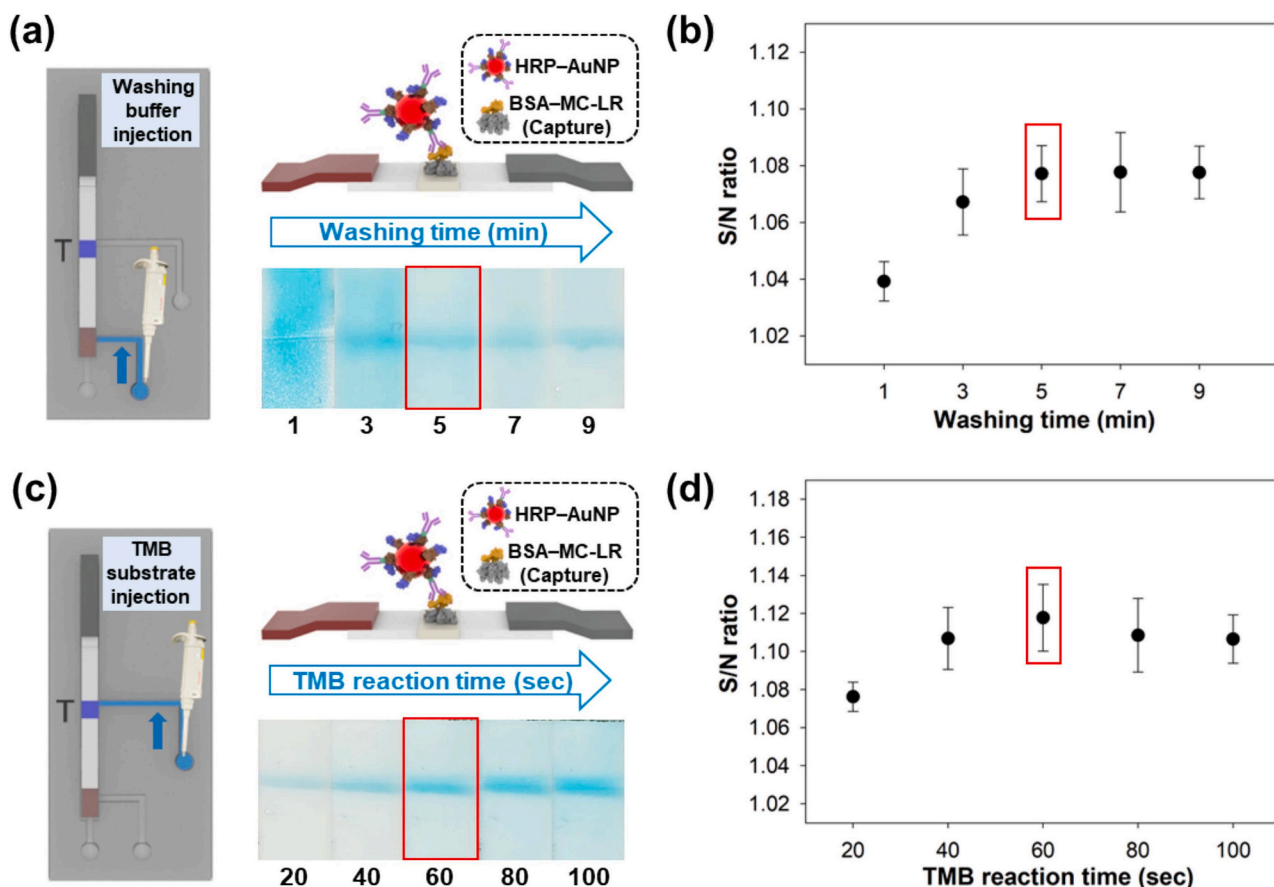


Fig. 4. Optimization of colorimetric detection conditions for the paper-based LFA device. (a) Schematic illustration of the washing step and photographs of the TL at various washing times (1, 3, 5, 7, and 9 min). (b) The corresponding signal-to-noise (S/N) ratios as a function of washing time. (c) Schematic of the TMB substrate injection and photographs of the TL at different TMB reaction times (20, 40, 60, 80, and 100 s). (d) The corresponding S/N ratios as a function of the TMB reaction time. The red boxes indicate the selected optimal conditions (5 min for washing and 60 s for the TMB reaction). All data represent the mean $\pm$ standard deviation of three independent experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

reaction times resulted in excessive pigmentation due to continuous TMB oxidation, leading to nonspecific background signals. When reaction times were extended further, the color began to blur due to diffusion within the membrane, rendering the analysis impossible. Therefore, determining the TMB reaction time that would provide the highest S/N ratio was necessary. This experiment was conducted using the same procedure as in the washing optimization; after completion of the washing process, the TMB solution was injected, and the S/N ratios were measured at 20, 40, 60, 80, and 100 s. As shown in Fig. 4d, the highest S/N ratio was achieved at 60 s. Consequently, 60 s was selected as the optimal TMB reaction time.

3.5. Analytical performance of the fabricated MC-LR detection platform

The MC-LR detection performance was evaluated using the optimized detection system and device. First, Fig. 5a illustrates the MC-LR detection mechanism. Upon injection of the MC-LR sample and ELNPs, fluid migration occurred through capillary action and lateral flow, initiating a competitive immunoassay. Following subsequent washing steps and a TMB reaction for color development, the mean gray value of the colorimetric signal was determined using the signal analyzer. Fig. 5b presents the detection images at various MC-LR concentrations (0, 50, 100, 200, and 400 pg/mL). According to the competitive immunoassay principle and enzyme-substrate reaction, an increase in the MC-LR concentration caused the blue band on the TL to fade, which consequently increased the corresponding mean gray value of the image. Based on these MC-LR detection results, a linear calibration curve was

derived, as shown in Fig. 5c. Using the logarithmic scale of the MC-LR concentration, the linear regression equation was determined to be $y = 15.9x + 126.33$ ($R^2 = 0.9698$). Based on this linearity, the limit of detection ($LOD = 3\sigma/S$) was calculated to be 85 pg/mL, and the limit of quantification ($LOQ = 10\sigma/S$) was determined to be 283 pg/mL. Consequently, the valid detection range of the proposed sensor is defined as 85 to 400 pg/mL. Notably, our calculated LOQ is well below the WHO drinking water guideline of 1 ng/mL, indicating that the proposed sensor possesses not only sufficient sensitivity but also practical quantitative reliability for assessing MC-LR below regulatory thresholds.

The dynamic detection range could also be effectively controlled by adjusting the amount of the detection probe (ELNPs), as demonstrated on a bare strip. In the competitive immunoassay, employing a highly diluted detection probe (5×10^9 particles/mL of ELNP) on the paper-based LFA device was essential for ultra-trace (pg/mL) detection. Because the absolute amount of the probe was limited, its binding sites were rapidly saturated even by trace amounts of MC-LR. This rapid saturation caused a sharp decrease in the number of probes binding to the test line, thereby maximizing sensitivity. Conversely, when the probe concentration was increased fourfold (2×10^{10} particles/mL of ELNPs) on a bare strip, the abundant enzyme-labeled antibodies required significantly more MC-LR (ng/mL) to meaningfully reduce the signal. This shift moved the dynamic range toward higher concentrations (0, 12.5, 25, 50, and 100 ng/mL), yielding distinct blue bands (Fig. 5d) and excellent linearity ($R^2 = 0.9706$) for MC-LR quantification (Fig. 5e). Supported by the robust color development in this higher

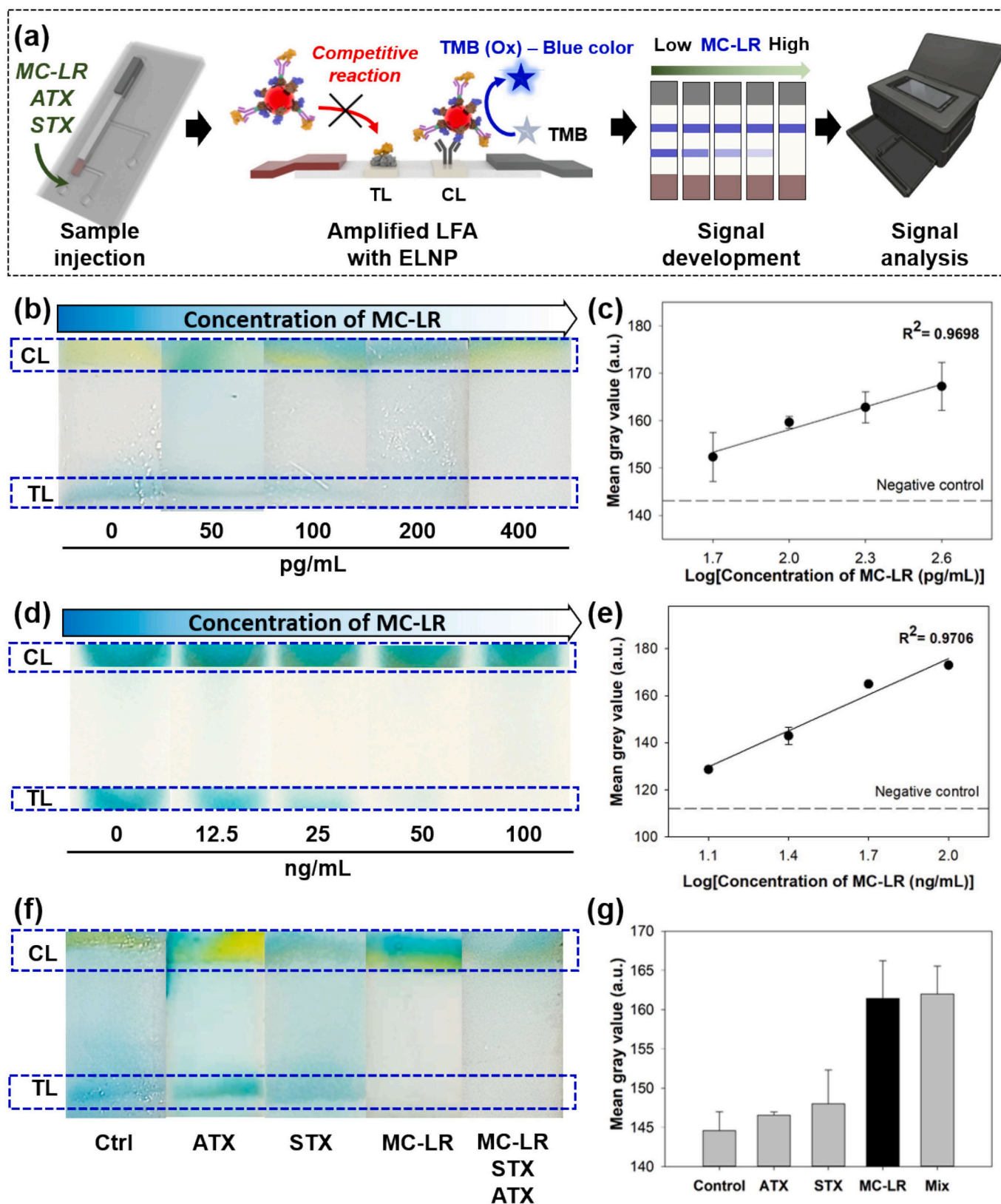


Fig. 5. Analytical performance of the fabricated detection platforms for MC-LR. (a) Schematic of the overall detection process. (b) Real images of the colorimetric response on the TL and CL at varying MC-LR concentrations (0–400 pg/mL) using the paper-based LFA device. (c) Calibration curve plotted against the logarithm of the MC-LR concentration. (d) Real images of the colorimetric response on the TL and CL at high MC-LR concentrations (0–100 ng/mL) using the bare strip. (e) Calibration curve plotted against the logarithm of the high MC-LR concentration. (f) Real images of the colorimetric response on the TL and CL and (g) quantitative intensity results for the selectivity assay against ATX, STX, and their mixture on the paper-based LFA device. All data represent the mean $\pm$ standard deviation of three independent experiments.

concentration range, the average relative standard deviation (RSD) for the calibration points was calculated to be as low as 1.05%, further validating the high intrinsic precision and stability of the sensing platform. These results clearly demonstrate that the faint signal in the low-concentration range is a functional requirement for achieving high sensitivity in this competitive format, rather than a lack of colorimetric capability in the proposed system. Following the sensitivity evaluation, a selectivity study of the fabricated device was conducted to verify its specific detection capability for MC-LR in real freshwater samples. Real freshwater samples, previously verified by LC-MS/MS analysis to be free of the harmful toxins MC-LR, STX, and ATX, were used to prepare spiked samples with a final concentration of 5 ng/mL for each toxin. As shown in Fig. 5f, the representative measurement images from the selectivity evaluation confirm that the blue band disappeared only in the presence of the MC-LR sample. These results were quantitatively evaluated by comparing the mean gray values in Fig. 5g. Consequently, the fabricated device exhibited high selectivity for MC-LR, enabling the specific detection of the target analyte without significant signal interference or matrix effects from various harmful toxins, organic matter, and other complex background components in real freshwater environments. Furthermore, to evaluate the practical robustness of the fabricated platform, intra-assay repeatability, storage stability, and batch-to-batch reproducibility were systematically assessed. Replicate analyses of the MC-LR standard (100 pg/mL) within a single batch demonstrated excellent repeatability with a relative standard deviation (RSD) of 0.96%. Storage stability was evaluated over a 10-day period under optimal conditions (ELNPs at 4 °C; LFA strips in a desiccator at room temperature). Both the probes and test strips maintained highly consistent colorimetric signals without significant background amplification, keeping RSDs well below 5% across all time points (Fig. S5). Finally, measurements using three individual test strips from each of the five independently fabricated batches yielded a low inter-batch RSD of 2.68% (Fig. S6). These results collectively confirm the inherent precision, structural consistency, and stability of the proposed sensing platform.

3.6. Validation with real freshwater samples

To verify the practical applicability and reliability of the proposed platform for environmental monitoring, four real freshwater samples (including Mansu Reservoir and Sangju Weir) were analyzed, and the results were compared with those obtained from LC-MS/MS and a commercial ELISA kit (Table 1). For this comparative analysis, the standard reference values were obtained using LC-MS/MS following the

Table 1

Detection of MC-LR in real freshwater samples. Comparative analysis of MC-LR detection results obtained from fabricated platform and standard LC-MS/MS, and a commercial ELISA kit. All data represent the mean $\pm$ standard deviation of three independent experiments.

Freshwater sample	Detection method			Recovery (%)	RSD (%)
	LC-MS/MS (ng/mL)	ELISA kit (ng/mL)	Our platform (ng/mL)		
1. Mansu Reservoir	N.D.	N.D.	N.D.		
2. Mansu Reservoir	N.D.	N.D.	N.D.		
3. Sangju Weir	0.108	0.083	0.104 $\pm$ 0.029*	96.3	27.88
4. Sangju Weir	N.D.	N.D.	N.D.		

N.D.: Not detected.

* : The detected concentration lies above the LOD but below the LOQ; therefore, absolute quantification is not possible. The value represents only a semi-quantitative estimation.

comprehensive sample pre-treatment described in Section 2.7. Conversely, to evaluate practical on-site applicability, the raw freshwater samples were directly applied to both our platform and the commercial ELISA kit without any specialized pre-treatment or laboratory-based extraction. First, for the uncontaminated samples (Samples 1, 2, and 4), the fabricated platform consistently yielded “not detected” (N.D.) results that showed complete agreement with LC-MS/MS and the ELISA kit, demonstrating reliable selectivity against blank matrices without producing false positives. Second, in terms of quantitative accuracy, the fabricated platform also exhibited excellent performance by producing results that closely matched the reference LC-MS/MS measurements. Specifically, for Sample 3, where LC-MS/MS detected an MC-LR concentration of 0.108 ng/mL, the proposed platform detected a concentration of 0.104 ± 0.029 ng/mL. A one-sample *t*-test comparing the fabricated platform's measurements (*n* = 3) to the LC-MS/MS reference value (0.108 ng/mL) yielded *t* = −0.239 and a *p*-value of 0.82 (*p* > 0.05), indicating no statistically significant difference between the two methods. For Sample 3, the calculated recovery rate was 96.3%, with an RSD of 27.88%. The measurement from the commercial ELISA kit for this sample was 0.083 ng/mL, closely comparable to the values obtained using both LC-MS/MS and the proposed platform. This relatively high RSD is primarily attributed to the ultra-trace concentration of the target analyte; the detected MC-LR level (~0.1 ng/mL) lies above the calculated limit of detection (LOD = 85 pg/mL) but below the strict limit of quantification (LOQ = 283 pg/mL). In this concentration regime, the analyte is reliably detectable, but precise quantification is inherently challenging. Furthermore, this variance is exacerbated by matrix effects, such as natural organic matter and suspended microparticles, which are inevitably encountered when analyzing completely raw freshwater without laboratory-based pre-treatment. To further investigate these matrix effects, the signal from 100 pg/mL of MC-LR spiked into a raw freshwater matrix (previously confirmed to be MC-LR-free) was compared with the corresponding 100 pg/mL point of the buffer-based calibration curve as a matrix-free reference. As shown in Fig. S7, while the freshwater sample exhibited a slightly larger variance and a marginally reduced mean signal compared to the standard buffer, this difference did not disrupt the competitive assay mechanism. This confirms that, at this trace-level concentration, the optimized microfluidic integration and signal amplification system remain sufficiently stable to tolerate matrix-induced variance while reliably distinguishing the target analyte from the background. Given that these detected concentrations (~0.1 ng/mL) are well below the WHO guideline limit of 1 ng/mL, this degree of RSD remains acceptable for a rapid on-site analysis. Therefore, the proposed platform is strategically positioned as a first-line, semi-quantitative screening tool that prioritizes logistical simplicity and complements, rather than replaces, standard LC-MS/MS. Collectively, these validation results confirm that the developed platform serves as a practical and robust alternative for the rapid on-site screening of real freshwater samples, effectively overcoming the logistical restrictions of conventional laboratory-based analytical methods.

3.7. Comparative analysis of sensing performance

As summarized in Table 2, the analytical performance of the developed platform was comprehensively compared with recently reported MC-LR detection methods in terms of the analytical method, readout system, detection time, and LOD. The proposed ELNP-based LFA platform achieved a highly competitive LOD of 85 pg/mL (0.085 ng/mL) within 15 min by utilizing a fully portable, smartphone-based readout system. A particularly notable achievement is the substantial improvement in analytical sensitivity. While previous colorimetric assays requiring expensive microplate readers or spectrophotometers generally exhibited LODs in the range of 0.38 to 1 ng/mL, the present platform achieved up to a 12-fold enhancement in sensitivity by introducing the HRP-mediated enzymatic signal amplification mechanism.

Table 2

Comparison of MC-LR detection using optical detection methods.

Material	Method	Readout system	Dynamic range (ng/mL)	LOD (ng/mL)	Detection time (min)	Reference
ZnO@MIP	Fluorescence	Portable fluorescence detector	0.2–20	1	10	[35]
QDs/microfluidic chip	Fluorescence	Fluorescence microscope	0.1–1000	0.097	45	[36]
Bioluminescent bacteria	Bioluminescence	Smartphone-based platform	1.5–40	0.23	15	[37]
MOF-818 nanozyme	Colorimetry	Microplate reader	1–200	0.849	312	[38]
PDA-DNA	Colorimetry	Microplate reader	5–100	1	60	[39]
Au nanobipyramids	Colorimetry	Microplate reader	0.5–20	0.82	150	[40]
AuNP-aptamer	Colorimetry	UV-vis spectrophotometer	10–150	0.38	–	[41]
Aptamer/AuNPs LFA	Colorimetry	Portable strip reader	1–50	0.84	5	[42]
ELNPs/paper LFA-based	Colorimetry	Smartphone-based platform	0.085–0.4	0.085	15	This work

Furthermore, even when compared to conventional AuNP-based LFAs that rely solely on the intrinsic plasmonic color of AuNPs, the catalytic reaction of the ELNPs significantly amplified the detection performance by approximately 10-fold. In addition, the proposed platform demonstrated distinct advantages regarding detection time and practicality. Unlike other optical analytical methods that require tens of minutes to several hours to achieve high sensitivity, the present system enabled rapid quantification within 15 min, thereby achieving an excellent balance between rapid analysis and high sensitivity. Finally, the platform overcame the limitations of existing methods that require specialized laboratory environments and bulky, expensive instrumentation, such as fluorescence microscopes and microplate readers. By adopting a widely accessible smartphone as the readout system, this sensor represents a highly practical and integrated platform optimized for the immediate on-site monitoring of MC-LR in aqueous environments.

4. Conclusions

In this study, a highly sensitive, semiautomated paper-based LFA platform was successfully developed for the rapid on-site screening of MC-LR. The incorporation of ELNPs as signal-amplifying probes significantly enhanced the analytical sensitivity, enabling reliable trace-level detection well below the WHO regulatory guideline. Furthermore, by coupling the 3D microfluidic paper chip with an inverse RGB-based portable signal analyzer, a user-friendly detection system was established that facilitated intuitive, semiquantitative readouts of the on-chip colorimetric reaction. Consequently, this system achieved a low LOD of 85 pg/mL within 15 min. Despite the inherent matrix effects encountered when analyzing completely raw freshwater samples, the platform demonstrated excellent practical applicability and specificity, successfully identifying ultra-trace MC-LR without requiring complex laboratory pretreatment procedures. Although the current antibody exhibits broad cross-reactivity among different microcystin variants, future integration of more specific bioreceptors could further mitigate this limitation and enable precise, variant-specific quantification. By trading a degree of absolute quantitative precision for substantial logistical simplicity, the proposed platform effectively balanced analytical rigor with field deployability, ultimately providing a cost-effective and robust solution for first-line water-quality screening.

CRedit authorship contribution statement

Jeong-Hyeop Shin: Writing – review & editing, Writing – original draft, Visualization, Validation, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Myeong-Jun Lee:** Writing – review & editing, Writing – original draft, Visualization, Validation, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Seung-Hun Jung:** Methodology, Investigation. **Tae-Hwan Kim:** Writing – review & editing, Investigation. **Hyuncheol Kim:** Writing – review & editing, Investigation. **Byung-Keun Oh:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Funding acquisition,

Conceptualization.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.microc.2026.119081>.

Data availability

Data will be made available on request.

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