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Development and Optimization of a Quantum Dot-Based Lateral Flow Assay for Hepatitis B Surface Antigen Detection

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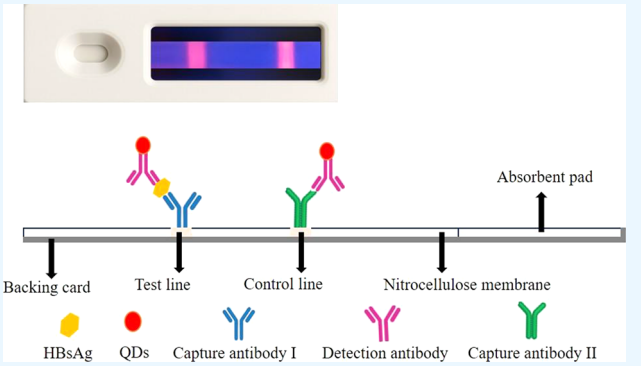
ABSTRACT: Early detection of hepatitis B virus (HBV) is essential for effective disease management, particularly in cases involving low viral loads where timely intervention can prevent chronic progression and liver-related complications. Although enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR) are considered the gold standards for HBV detection, their reliance on specialized infrastructure limits their accessibility in resource-limited settings. This study reports the development and optimization of a cadmium quantum dots-based lateral flow assay (cadmiumQDs-LFA) for the detection of hepatitis B surface antigen (HBsAg), harnessing the superior fluorescence properties of cadmiumQDs to improve analytical performance. Compared to conventional gold nanoparticles (AuNPs)-based LFA, cadmiumQDs exhibit higher fluorescence intensity and stability, enabling improved sensitivity for detecting low-abundance biomarkers. Commercially sourced cadmiumQDs were functionalized with mercaptopropionic acid (MPA) to introduce carboxyl (−COOH) functional groups for bioconjugation. The optimized cadmiumQDs conjugates were integrated into an LFA, achieving a limit of detection (LOD) of 15.6 ng/mL for HBsAg. Performance evaluation using 244 clinical serum samples yielded a sensitivity of 86.5%, specificity of 95.9%, positive predictive value (PPV) of 98% and negative predictive value (NPV) of 75.3%. Comparative analysis with a commercial LFA demonstrated enhanced detection of low HBsAg levels, addressing a key limitation of conventional methods.



1. INTRODUCTION

Hepatitis B virus (HBV) infection remains a major global public health concern, contributing significantly to morbidity and mortality. Despite the availability of effective vaccines and antiviral therapies, HBV continues to affect an estimated 253 million individuals worldwide, with a global prevalence of 3.2% as of 2023.[1] In Malaysia alone, approximately one million individuals are chronically infected with HBV. Early and accurate detection is crucial for timely clinical intervention, reducing disease transmission, and preventing severe complications such as liver cirrhosis and hepatocellular carcinoma.[2,3] Diagnosis of HBV typically involves the detection of viral biomarkers such as hepatitis B surface antigen (HBsAg) and HBV DNA in blood or serum samples.[4]

Current detection strategies encompass both serological and molecular assays, each with distinct advantages and limitations. Serological assays, such as enzyme-linked immunosorbent assay (ELISA),[5] chemiluminescent microparticle immunoassay (CMIA),[6] and electrochemiluminescence immunoassay (ECLIA)[7,8] are widely employed in clinical diagnostics due to their cost-effectiveness, operational simplicity, and high-throughput capabilities. ELISA remains the gold standard for HBV screening, offering high sensitivity and specificity. However, its performance may be compromised in cases of early infection or low viral loads, where HBsAg remains undetectable.

In contrast, molecular assays such as polymerase chain reaction (PCR)-based methods enable direct detection of HBV DNA with superior analytical sensitivity. Quantitative real-time PCR (qPCR) is the reference standard for HBV DNA quantification and plays a pivotal role in disease monitoring and treatment evaluation. Despite these advantages, qPCR requires sophisticated laboratory infrastructure, highly trained personnel, and expensive reagents, which limit its accessibility in resource-constrained settings. Commercial platforms, such as the COBAS Ampliprep/COBAS TaqMan system (Roche Diagnostics) and the Xpert HBV Viral Load Test (Cepheid), further underscore the cost and logistical barriers associated with molecular diagnostics. While qPCR remains indispensable for viral load monitoring, serological assays continue to serve

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as the primary method for HBV screening and initial diagnosis in most clinical settings.

Despite technological advancements, HBV diagnostics continue to face challenges related to accessibility, affordability, and sensitivity. Serological assays, though widely available, may produce false-negative results in early infections or low antigenemia cases, delaying diagnosis and treatment. Conversely, molecular assays, though highly sensitive, are often impractical for large-scale screening due to their high cost and operational complexity. Therefore, there is an urgent need for affordable, rapid, and sensitive point-of-care testing (POCT) solutions, particularly for decentralised and resource-limited settings. The development of POCT devices that align with the ASSURED (Affordable, Sensitive, Specific, User-friendly, Rapid, Equipment-free, and Deliverable) criteria defined by the World Health Organization (WHO) would significantly enhance HBV screening capabilities in such settings.

Nanotechnology has emerged as a transformative approach in biosensing, with quantum dots (QDs) offering significant advantages over conventional fluorophores due to their exceptional brightness, photostability, and tunable emission spectra. These properties make QDs highly attractive for diagnostic applications, particularly for the detection of low-abundance biomarkers.[9,10] Lateral flow assays (LFA) are widely adopted POCT platforms, owing to portability, speed, and minimal training requirement.[11] However, traditional LFA that utilized gold nanoparticles (AuNPs) as labels suffer from limited sensitivity, particularly when detecting low concentrations of HBsAg in early stage infections.[12]

The incorporation of fluorescence-based QDs in LFA (QDs-LFA) has been shown to enhance sensitivity and enable semiquantitative detection capabilities, outperforming conventional AuNP-based LFA.[13,14] QDs provide superior brightness, long-term stability, and multiplexing potential, making them a promising alternative for improving HBV diagnostics.[15] Nevertheless, challenges persist, including suboptimal antibody conjugation efficiency, QDs instability, and aggregation-induced signal loss, all of which can impact assay reproducibility and clinical reliability.[16−18] Moreover, the performance of QDs-LFA is influenced by assay parameters such as antibody concentration, incubation time, and nitrocellulose membrane type, which require careful optimization.

Considering these challenges, this study aims to develop and optimize a QDs-based lateral flow assay (QDs-LFA) for HBV detection, with an emphasis on enhancing analytical sensitivity while maintaining the simplicity and speed of conventional LFA platforms. By addressing the limitations of current diagnostic methods, this work provides a promising fluorescence-based platform that can be further advanced toward point-of-care testing through integration with portable readers and larger-scale validation.

2. MATERIALS AND METHODS

2.1. Materials

The materials used in the experiment were cadmiumQDs, bovine serum albumin (BSA), phosphate buffer saline (PBS), 3-mercaptopropionic acid (MPA), polyethylene glycol 2000 (PEG), casein, Triton X-100, Tween-20, *N*-(3-(dimethylamino)propyl)-*N*′-ethylcarbodiimide hydrochloride (EDC), and *N*-hydrosuccinimide (NHS), all of which were obtained from Sigma-Aldrich, USA. The skimmed milk was purchased from Sunlac, New Zealand, and 95% ethanol from Thermo Fisher, USA. The HBsAg mouse monoclonal antibody (test line capture antibody) was sourced from FineTest, China, while the Magic anti-HBV surface antigen monoclonal antibody (detection antibody) and recombinant HBV surface antigen were acquired from Creative Diagnostics, USA. The goat antimouse IgG antibody (control line capture antibody) was obtained from Elabscience, USA. Nitrocellulose membranes for LFA development were purchased from Cybeles, Thailand. All chemicals used were of analytical grade, and buffers were prepared in deionized water obtained from a PURELAB OptionQ-7BP MK 1 purification system (ELGA, UK).

2.2. Methodology

2.2.1. Ethical Approval and Study Samples. Ethical approval for this study was granted by the Human Research Ethics Committee, Universiti Sains Malaysia (JEPeM-USM), under protocol code USM/JEPeM/21120844. Discarded serum samples with a volume exceeding 500 μL were collected from Hospital Pakar Universiti Sains Malaysia for disease evaluation. The presence of HBsAg was initially screened using the Elecsys HBsAg II (Roche Diagnostics) ECLIA.

For specificity testing, serum samples were obtained from healthy individuals and patients positive for dengue (DENV), hepatitis C virus (HCV), syphilis (SYP), or human immunodeficiency virus (HIV). Sample size determination was based on the Hajian-Tilaki formula,[19] assuming a 95% confidence interval and a 4% prevalence rate.[20] The calculated sample size was 380, increased to 418 to account for a 10% dropout rate. After screening, 244 samples met inclusion criteria, comprising 171 positive-HBsAg samples and 73 negative-HBsAg samples, the latter comprising healthy individuals and those positive for other infectious diseases (Table 1).

Table 1. Details of the Serum Sample Used for the Diagnostic Evaluation

serum sample type		quantity (n)
Positive-HBsAg		171
Negative-HBsAg	Uninfected individual	20
	Positive for HCV	20
	Positive for SYP	3
	Positive for HIV	20
	Positive for DENV	10
Total		244

2.2.2. Functionalization of Quantum Dots. Water-soluble cadmiumQDs were prepared by modifying their hydrophobic surface to a hydrophilic one using thiol-terminated ligand functionalization. Initially, cadmiumQDs was dissolved in toluene at a concentration of 5.0 mg/mL. To induce precipitation, ethanol was added in a 1:2 ratio, and the mixture was centrifuged at 3000 rpm for 15 min. The precipitate was washed three times with ethanol to remove excess solvent and impurities before being redissolved in 1.0 mL of toluene. To initiate functionalization, 0.5 mL of MPA was added to the solution, and the mixture was sonicated for 30 min. The reaction was maintained at ambient temperature for 24 h, after which the MPA-modified cadmiumQDs were then centrifuged at 5000 rpm for 5 min. The resulting precipitate was dried in a vacuum desiccator for 1 h and resuspended in 10 mM PBS (Figure 1a). Additional

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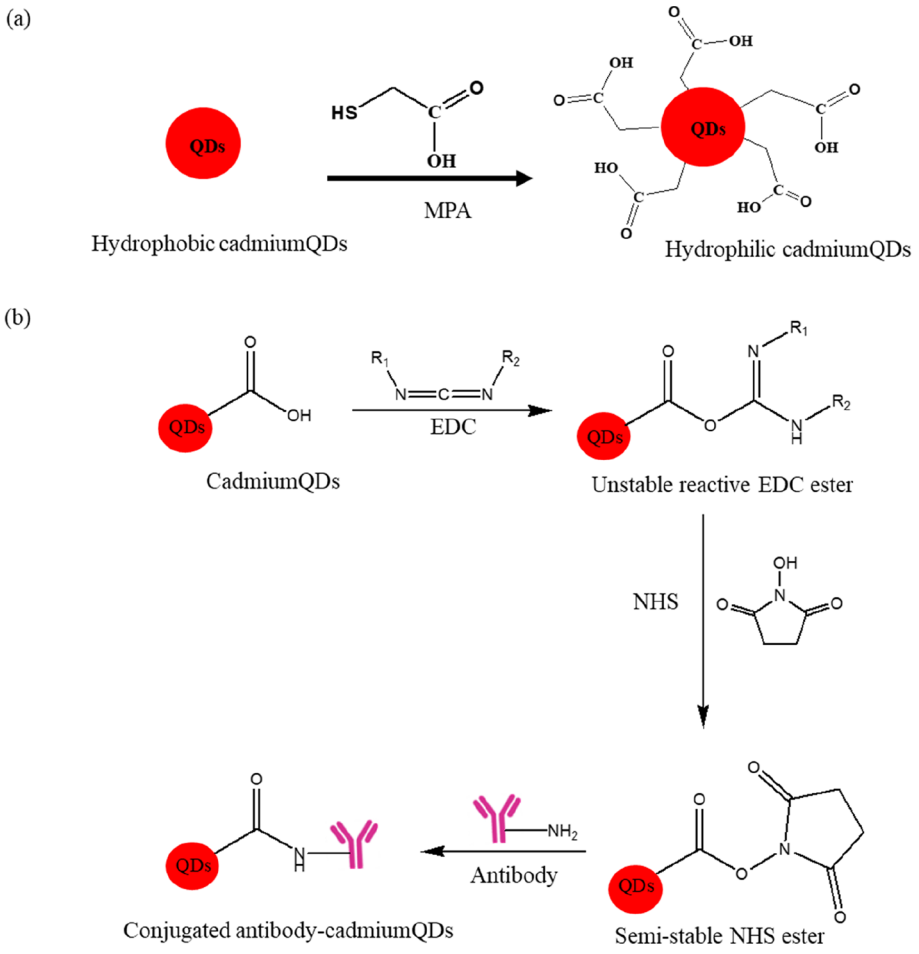

Figure 1. Schematic mechanism on functionalization and bioconjugation of cadmiumQDs with an antibody. (a) cadmiumQDs were modified with MPA to introduce carboxyl (−COOH) groups, enhancing the solubility in aqueous solution and providing reactive sites for bioconjugation. (b) Carboxyl-modified cadmiumQDs were covalently linked to antibodies using carbodiimide chemistry with EDC and NHS, forming stable amide bonds between cadmiumQDs and antibody molecules.

centrifugation and washing steps were carried out to remove unbound ligands and confirm successful phase transfer into water.

2.2.3. Bioconjugation of CadmiumQDs with Antibodies. Antibody conjugation to cadmiumQDs was carried out using EDC/NHS chemistry to form covalent bonds between carboxyl functionalized QDs and the amine groups of antibodies. Prior to conjugation, the cadmiumQDs were sonicated for 15 min to reduce aggregation. Surface activation was initiated by adding 100 μL of 100 mM EDC to 0.5 mL of 1% Tween-20, followed by incubation for 5 min. Subsequently, 100 μL of 100 mM NHS was added and stirred for 20 min. The detection antibody (1 μL) was then added to the activated cadmiumQDs and incubated at 4 °C for 4 h to facilitate covalent attachment (Figure 1b). The conjugates were subsequently centrifuged at 8000*g* for 10 min, and the pellet was resuspended in 400 μL of Tween-20. The conjugates were then blocked with 1% BSA to prevent nonspecific interactions and stored at 4 °C. The conjugation efficiency was assessed using UV−vis spectroscopy, fluorescence emission analysis (TECAN Infinite M-200), high-resolution transmission electron microscopy (HR-TEM) (JEM-2100F, USA), zeta potential analysis (Malvern, UK), and agarose gel electrophoresis (1.0% gel in 0.5X TBE buffer, 70 V, 60 min).

2.2.4. LFA Construction. Following the successful synthesis and characterization of the antibody-cadmiumQDs conjugates, the LFA strips were assembled. The test line, positioned 5 mm from the upstream end of the membrane, was coated with HBsAg mouse monoclonal antibody, which specifically captured HBsAg from the serum sample. The control line, located at 10 mm from the upstream end, was coated with goat antimouse IgG antibody to capture unbound antibody-cadmiumQDs conjugates, ensuring assay validity (Figure 2a). Upon application, the serum migrated through the membrane, allowing HBsAg to bind with the detection antibody-cadmiumQDs complex at the conjugation pad. The complex was subsequently captured at the test line, generating a fluorescent signal, while excess conjugates were retained at the control line (Figure 2b).

2.2.5. Optimisation of LFA. Several experimental parameters were systematically optimized to enhance the sensitivity, specificity, and overall performance of the developed lateral flow assay (LFA). Two nitrocellulose membranes, CN95 and CN140, were selected based on their distinct capillary wicking rates, which directly influence fluid migration kinetics and antigen−antibody interaction efficiency. According to manufacturer specifications, the capillary flow rate of CN140 is 118.2 s/4 cm, while CN95 exhibits a faster flow rate of 84.8 s/4 cm. Both membranes were evaluated for

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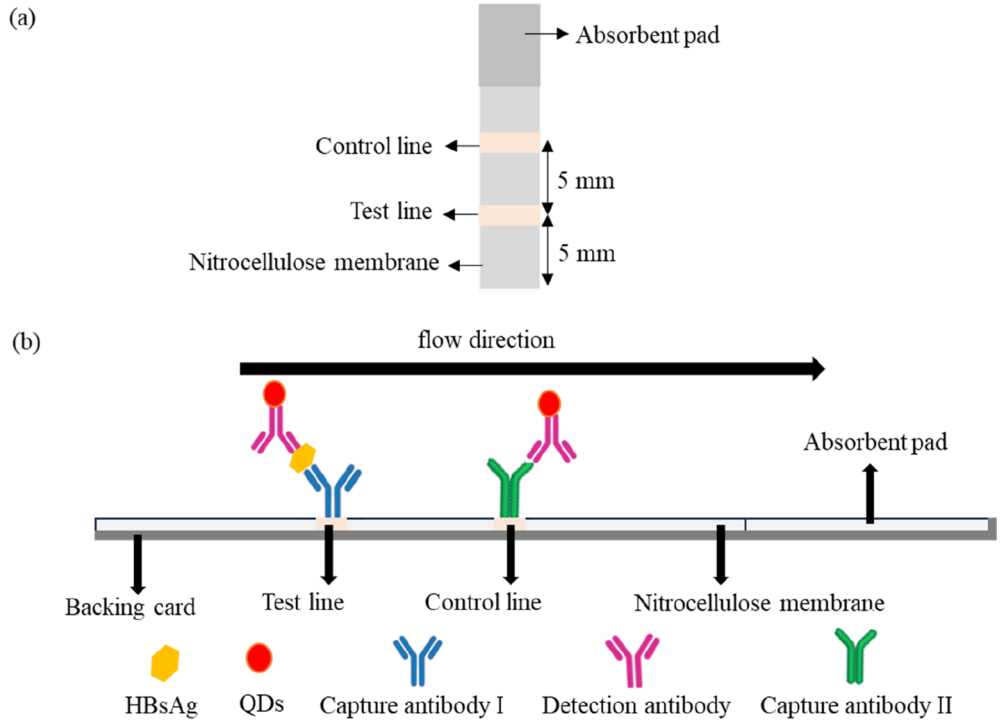

Figure 2. Schematic dual-view representation of the developed LFA. (a) The top-down view shows the structural assembly, consisting of an absorbent pad and a membrane pad, overlapping onto the backing pad. The test line and control line are placed 5 mm apart on the membrane where HBsAg mouse antibody and goat antimouse IgG antibody are dispensed, respectively. (b) Side view reveals the flow of serum through the membrane. In the presence of HBsAg, the capture antibody I at the test line will capture the HBsAg and form a complex with antibody-cadmiumQDs, producing a fluorescence signal. Meanwhile, the capture antibody II at the control line captures excess conjugated antibody-cadmiumQDs, ensuring proper fluid flow and reaction completion.

their impact on fluorescence signal intensity and flow consistency.

The concentration of the capture antibody immobilized at the test line was optimized across a range of 5.0 to 0.156 mg/mL, while the control line was prepared with goat antimouse antibodies at 1.0, 0.5, and 0.25 mg/mL. The concentrations of the antibody-cadmiumQDs conjugates were also optimized to ensure strong fluorescence signals while avoiding nanoparticle aggregation. To facilitate efficient bioconjugate migration, the running buffer composition was evaluated using varying concentrations of Triton X-100 and Tween-20 (0.1%, 0.5%, 1.0% v/v in PBS). In parallel, a panel of blocking buffers, including casein, PEG, skimmed milk, and BSA, was screened at 1% (w/v) to minimize nonspecific binding. The optimal incubation time for signal development was determined by incubating the developed strips at 5, 10, 15, and 20 min.

2.2.6. Analytical and Diagnostic Evaluation. The analytical performance of the developed LFA was assessed for repeatability, sensitivity, and specificity. Repeatability was assessed by testing the same set of strips once per day over three consecutive days. Sensitivity was determined using serial dilutions of recombinant HBsAg (1000 ng/mL to 15.6 ng/mL). The LOD was defined as the lowest antigen concentration at which a visible test line could be consistently observed across replicates. Specificity was evaluated by testing the LFA against serum samples positive for HCV, SYP, HIV, or DENV, as well as serum from healthy individuals. The performance of the developed LFA was compared against the commercial LFA (Mediven, Malaysia). Diagnostic sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were calculated using standard formulas. A test was considered a positive control (C) and test (T) lines appeared, and negative if only the C line appeared.

$$\text{Diagnostic sensitivity} = \frac{\text{number of true positives}}{\text{number of true positives} + \text{number of false negatives}} \times 100$$

$$\text{diagnostic specificity} = \frac{\text{number of true negatives}}{\text{number of true negatives} + \text{number of false positives}} \times 100$$

$$\text{PPV} = \frac{\text{number of true positives}}{\text{number of true positives} + \text{number of false positives}} \times 100$$

3. RESULTS AND DISCUSSION

3.1. Characterization of CadmiumQDs and the Bioconjugates

The synthesized cadmiumQDs were characterized to confirm their physicochemical properties, including fluorescence intensity, colloidal stability, and morphology. Functionalization with 3-mercaptopropionic acid (MPA) introduced carboxyl (−COOH) groups onto the cadmiumQDs surface, enabling covalent antibody conjugation via EDC/NHS cross-linking chemistry.[21,22] This covalent linkage ensured stable antibody immobilization, enhancing the reliability of cadmiumQDs as fluorescent labels in LFA applications.[23] Additionally, the presence of −COOH groups reduced steric hindrance and improved biomolecular compatibility, thereby increasing conjugation efficiency.[24]

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The MPA-functionalized cadmiumQDs exhibited distinctive optical characteristics that confirmed successful modification. Under daylight, the cadmiumQDs solution appeared a pale orange-red solution, while under UV illumination (365 nm), it emitted bright red fluorescence. Fluorescence spectroscopy revealed a strong emission peak at 610 nm, confirming the photoluminescence of the cadmiumQDs (Figure 3). These features support the potential of cadmiumQDs as robust signal reporters for qualitative and sensitive detection in LFA applications.

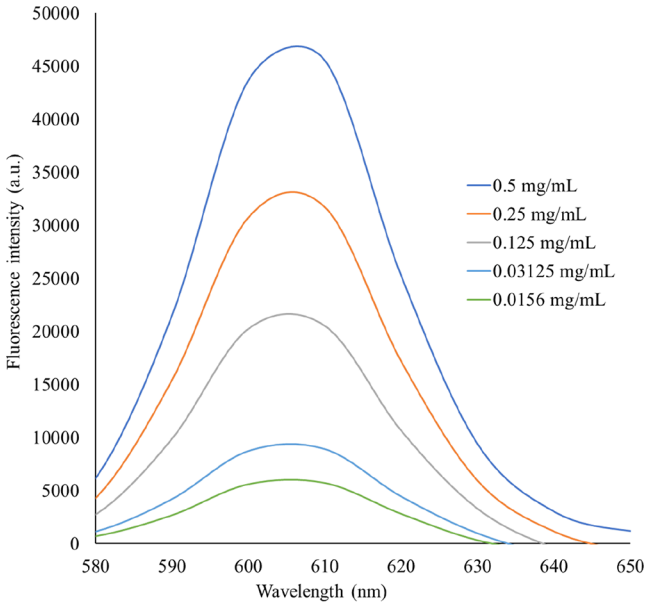

Figure 3. Fluorescence intensity spectra of cadmiumQDs at varying concentrations under excitation at 365 nm. The fluorescence intensity increased consistently with concentrations, indicating a concentration-dependent fluorescence response.

High-resolution transmission electron microscopy (HR-TEM) analysis revealed that the cadmiumQDs exhibited a uniform spherical morphology with an average particle diameter of 3.8 nm, confirming their nanoscale dispersion (Figure 4a). The particles showed good colloidal stability with minimal aggregation, as an essential characteristic for maintaining their optical and functional performance in diagnostic applications. The successful conjugation of cadmiumQDs with antibodies was further verified via agarose gel electrophoresis. The size, surface charge, and molecular weight of the nanoparticles influenced their electrophoretic mobility.[25] Unconjugated cadmiumQDs, due to their small and uniformly negatively charged, migrated readily through the gel under an electric field. Their bright fluorescence under UV illumination resulted in distinct bands, indicating efficient movement through the gel matrix.

In contrast, the antibody-cadmiumQDs conjugates displayed significantly reduced mobility or remained near the sample wells, reflecting their increased hydrodynamic size following antibody attachment (Figure 4b). This shift supports successful conjugation, attributed to the MPA-mediated surface modification that introduced abundant −COOH groups on their surface. These reactive groups facilitated stable covalent bonding with the $-NH_2$ groups of antibodies via EDC/NHS chemistry, ensuring efficient and specific conjugation.[26]

Zeta potential analysis was conducted to assess changes in surface charge during conjugation. The unconjugated cadmiumQDs exhibited a zeta potential of −6.84 mV, while the unconjugated antibody (Ab) exhibited a near-neutral charge of −4.05 mV. Upon conjugation, the Ab-cadmiumQDs displayed a slightly less negative surface charge of −5.05 mV (Figure S1). The initial negative charge of cadmiumQDs is attributed to the oxygen-containing functional groups introduced during MPA passivation, which contribute to colloidal stability. The observed shift toward a less negative value upon conjugation indicates the surface contribution of the near-neutral antibody, thereby confirming successful conjugation.[27,28]

3.2. Optimisation of Fluorescence Intensity for LFA Application

To enhance fluorescence signal amplification in the LFA, the effects of varying antibody and cadmiumQDs concentrations were systematically evaluated. CadmiumQDs served as fluorescent labels, while antibodies provided specific binding to the target HBsAg.

The impact of antibody concentration was assessed by conjugating cadmiumQDs (2.5, 5.0, and 10.0 μg/mL) with antibodies at different concentrations (1.0, 2.5, and 5.0 μg/mL). An increase in antibody concentration resulted in stronger fluorescence signals, likely due to a greater number of available antigen-binding sites. Similarly, increasing the cadmiumQDs concentration further enhanced fluorescence emission, indicating improved signal intensity.

The observed fluorescence intensities were visually confirmed (Figure S2a,b), showing a positive correlation between cadmiumQDs concentration and fluorescence signal strength. However, at excessively high cadmiumQDs concentrations, aggregation occurred upon reconstitution in surfactant (Figure S2c). This aggregation led to precipitation, which diminished fluorescence intensity and disrupted antibody conjugation. Consequently, ratios that induced aggregation were considered invalid, as their fluorescence signals were attributed to nonspecific aggregation rather than effective bioconjugation. These findings underscore the need for precise control of cadmiumQDs concentration to prevent aggregation-induced fluorescence losses.

Through systematic optimization, the optimal antibody-to-cadmiumQDs ratio was determined as 5.0:10.0, which produced the highest fluorescence intensity (5730.15 ± 507.25 au) (Table 2). This ratio ensured efficient antigen−antibody interactions while minimizing interference from unbound or aggregated components.

3.3. Optimisation of LFA

The LFA was systematically optimized to improve analytical sensitivity and minimize background noise. The selection of nitrocellulose membranes played a pivotal role in assay performance. Among the membranes evaluated, CN95 exhibited superior capillary wicking properties and more uniform reagent distribution compared to CN140, thereby making it the preferred substrate for LFA assembly (Figure S3a).[29] These characteristics enhanced flow consistency and contributed to more explicit signal definition.

Optimisation of the capture antibody concentration at the test line revealed that 0.5 mg/mL produced the highest fluorescence intensity with consistent signal reproducibility (Figure S3b). Lower concentrations resulted in weaker signals due to insufficient antigen binding, while higher concentrations

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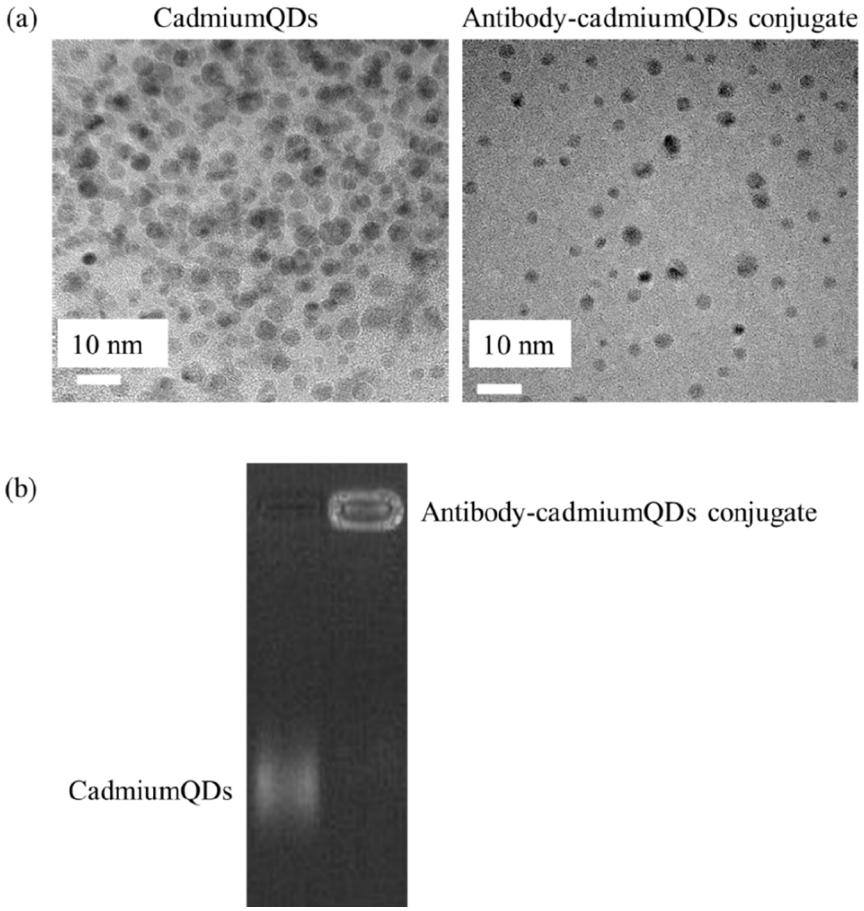

Figure 4. Characterization of antibody-cadmiumQDs conjugates. (a) Morphological images of cadmiumQDs (left) and antibody-cadmiumQDs conjugate (right) and (b) gel electrophoresis under UV light for cadmiumQDs and bioconjugate. The characterisations confirm the successful antibody attachment to cadmiumQDs.

Table 2. Fluorescence Intensity at 610 nm for Antibody-cadmiumQDs Conjugates Prepared at Various Concentration Ratios[a]

ratios of Ab/cadmiumQDs (ug/mL)	fluorescence intensity (a.u)
1.0:2.5	468.22 ± 75.95
2.5:2.5	741.20 ± 93.92
5.0:2.5	1215.92 ± 207.87
1.0:5.0	1215.92 ± 207.87
2.5:5.0	1458.57 ± 384.47
5.0:5.0	1941.15 ± 320.51
1.0:10.0	3144.61 ± 188.97
2.5:10.0	4209.73 ± 398.52
5.0:10.0	5730.15 ± 507.25

[a]Values represent the mean ± standard deviation from triplicate measurements.

did not enhance performance and increased the risk of background noise. For the control line, a concentration of 0.625 mg/mL was optimal, ensuring a robust and reliable internal control signal without oversaturation (Figure S3c).

The optimal conjugation ratio of antibody to cadmiumQDs was determined to be 5.0:10, which provided the best balance between fluorescence signal amplification and colloidal stability (Figure S3d). Excess cadmiumQDs increased the risk of nanoparticle aggregation, leading to reduced assay performance, while insufficient cadmiumQD concentrations limited signal intensity.[30]

The composition of the blocking buffer was also a critical determinant in minimizing nonspecific binding. Among the various blocking agents tested, 1% bovine serum albumin (BSA) resulted in the highest signal-to-noise ratio by effectively masking unreacted binding sites and minimizing background interference. For the running buffer, 0.1% Triton X-100 in 10 mM phosphate-buffered saline (PBS) was identified as optimal, as it promotes efficient bioconjugate migration and reduces surface tension, thereby enhancing flow and signal consistency across the membrane (Figure S3e).

The effect of incubation time on signal formation was evaluated at 5, 10, 15, and 20 min. An incubation time of 15 min was identified as optimal, yielding a strong and sharply defined fluorescence signal at the test line with minimal background fluorescence. Shorter incubation times resulted in incomplete antigen−antibody interactions, leading to a weak signal development. In contrast, longer durations beyond 15 min led to signal diffusion, potentially compromising the clarity and interpretability of the results (Figure S3f).

3.4. Analytical Performance

The analytical performance of the developed LFA was evaluated in terms of its repeatability, sensitivity, and specificity. Repeatability was assessed across three independent testing days, yielding consistent results, as shown in Figure S4. The fluorescence intensity remained stable across all tested concentrations, with clearly defined test lines observed on each day, indicating high assay reproducibility.

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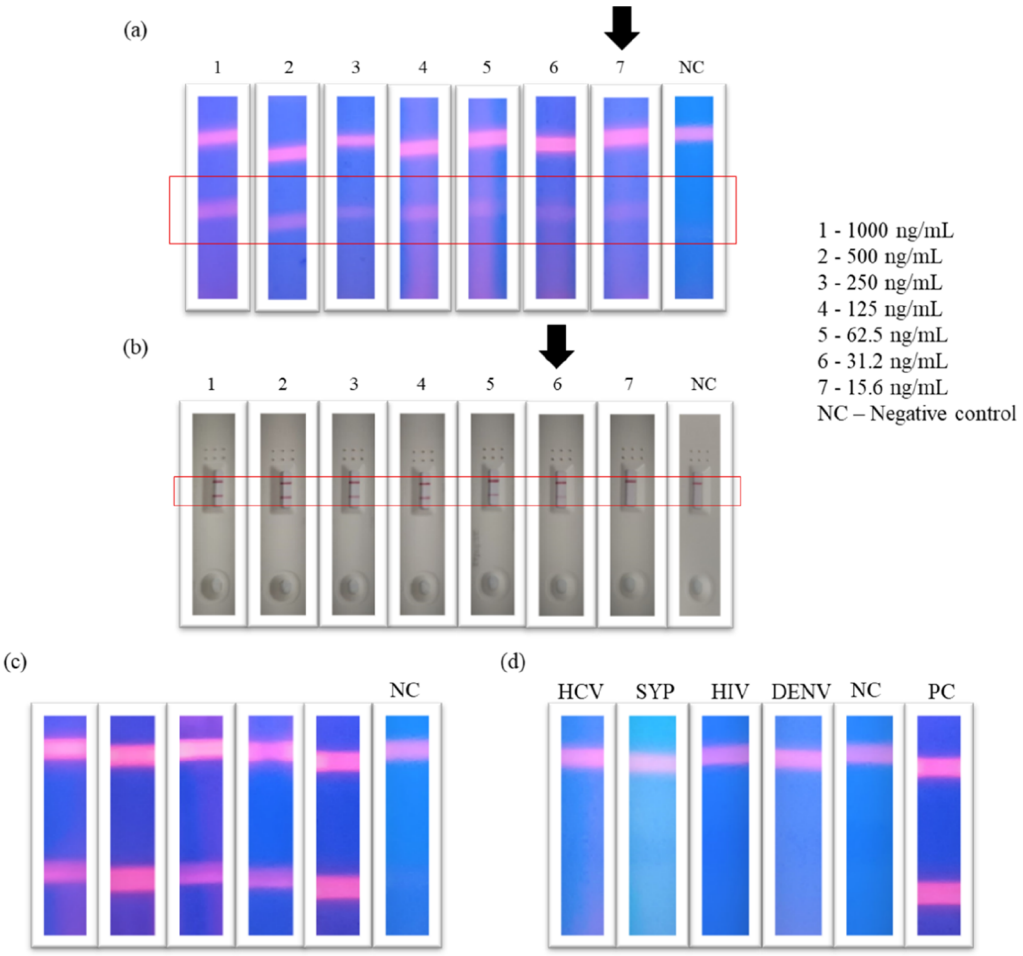

Figure 5. Analytical and diagnostic evaluation of the developed LFA. (a) Representative strip images at 1000, 500, 250, 125, 62.5, 31.2, and 15.6 ng/mL HBsAg concentrations, showing the sensitivity analysis. The LOD was defined as the lowest concentration at which a visible test line was consistently observed (15.6 ng/mL); (b) comparison with a commercial LFA, which exhibited a higher LOD of 31.2 ng/mL; (c) detection of HBsAg in positive serum samples showing clear fluorescence signals at both test and control lines, and (d) specificity test using samples positive for HCV, SYP, HIV, and DENV, as well as serum from a healthy individual as negative control (NC) and serum that is HBsAg-positive as positive control (PC). Only the serum samples that are HBsAg-positive produced a detectable fluorescence signal, confirming assay specificity.

The limit of detection (LOD) was determined at 15.6 ng/mL, based on fluorescence intensity measurements of serially diluted HBsAg samples ranging from 1000 ng/mL to 15.6 ng/mL. Fluorescence signals were consistently detected across all tested HBsAg concentrations, with signal intensity increasing proportionally to the antigen concentration.

Specificity testing was performed using serum samples positive for unrelated pathogens, including HCV, SYP, HIV, and DENV. No fluorescence signals were observed for these non-HBV samples, confirming the specificity of the assay. In contrast, the positive HBsAg serum samples consistently produced strong fluorescence signals, which were clearly distinguishable from those of the negative control (Figure 5).

3.5. Stability of Antibody-CadmiumQDs (Ab-QDs) Conjugates

The stability of Ab-QDs on LFA strips was assessed under two storage conditions: refrigerated (4 °C) and ambient room temperature (~25 °C). For refrigerated storage, the strips were monitored over 90 days. Fluorescence intensity was measured at 7 day intervals during the first 28 days and subsequently at 20 day intervals until day 90. The Ab-QD retained strong fluorescence signals throughout the 90 days, indicating good long-term stability under cold storage conditions (Figure 6a).

For ambient temperature storage, the strips were evaluated weekly over a four-week period. Fluorescence remained stable during the initial weeks, with signal visibility maintained up to week 4. However, a gradual decline in signal intensity was observed thereafter (Figure 6b). These findings suggest that Ab-QDs demonstrate satisfactory short-term stability at room temperature, but refrigerated storage is recommended for maintaining long-term functionality.

3.6. Diagnostic Performance

The diagnostic performance of the developed LFA was assessed using a cohort of 244 clinical serum samples, comprising 171 confirmed HBsAg-positive and 73 HBsAg-negative samples, with electrochemiluminescence immunoassay (ECLIA) serving as the reference method. As presented in Table 3, the developed LFA successfully detected 148 out of 171 HBsAg-positive samples, yielding a sensitivity of 86.5%. Among the 73 confirmed negative samples by ECLIA, 70 were correctly classified as true negatives, while three samples produced false-positive results, resulting in a specificity of 95.9%. The positive predictive value (PPV) and negative predictive value (NPV) were calculated as 98.0% and 75.3%, respectively. The overall diagnostic accuracy of the assay was 89.3%.

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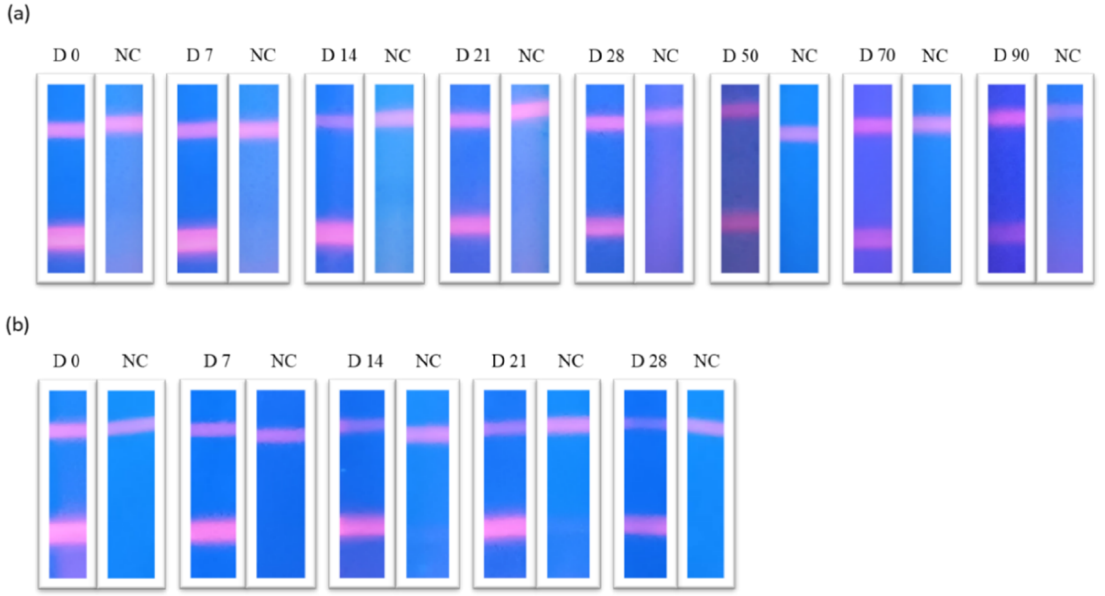

Figure 6. Stability evaluation of antibody-cadmiumQDs (Ab-QDs) conjugates on LFA strips stored under two conditions: (a) refrigerated (4 °C) over a 90 day period, and (b) ambient room temperature (~25 °C) over 4 weeks. Fluorescence signals at the test line remained stable under refrigerated conditions, while a gradual decrease in signal intensity was observed under room temperature storage beyond week 4.

Table 3. Diagnostic Classification of Clinical Serum Samples of the Developed LFA in Comparison with ECLIA

	developed LFA		
confirmed using ECLIA	positive	negative	total
HBsAg-positive	148 (TP)	23 (FN)	171
HBsAg-negative	3 (FP)	70 (TN)	73
uninfected individual	1	19	
HCV-positive	1	19	
SYP-positive		3	
HIV-positive	1	19	
DENV-positive		10	
total	151	93	244

To further benchmark the developed LFA, its performance was compared to that of a commercially available LFA (Mediven, Malaysia), which also utilized serum samples confirmed by ECLIA for evaluation. As shown in Table 4, the commercial LFA exhibited slightly higher diagnostic sensitivity (88.3%) and specificity (100.0%) relative to the developed LFA. Notably, the commercial LFA produced no false-positive results, achieving a perfect PPV (100.0%) and an NPV of 78.5%, resulting in an overall diagnostic accuracy of 91.8%.

Table 4. Comparative Diagnostic Performance of the Developed LFA and Commercial LFA for HBsAg Detection

parameter	developed LFA	commercial LFA
true positives (TP)	148	151
false negatives (FN)	23	20
true negatives (TN)	70	73
false positives (FP)	3	0
sensitivity (%)	86.5	88.3
specificity (%)	95.9	100.0
accuracy (%)	89.3	91.8
positive predictive value (PPV, %)	98.0	100.0
negative predictive value (NPV, %)	75.3	78.5

While the commercial assay displayed marginally higher diagnostic accuracy, the developed LFA produced a brighter fluorescence signal, which contributed to visual clarity and offered the potential for semiquantitative or quantitative analysis. This attribute is particularly beneficial for detecting low-abundance targets, where conventional AuNPs-based colorimetric LFA may underperform in sensitivity and visual detection.

4. DISCUSSION

The findings of this study demonstrate the diagnostic advantages of employing cadmiumQDs as fluorescent reporters in LFA for the detection of HBsAg. Compared to conventional AuNPs-based systems, cadmiumQDs offer superior photostability, narrow emission spectra, and significant brightness, which collectively enhance sensitivity and reliability in detecting low antigen concentrations.

QDs have been extensively studied in immunoassays due to their unique optical properties and multiplexing capabilities. In this study, incorporating cadmiumQDs enhanced the sensitivity of HBsAg detection and lowered the rate of false-negative results often seen with colorimetric LFA. This aligns with earlier research that has reported improved performance of QD-based LFA in detecting disease biomarkers such as cardiac troponin I and prostate-specific antigen. The use of QDs in clinical diagnostics faces several challenges, including the potential cytotoxicity of heavy metal-based QDs, complex bioconjugation steps, and the necessity for external fluorescence readers, which may limit their application in low-resource settings. However, the benefits may outweigh the limitations. Although cadmiumQDs are generally more expensive than traditional AuNPs, the quantities required per test are minimal, and the synthesis method applied in this study is relatively low-cost and scalable. Moreover, the enhanced sensitivity achieved using QDs may allow for reduced sample and reagent volumes, improving overall assay efficiency and offsetting the initial material cost.

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The optimization of assay components significantly influenced the performance of the developed LFA. The selection of CN95 as the nitrocellulose membrane enabled efficient capillary flow and uniform analyte migration, thus aiding reliable antigen−antibody interactions. Additionally, the inclusion of 1% BSA in the blocking buffer effectively minimized nonspecific binding. At the same time, the optimized antibody-to-cadmium QDs ratio (5.0:10.0) ensured stable bioconjugation, balancing fluorescence signal enhancement with reduced aggregation-induced signal loss. Analytical validation demonstrated a detection limit of 15.6 ng/mL, representing a substantial improvement over traditional AuNPs-based LFA, which depend on visual color changes and inherently have lower sensitivity. Specificity testing further verified the assay's accuracy, as no fluorescence signals were observed in cross-reactivity tests involving HCV, SYP, HIV, or DENV. This demonstrates that the developed LFA can accurately differentiate between HBV infections and other viral infections, thereby reducing the risk of misdiagnosis.

Despite these advantages, the diagnostic performance of the developed LFA did not exceed that of the commercial LFA, as reflected by a higher false-negative rate (13.5% versus 1.7%). Several factors may have contributed to this discrepancy. The affinity and specificity of the detection antibody employed in the bioconjugate may affect antigen binding efficiency, particularly at low HBsAg concentrations. Moreover, steric hindrance or suboptimal orientation of antibodies upon conjugation to cadmiumQDs may reduce the accessibility of antigen-binding sites. The matrix effects in clinical serum samples, including viscosity, protein interference, or nonspecific interactions, can influence capillary flow or inhibit antigen−antibody interactions. As detection in this assay is qualitative and based on fluorescence visible to the naked eye, low HBsAg levels approaching the detection limit (15.6 ng/mL) may result in weak signals that are challenging to interpret, especially without a reader. In contrast to conventional LFA that rely on subjective colorimetric interpretation, fluorescence-based detection can be further integrated with portable fluorescence readers to provide semiquantitative or fully quantitative measurements.

To mitigate these limitations in future assay iterations, the use of higher-affinity monoclonal antibodies, site-directed conjugation strategies, or fluorescence intensity quantification via portable readers could enhance detection sensitivity and reduce false-negative outcomes. Furthermore, pretreatment of serum or buffer modifications could help minimize matrix interference and improve overall reliability. This would enable clinicians to monitor disease progression and treatment efficacy more effectively.

The platform's adaptability makes it a strong candidate for multiplexed detection as an increasingly valuable feature in syndromic testing and epidemiological surveillance. Future studies should focus on expanding the clinical validation of the developed assay using larger sample sizes from diverse patient populations. Additionally, stability studies should be conducted to evaluate the shelf life of the assay under different storage conditions. Investigating the potential of multiplexed detection, where cadmiumQDs could be used for the simultaneous detection of multiple viral infections, could further enhance the diagnostic applicability of this platform. Ultimately, while the present work demonstrates a promising proof of concept, translation into point-of-care testing will require integration with portable fluorescence readers and larger-scale clinical validation.

5. CONCLUSION

This study establishes the feasibility and diagnostic potential of a cadmiumQDs-based LFA for the detection of HBsAg, offering a compelling alternative to conventional colorimetric assays. By integrating fluorescent nanomaterials into the LFA platform, the assay achieves an enhanced fluorescence signal, providing greater sensitivity and paving the way for semiquantitative or fully quantitative detection in point-of-care settings. The findings reflect a broader shift in rapid diagnostics, moving from visually interpreted formats toward fluorescence-based approaches with improved accuracy and analytical performance. While limitations such as sensitivity variation and bioconjugate stability warrant further optimization, the overall assay performance supports its potential for clinical application, particularly in early stage detection.

In conclusion, the cadmiumQDs-based LFA platform presents promising opportunities for expansion into multiplexed detection systems, enabling comprehensive diagnostic solutions for coinfections or syndromic testing. Its compatibility with portable reader integration further enhances its utility in decentralised or resource-limited environments. Future work will include evaluating the long-term stability of the complete LFA strip under various storage conditions to ensure performance reliability for real-world use. Additionally, ongoing efforts should focus on large-scale clinical validation, the biocompatibility of QDs, and user-friendly device integration, which will be essential to advancing this platform toward real-world applications. Continued exploration of safer QDs alternatives and robust device development will be crucial for translating this promising approach into scalable, field-ready technologies.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.5c04527.

> Zeta potential measurements of cadmiumQDs at different surface modification stages, fluorescence images of antibody-cadmiumQDs conjugates at various ratios, fluorescence signal detection of LFA strips under different optimization parameters, and repeatability assessment of the developed LFA for HBsAg detection over 3 days (PDF)

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N. A. research planning and management, project supervision, synthesis, characterization and bioconjugation, analysis and draft manuscript, N. S. bioconjugation, LFA assembly, parameter optimization and analysis, J. A. project supervision and manuscript review, R. H. S. research planning, project supervision, analysis and manuscript review.

Notes

The authors declare no competing financial interest.

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