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A Highly Sensitive Lateral-Flow Strip Using Latex Microspheres to Detect NGAL in Urine Samples

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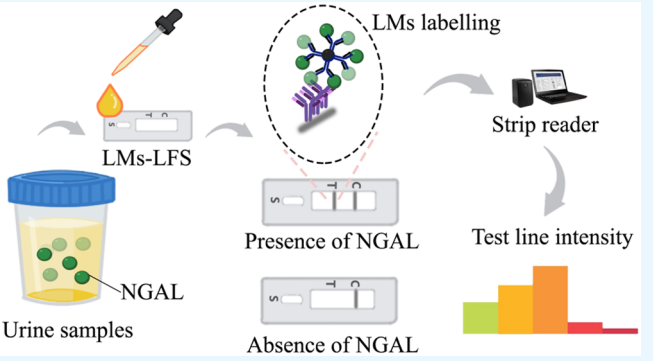
ABSTRACT: The incidence of kidney disease is increasing worldwide. Rapid and cost-effective approaches for early detection help prevent this disease. Neutrophil gelatinase-associated lipocalin protein (NGAL) is a novel biomarker for acute kidney injury (AKI) and chronic kidney disease (CKD). We aimed to develop a lateral flow strip (LFS) based on a lateral flow immunoassay method (LFIA), using latex microspheres (LMs) as a color labeling to detect NGAL in urine. The performance and potential of the developed LMs-LFS at a point-of-care (POC) testing were evaluated. The results showed that LMs-LFS successfully detected urinary NGAL within 15 min with high specificity without cross-reactivity to or interference from other endogenous substances in urine. The visual limit of detection (vLOD) was 18.75 ng/mL, and the limit of detection (LOD) was 1.65 ng/mL under the optimum condition. The LMs-LFS developed in this study showed a high correlation with the enzyme-linked immunosorbent assay (ELISA) method (R^2 = 0.973, n = 60 urine specimens) for detecting NGAL in urine. The LMs-LFS remained stable for at least six months at room temperature. The LMs-LFS can be a rapid, sensitive, and specific tool for the diagnosis and follow-up of renal disorders at the POC.



1. INTRODUCTION

Acute kidney injury (AKI, formerly known as acute renal failure) and chronic kidney disease (CKD) are becoming global health problems. Early intervention can significantly improve the prognosis in both cases. AKI and CKD are currently diagnosed by measuring the serum creatinine levels. However, serum creatinine levels remained stable until approximately 50% of the renal function had been lost. A variety of factors, such as age, malnutrition or obesity, vegetarian diet, and paraplegia, can influence serum creatinine levels. Neutrophil gelatinase-associated lipocalin protein (NGAL) is a novel biomarker to detect early tubular injury in AKI investigation.[1] NGAL rises within 3 h,[2] peaks at about 6−12 h, and remains present in blood and urine for up to 5 days.[3,4] Hence, NGAL plays a role in predicting clinical situations leading to AKI (such as cardiac surgery, kidney transplantation, contrast nephropathy, hemolytic uremic syndrome, and in the intensive care setting) or CKD (such as lupus nephritis, glomerulonephritis, blockage, dysplasia, polycystic kidney disease, and IgA nephropathy). Patients with progressive CKD had significantly higher serum and urinary NGAL levels compared to those without progression.[5] Thus, monitoring serum or urinary NGAL levels can help to improve the management of AKI and CKD to prevent progression of renal disease to end-stage renal disease (ESRD).

There are various methods to measure NGAL.[6−9] Enzyme-linked immunosorbent assay (ELISA) takes about 4 h to process and requires specialists to operate.[6] Chemiluminescence microparticle immunoassay (CMIA) has high sensitivity, but is costly and requires a specific chemiluminescence detector.[9] Radioimmunoassay (RIA) is precise and reliable, but its use is limited in application due to the perceived health risk and short half-life of radioisotopes.[7] Thus, these methods commonly have limitations when used as point-of-care testing (POCT) tools. To overcome these problems, a lateral flow immunoassay (LFIA) technique, which is a rapid and simple tool, was developed for NGAL detection as POCT.

LFIA is a one-step tool for clinical detection in which a sample containing the analyte of interest moves through the strips attached to the molecule that can interact with the

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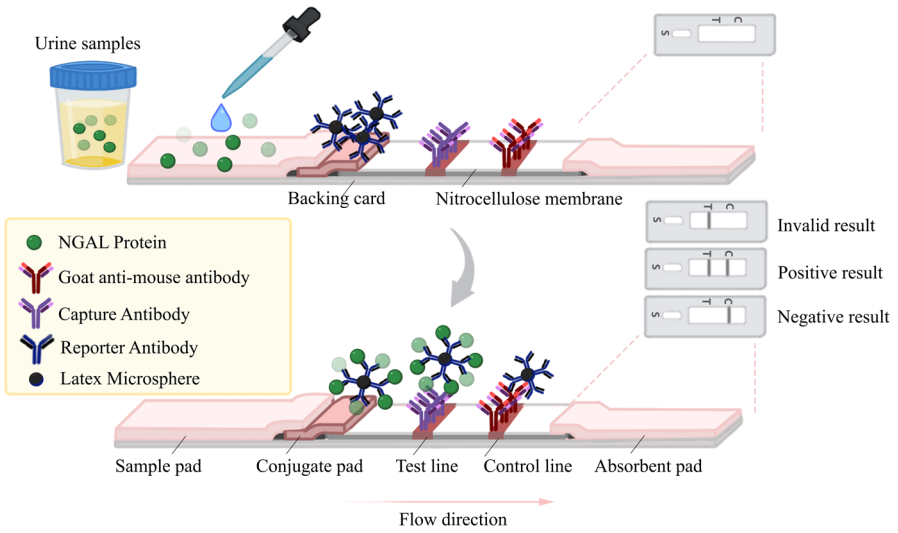

Figure 1. Schematic diagram of LMs-LFS consists of five parts, including the sample pad, conjugate pad, nitrocellulose membrane (test line, capture antibody and control line, goat antimouse antibody), backing card, and absorbent pad. The expected results of a urine sample dropped onto LMs-LFS are as follows.

analyte. Due to its rapid analysis and user-friendly operation, LFIA systems for NGAL detection with various detection labels have been demonstrated.[10−12] The common-colored particle detection labels that are often used to conjugate with the detecting antibodies are gold nanoparticles (GNPs),[13] latex microspheres (LMs),[14] carbon nanotubes,[15] luminescent particles (quantum dots (QDs), upconverting phosphor nanoparticles,[11] gold nanobeads,[16] and magnetic nanoparticles (MNP).[17] Among these, GNPs are commonly used as label detectors in LFIA strips. However, LMs offer several advantages in the detection of analytes in urine samples. For instance, they have the ability to covalently bond with detection antibodies and maintain stability across various pH levels.[18] Moreover, LMs provide strong visual signals that can be easily interpreted without the need for specialized equipment. They are available in uniform sizes and in a wide range of sizes.[19] The surface chemistry of LMs can also be adjusted to enhance the sensitivity and specificity in the detection of analytes. Additionally, LMs are a relatively inexpensive option compared to fluorescent substances and GNPs, making them a cost-effective choice for diagnostic assays.[20] In this study, we developed a latex microspheres-lateral flow strip (LMs-LFS) for rapid and sensitive NGAL detection based on the immune-complex flow-through nitrocellulose membrane using LMs as the color-labeling particles. The LMs-LFS has been extensively optimized, resulting in a high specificity of 91.7% and sensitivity of 95.2% for NGAL detection. It is also unaffected by common urine interferents. Additionally, it exhibits a strong correlation with established methods, such as ELISA. The detection time is only 15 min, and clinical specimens have been successfully tested. Based on our study's ROC analysis, a cutoff value of 26 ng/mL was suggested for CKD. The LMs-LFS developed here allows a one-step, rapid, inexpensive, and user-friendly detection of NGAL to be used at POC for the evaluation of kidney damage.

2. MATERIALS AND METHODS

2.1. Reagents. C-terminal polyhistidine-tagged recombinant human lipocalin-2 (NGAL) was purchased from Sino Biological (Beijing, China). Mouse monoclonal antihuman NGAL antibody (mAbNGAL) as the capture antibody (cat.10−1575) to coat the test line of the LFIA and reporter antibody (cat.10−1576) to conjugate with LMs and place in the conjugate pad were purchased from Fitzgerald Industries International (North Acton, MA, USA). Goat antimouse antibody was purchased from Lampire Biological Laboratories, Inc. (Ottsville, PA, USA). Nitrocellulose membranes (UniSart CN140 and CN95) were obtained from Sartorius Stedim Biotech SA, (Goettingen, S.A., Germany). The backing card, absorbent pad (Whatmann CF5), and sample pad (Whatmann GR470) were obtained from Global Life Sciences Solutions USA LLC (Marlborough, MA, USA). Globulin, bilirubin, hemoglobin, albumin, bovine serum albumin (BSA), chloroauric acid ($HAuCl_4.3H_2O$), and human serum albumin (HSA) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Glucose and ascorbic acid were purchased from KemAus (Cloisters Cherrybrook, Australia). A latex conjugation kit (Abcam, UK), an ELISA kit for NGAL, and synthetic urine were obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Sample Collection and Selection Criteria. Urine samples were collected from a total of 60 individuals, including 30 healthy individuals and 30 CKD patients, all over 18 years old. The CKD group consisted of individuals diagnosed with CKD according to the KDIGO clinical practice guidelines[21] at Srinagarind Hospital, Khon Kaen University, Thailand, between May 2023 and June 2023. Their blood creatinine levels and glomerular filtration rate (GFR) were measured to confirm CKD, defined as a GFR of 60 mL/min or less. Patients with acute renal damage were excluded from the study. The control groups were healthy individuals (n = 30). A thorough clinical examination and history were recorded for both the CKD and healthy control groups. All the participants were informed of the necessary information and were asked to sign a written informed consent form. Only participants who gave their consent were enrolled in this study. Midstream random urine samples from both groups were collected at the Clinical Chemistry Unit, Srinagarind Hospital, Khon Kaen University, Thailand. Each urine sample was aliquoted into three separate tubes (1 mL each) and stored at −20 °C without preservatives.

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2.3. Ethics Statement. This study was approved by the Human Research Ethics Committee of Khon Kaen University (HE641124).

2.4. Conjugation of Latex Microsphere (LMs) with the Antibody (mAbNGAL). The conjugation of LMs with antibodies was performed according to the methods described by Abcam Co., Ltd. (Cambridge, UK) with some modification.[22] In brief, the LMs in the Latex Conjugation Kit (ab269894 Abcam Co., Ltd., Cambridge, UK) were conjugated with the reporter antibody mAbNGAL using an active ester method. A 40 μL portion of 1 mg/mL of mAbNGAL diluted in reaction buffer was resuspended in the lyophilized LMs with gentle mixing for 15 min at room temperature. Then, 1 mL of a quencher was added to the conjugate solution. After incubation for 5 min at room temperature, the conjugate solution was centrifuged at 6,000 *g* for 20 min and 850 μL of supernatant was removed. After that, the mixture was centrifuged again at 6,000 *g* for 10 min, the supernatant was removed, and 40 μL of resuspension buffer was added to the mixture. Finally, 2% BSA was added to the conjugate solution to obtain a final concentration of 1% BSA. The mAbNGAL-LMs conjugate was stored at 4 °C until use.

2.5. Characterization of the Antibody-Latex Microsphere Conjugates (mAbNGAL-LMs). The optical properties of bare LMs and mAbNGAL-LMs (reporter antibody) were characterized by using a UV−visible spectrophotometer (Eppendorf BioSpectrometers, Hamburg, Germany). The zeta potentials and hydrodynamic diameters of the bare LMs and mAbNGAL-LMs were measured using a Malvern Zetasizer Nano ZS instrument (Zetasizer Nano S90, Malvern, UK). Attenuated total-reflection Fourier-transform infrared spectroscopy (ATR-FTIR) (Bruker, Germany) was used to characterize the chemical structure and bonding formation of the mAbNGAL-LMs.

2.6. Preparation and Optimization of Latex Microsphere Lateral Flow Strips (LMs-LFS). To prepare the LMs-LFSs, five parts, including the conjugate pad, nitrocellulose membrane, backing card, absorbent pad, and sample pad, were assembled (Figure 1). To optimize the LMs-LFSs, two concentrations (1 and 2 mg/mL) of mAbNGAL were tested as capture antibodies for the test lines. Goat antimouse antibody (1 mg/mL) was used for the control line using a KinBio Platform dispenser (Shanghai KinbioTech. Co., Ltd., Shanghai, China). The dispensing condition was at a speed of 50 mm/s (X = 18, Y= 26, Z= 16) on a nitrocellulose membrane (CN140, CN95); X, Y, and Z are abbreviations of X- (horizontal), Y- (vertical position), and Z-axis (height between dispensing tip and platform). After dispensing, the membrane was dried at 37 °C for 1 h. To optimize the type of nitrocellulose membrane, UniSart CN140 and CN95 were tested at the optimum concentration of the capture antibody. After that, adsorbent pads (Whatman CF5 and GR470) were also evaluated for LMs-LFS performance at the optimal concentrations of the capture antibody and the optimal type of nitrocellulose membrane.

The urine sample was dropped onto the developed LMs-LFS. If there is NGAL in the urine sample, then it binds to the mAbNGAL in the conjugate solution and then migrates. The sandwich structure is formed in this area because NGAL is captured by the capture antibody. After that, the excess mAbNGAL is bound with a goat antimouse antibody. The positive result is indicated by the color at both the test and control lines. The color intensity of the test line varied according to the amount of NGAL. The negative result is shown by the color appearing at the control line only. No color at the control line means an invalid result (Figure 1).

Positive = Black color appears at the test and control lines.
Negative = Black color appears at the control line only.
Invalid = No black color appears at the control line.

2.7. LMs-LFS Testing Procedure. NGAL, at the concentration range of 0−150 ng/mL, diluted with 1X phosphate buffer saline (PBS) (pH 7.4) containing 0.5% BSA and tested with the developed LM-LFSs. A 10 μL portion of the urine sample was mixed with 0.5 μL of mAbNGAL-LMs and loaded onto the conjugate pad. Then, 110 μL of the running buffer was added to the sample pad. The intensity of the test line was read by using a RapidScan ST5 lateral flow assay reader (Eurofins Shanghai Co., Ltd.). The photos of these strips were taken with a smartphone (Samsung S10+, Samsung, Seoul, South Korea). The distance between the smartphone and LMs-LFS was 21 in. and 90° angle for controlling perspective in images.

2.8. Specificity of LMs-LFS. To determine the specificity of LMs-LFS to NGAL, the substances commonly found in urine samples, including 10 mg/dL albumin, 10 mg/dL globulin, 40 mg/dL ascorbic acid, 4 mg/dL bilirubin, 500 mg/dL glucose, and 10 mg/dL hemoglobin, were tested on the LMs-LFS compared to the 18.75 ng/mL standard NGAL. The intensity of the bands at the membrane was recorded using the RapidScan ST5 reader software.

2.9. Interference of LMs-LFS by Contaminants in Urine. To determine the interference by the substances commonly found in urine on the developed LMs-LFS, the substances at the same concentration as those given in Section 2.8, were mixed with 37.5 ng/mL standard NGAL at the ratio of 1:1 to give a final concentration of NGAL of 18.75 ng/mL. The mixtures were applied to the LMs-LFS, and the intensity of the bands was recorded using the RapidScan ST5 reader software.

2.10. Stability and Reproducibility of LMs-LFS. The LMs-LFSs were kept at 25 °C for up to 6 months, and the stability of these strips was tested every month using the standard NGAL concentration of 18.75 ng/mL. The percentage of LMs-LFS stability was calculated using the value at month 0 as the control. The purpose of assessing the reproducibility of LMs-LFSs of different batches was to evaluate the consistency and reliability of the assay. Three different batches of LMs-LFSs were tested with NGAL at various concentrations (ranging from 0 to 150 ng/mL). Each batch of test strips was tested in triplicate at each concentration and assessed using the coefficient of variation (CV).

2.11. NGAL Binding Capacity of LMs-LFS. The binding capacity of the developed LMs-LFS to NGAL in urine was determined. Various concentrations (18.75, 37.5, 75, and 150 ng/mL) of NGAL were spiked to the synthetic urine. The percent recovery was calculated by the formula below

$$\%\text{recovery} = \frac{\text{concentration of NGAL determined by LMs-LFS} \times 100}{\text{concentration of the spiked NGAL}}$$

2.12. Comparison Between LMs-LFS and ELISA to Measure Urinary NGAL. NGAL levels in clinical urine samples (n = 60) were measured using a commercial ELISA kit (Sigma-Aldrich, St. Louis, MO, USA). Each urine sample was diluted 1:100−2000 with diluent and then analyzed in

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triplicate according to the procedure provided by the ELISA kit. In brief, 100 μL of various concentrations (0, 4.1, 10.24, 25.6, 64, 160, 400, 1000 pg/mL) of NGAL and urine samples were added into each well of a 96-well plate and incubated for 2.5 h at room temperature, followed by washing for 4 times with 1*X* PBS. Next, 100 μL of biotinylated detection antibody was added to each well and incubated for 60 min washed again for 4 times with 1*X* PBS. Horseradish peroxidase (HRP)-streptavidin (100 μL) was then added to each well. After washing for 4 times with 1*X* PBS, 100 μL of 3,3′,5,5′-tetramethylbenzidine (TMB) reagent was added to each well and incubated for 30 min in the dark. Finally, 50 μL of stop solution was added, and the absorbance at 450 nm was recorded immediately. The results from the ELISA method were compared to those of our developed LMs-LFS.

3. RESULTS

3.1. Characterization of mAbNGAL-LMs Conjugate. A UV−visible spectrophotometer was used to monitor the immobilization of mAbNGAL onto the LMs. The maximum absorption wavelength of the bare LMs was 572 nm, while the mAbNGAL-LMs showed a slight shift of the maximum absorption wavelength to 575 nm (Figure 2a). The hydro-

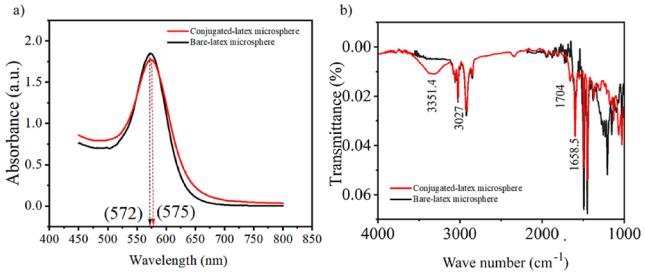

Figure 2. mAbNGAL-LMs conjugate results: a) UV−visible spectra of bare-latex microspheres (LMs) (black line) and mAbNGAL-LMs (red line). b) ATR-FTIR spectra of the bare LMs and mAbNGAL-LMs.

dynamic diameters and zeta potentials of the conjugates were measured to validate the effective antibody modification of the LM surface. Table 1 shows that the average hydrodynamic

Table 1. Hydrodynamic Diameters and Zeta Potentials of Bare-Latex Microspheres (LMs) and Conjugated Latex Microspheres (mAbNGAL-LMs)

particle type	particle diameter (nm)	zeta potential (mV)
bare-latex microspheres (LMs)	403 ± 7.55	-43.97 ± 1.59
conjugated-latex microspheres (mAbNGAL-LMs)	453 ± 3.61	-40.53 ± 0.61

diameters of the bare LMs and mAbNGAL-LMs were 403 ± 7.55 and 453 ± 3.61 nm, respectively. The observation of an increase in the hydrodynamic diameter distribution of LMs after conjugation was confirmed (Figure S1), which indicates the addition of conjugated mAbNGAL on the surface of LMs.The zeta potential values showed a decrease in magnitude, changing from −43.97 ± 1.59 to −40.53 ± 0.61 mV (Figure S1), which implied that the mAbNGAL-LMs were ready for further testing. The ATR-FTIR spectrum of the carbonylated polystyrene beads exhibited two distinct peaks at wavenumbers 1704 and 3027 cm^{-1} (Figure 2b), which correspond to C═O and O−H stretching of the free carboxylic acid groups, respectively. Furthermore, the contact between the carboxylic acid group and mAbNGAL results in the formation of an amide bond, which allows mAbNGAL to be attached to polystyrene beads. Maximum broad ranges at 3351.4 and 1658.5 cm^{-1} corresponding to N−H and C═O stretching bonds indicated amide bond formation.

3.2. Determination of the Optimum Condition of the LMs-LFS Developed to Detect NGAL. The concentration of the capture antibody (mAbNGAL) for the test line of LMs-LFS developed was optimized. When mAbNGAL concentration of 1 and 2 mg/mL were used, the concentrations of NGAL that could be observable by the naked eye on the strip were 37.5 ng/mL (Figure 3a) and 18.75 ng/mL (Figure 3b), respectively. Their intensities were measured using a RapidScan ST5 reader, as shown in Figure 3c. The results showed that the use of 2 mg/mL mAbNGAL gave better sensitivity than 1 mg/mL. Then, various types of nitrocellulose membranes and the adsorbent pads were evaluated for the LMs-LFS (Figure 3d). The results showed that the UniSart CN140 nitrocellulose membrane produced better signal intensities than the UniSart CN95 membrane. For the absorbent pad, the Whatman CF 5 membrane showed consistently outperformed compared to the Whatman CR 470, the latter showed strong background interference. The intensity, estimated by using a RapidScan ST5 reader, is shown in Figure 3e. Different types of buffers were also tested, and 50 mM Tris-HCl with 1% Tween 20 produced the highest intensity of the test lines (Figure 3f,g) compared to the other buffers. Taken together, the optimal conditions and components for the LMs-LFS developed were as follows: 2 mg/mL of mAbNGAL at the test line, UniSart CN140 nitrocellulose membrane, Whatman CF 5 adsorbent pad, and 50 mM Tris-HCl with 1% Tween 20 buffer.

3.3. Sensitivity and Limit of Detection of LMs-LFS. Various concentrations of NGAL (0, 4.69, 9.38, 18.75, 37.5, 75, 150, 300 ng/mL) were measured using the developed LMs-LFS under optimal conditions (Figure 4a). The visual limit of detection (vLODs), defined as the minimal concentration of NGAL required for a colorless test line, was 18.75 ng/mL. The standard curve, where the *x*-axis is the log of the NGAL concentration (ng/mL) and the *y*-axis is the test line intensity, shows the regression equation of $y = 474.69\log x - 314.12$ ($R^2 = 0.997$) (Figure 4b). A good linear correlation was observed at the range from 9.38 to 150 ng/mL of NGAL. The limit of detection (LOD) was 1.65 ng/mL, calculated using the equation: LOD = 3.3SD/*S*, where SD is the standard deviation of the blank signals (10 replicates) and *S* is the slope of the standard curve.

3.4. Specificity and Interference Test of LMs-LFS. To determine the specificity of the developed LMs-LFS, various contaminants commonly found in urine, including 10 mg/dL albumin, 10 mg/dL globulin, 40 mg/dL ascorbic acid, 4 mg/dL bilirubin, 500 mg/dL glucose, and 10 mg/dL hemoglobin, were tested for their reactivity to the LMs-LFS using 18.75 ng/mL NGAL as a positive control (Figure 5a). No black color, which was observed at the test lines with those urinary contaminants, implies that the system was specific to NGAL (Figure 5a). The intensity value was estimated using a RapidScan ST5 lateral flow reader and its software, as shown in Figure 5b. For the interference test, the substances commonly found in urine were mixed with 37.5 ng/mL of NGAL (final concentration of NGAL: 18.75 ng/mL) to test

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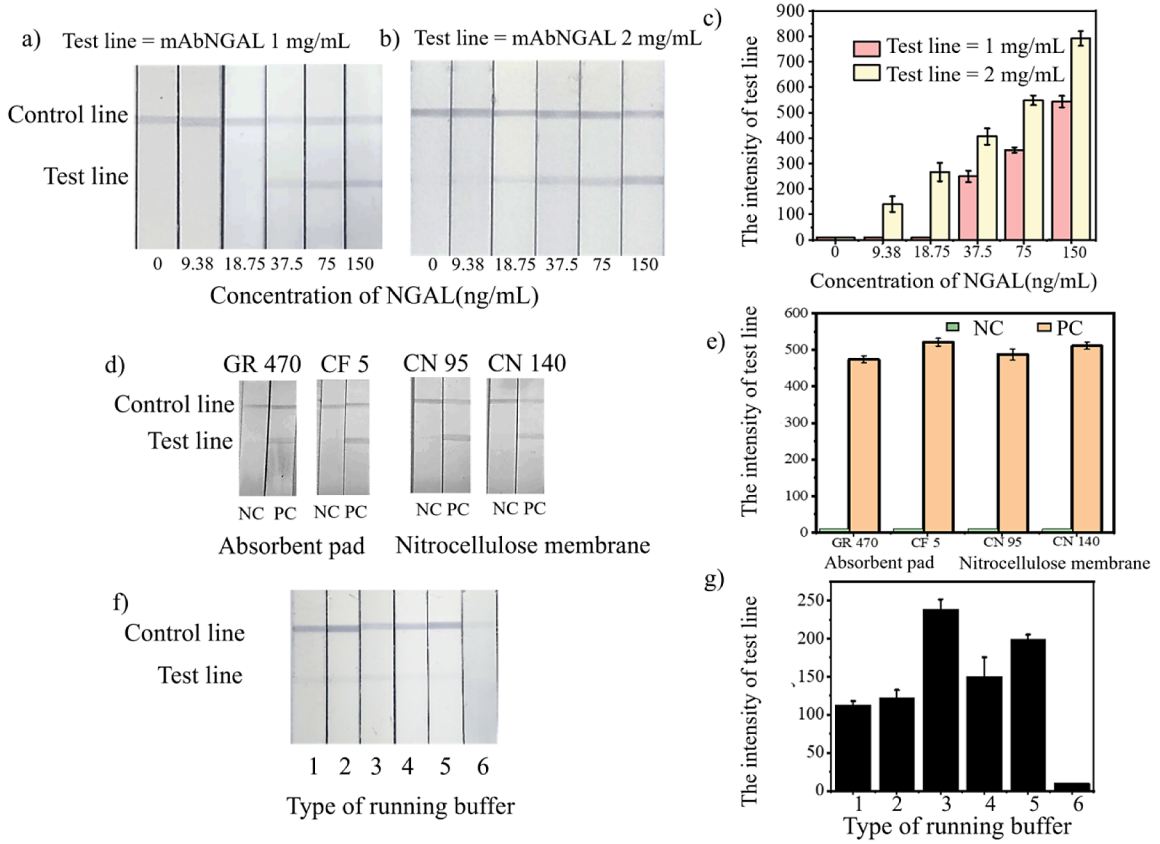

Figure 3. Optimization conditions for NGAL detection using the developed LMs-LFS. **a-c)** Optimization of mAbNGAL concentration at the test line: **a)** 1 mg/mL, **b)** 2 mg/mL of mAbNGAL, **c)** comparison of the test line intensity produced by 1 and 2 mg/mL of mAbNGAL. **(d, e)** Optimization of the nitrocellulose membrane and the absorbent pad: **d)** nitrocellulose membrane (UniSart CN140 and CN 95) and the absorbent pad (Whatman CF5 and GR 470) and **e)** comparison of the test line intensity using UniSart CN140 and CN 95 nitrocellulose membrane and Whatman CF5 and GR 470 absorbent pad(NC = Negative control (1*X* PBS pH 7.4 containing 0.5% BSA buffer), PC = Positive control (NGAL protein 75 ng/mL)). **(f,g)** Selection of running buffer: **f)** LMs-LFS results using five different types of running buffers (1 = 50 mM Tris-HCl with 200 mM NaCl and 1% Triton X-100; 2 = 50 mM Tris-HCl with 1% Triton X-100; 3 = 50 mM Tris-HCl with 1% Tween 20; 4 = 5 mM phosphate buffer pH 7.4 with 1% Triton X-100; 5 = 5 mM phosphate buffer pH of 7.4 and 1% Tween 20; 6 = distilled water. NGAL concentration was 18.75 ng/mL throughout, which is the lowest concentration detectable by the naked eye in the previous experiment. **g)** The intensity of the test line with five different types of running buffers and distilled water. Each number represents the average of three data points, with error bars indicating standard deviations.

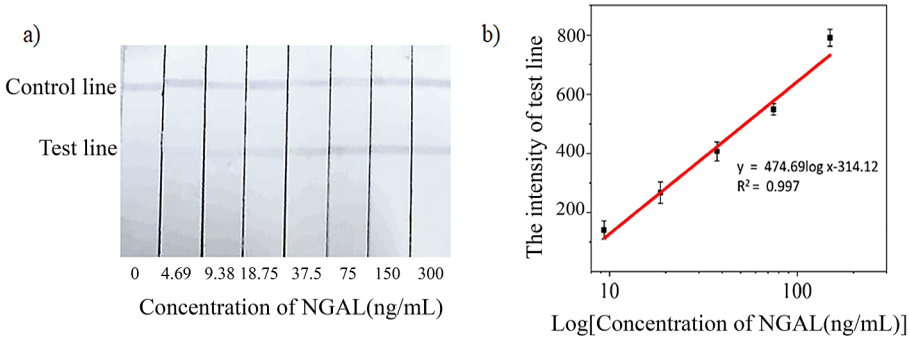

Figure 4. Sensitivity and limit of detection of LMs-LFS. a) The intensity of the test line of the LMs-LFS produced at various concentrations of NGAL. b) Standard curve for NGAL detection. The experiments were performed in triplicate for each NGAL concentration. Each number represents the average of three data points, with error bars indicating standard deviations.

the ability to interfare the binding of NGAL to the LMs-LFS (Figure 5c). The results are shown in Figure 5d. Obviously, the developed LMs-LFS showed specific binding to NGAL, and this specific binding was not interfered with by the substances possibly present in urine.

3.5. Binding Capacity of LMs-LFS to NGAL.

The binding capacity of the developed LMs-LFS to NGAL in synthetic urine samples was determined (Figure S2). Synthetic urine samples spiked with various concentrations of NGAL (0, 18.75, 37.5, 75, and 150 ng/mL) were tested with the developed LMs-LFS. The percent recovery ranged from 91.57

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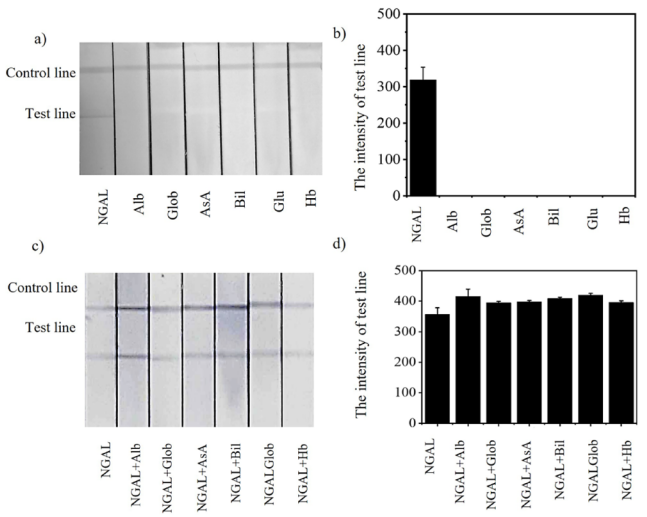

Figure 5. Specificity and the interference test of LMs-LFS. (a,b) Specificity test of LMs-LFS: a) various substances tested for specificity; b) intensity of the test lines of LMs-LFS for the specificity test (18.75 ng/mL NGAL; 10 mg/dL albumin (Alb); 10 mg/dL globulin (Glob); 40 mg/dL ascorbic acid (AsA); 4 mg/dL bilirubin (Bil); 500 mg/dL glucose (Glu); 7 = 10 mg/dL hemoglobin (Hb). (c,d) Interference test of LMs-LFS by various substances: c) Substances often found in urine were mixed with NGAL (to 37.5 ng/mL NGAL, 10 mg/dL albumin, 10 mg/dL globulin, 40 mg/dL ascorbic acid, 4 mg/dL bilirubin, 500 mg/dL glucose, 10 mg/dL hemoglobin). d) Intensity of the test lines of LMs-LFS with/without interfering substances. Each number represents the average of three data points, with error bars indicating standard deviations.

to 104.44%, with percent relative standard deviation (%RSD) values less than 5% (Table S1).

3.6. Comparison Between the Developed LMs-LFS and ELISA to Detect NGAL in Clinical Urine Samples. NGAL levels in urine samples of CKD patients (n = 60) were measured using the developed LMs-LFS and the results were compared to those of a commercial ELISA kit. The results revealed that both methods showed similar performance with high correlated outcomes (R^2 = 0.973), as shown in Figure 6a, suggesting the potential of the developed LMs-LFS for practical usage. Compared to the ELISA method, the developed LMs-LFIA requires a shorter time (within 15 min) than ELISA (4 h) to obtain the results. The capacity of NGAL levels (ng/mL) in urine for CKD screening was evaluated using receiver operating characteristic (ROC) curve analysis (Figure 6b). The ROC curve analysis demonstrated an AUC of 0.904, accurately predicting CKD by effectively distinguishing between the CKD group (n = 30) and the healthy group (n = 30). To evaluate the optimal cutoff point for NGAL levels, the Youden's Index was used to calculate the J-index with the formula: J = sensitivity + specificity−1.[23] The J-index determined that the optimal threshold for the NGAL value was 26 ng/mL, which yielded a sensitivity of 95.2% and a specificity of 91.7% for screening CKD (Table S2). The concentrations of NGAL in the CKD urine samples were significantly higher than those of the healthy groups at 95% confident interval with a p-value of <0.001 (Figure 6c). The median concentrations of NGAL in CKD and healthy groups were 117.03 (4.59−1506.37) ng/mL and 4.59 (4.59−62.01) ng/mL, respectively (Figure 6c).

3.7. Reproducibility and Stability of the Developed LMs-LFS. To evaluate the reproducibility of the developed LMs-LFS, three different batches of NGAL at various concentrations (0 to 150 ng/mL) were tested with LMs-LFS (Figure S3a) and the intensity of the bands was measured using a RapidScan ST5 reader (Figure S3b). The coefficient of variation (CV) varied from 1.70% to 4.41% (Table S3), which implied the high reproducibility of the LMs-LFS. To determine the stability, LMs-LFS were stored at 25 °C for up to 6 months, and their performance was evaluated every month by measuring the intensity of the LMs-LFS test line (Figure S3c) using a RapidScan ST5 reader (Figure S3d). The intensity of the test line at the lowest concentration of NGAL (18.75 ng/mL) detected with the naked eye showed a 14.24% decline from month 0 to month 6.

4. DISCUSSION

NGAL is a promising early biomarker to predict kidney injury.[24] An increase in urinary and plasma NGAL levels is observed as early as 1 to 2 h after renal injury, while the kidney function remains reversible.[25] Thus, it is well adapted to detect the onset of kidney injury and is a helpful biomarker for determining its severity, improving CKD diagnosis and prognosis, and assessing the efficacy of treatments.[26−28]

There are several methods to measure NGAL levels, e.g., chemiluminescent microparticle immunoassay (CMIA),[27,29−31] particle-enhanced turbidimetric immunoassay

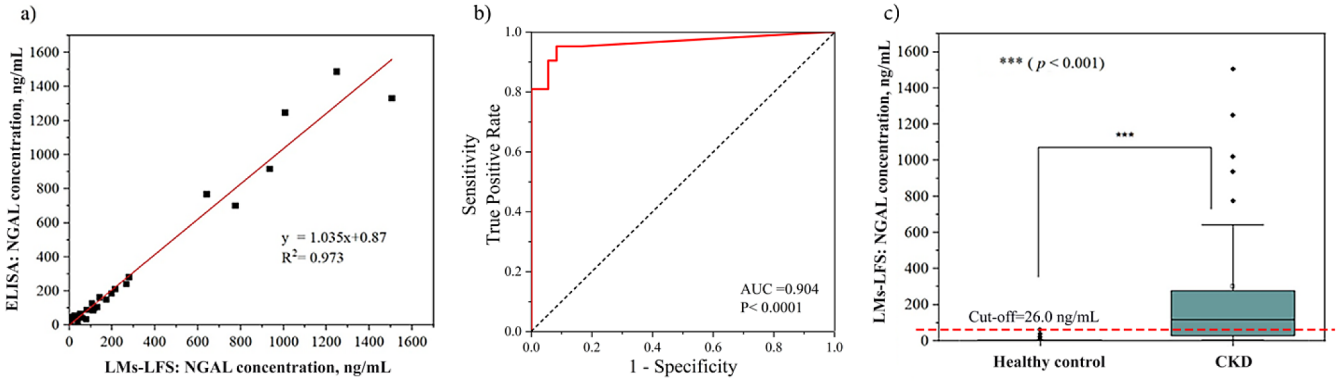

Figure 6. a) Correlation between NGAL levels in CKD urine samples (n = 60) determined by ELISA and LMs-LFS. b) Receiver Operating Characteristics (ROC) Analysis of the LFIA for Detection of CKD in Urine. c) Statistical comparison of median NGAL concentrations in the CKD group (n = 30) and healthy controls (n = 30).

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Table 2. LFS Research for NGAL Detection

LFS detection based on	sample types	detection	time (min)	linearity range (ng/mL)	LOD (ng/mL)	reference
europium	urine	fluorescence intensity reader	15	1−3000	0.36	12
upconverting nanoparticles	serum, urine	fluorescence intensity reader	30	30−1000	7.68	11
latex microspheres	urine	strip reader, naked eye	15	9.38−150	1.65	**the present study**

(PETIA),[32,33] fluorescence-based lateral flow immunoassay (FLFIA).[11,12] However, these methods need sophisticated analyzers, as well as specialists for operation, and are time consuming. Currently, NGAL can be detected using several methods employing antibodies, but there is no LFIA based on latex labels that has been investigated for the detection of NGAL.

In the present study, we developed a rapid test for NGAL detection based on the membrane immune-complex flow-through nitrocellulose principle (Figure 1). LMs were chosen as a color labeling in this study because they are more sensitive label for LFIA compared to conventional labels such as GNPs.[34−36] In a previous study on the use of GNPs as a color labeling, larger (40 nm) GNPs flow more slowly than the smaller ones (20 nm) across lateral flow membranes.[14] LMs were approximately 400 nm in size, and their mobility was much slower. Several investigations revealed that the slower mobility of bigger particles improves the sensitivity of the test.[14,37] Moreover, our work focused on using covalent LMs for signal amplification, which have unique properties that make them effective labeling detectors for detecting NGAL in urine samples. One advantage of using covalent LMs is their ability to form covalent bonds with detection antibodies. This reduces the occurrence of nonspecific binding and improves stability. These factors are crucial when working with urine samples to avoid false-positive results from nonspecific interactions. Furthermore, covalent LMs maintain stability across different pH levels, ensuring consistent and reliable assay performance.[18]

The conditions for the developed LMs-LFS, including the mAbNGAL concentration for the test line, type of membrane, and running buffer were optimized to obtain higher sensitivity of the developed LMS-LFS. Higher concentrations of the capture antibody mAbNGAL at the test line resulted in higher signal intensities (Figure 3a−c). Several studies have shown that the amount of mAbNGAL concentration for the test line has a significant impact on the strip color development.[38,39] Moreover, the UniSart CN140 membrane gave a stronger signal intensity at the test line and control line than the UniSart CN95 membrane (Figure 3d). The thickness and capacity of the absorbent pad are known to affect the washing of nonspecific components from the test and control lines and also reduce noise signals while enhancing assay sensitivity.[40,41] The reduction of background noise or interference is important to ensure the detection of the signals at the test and control lines. For the absorbent pad used in this study, Whatman CF5 had a signal intensity slightly higher than that of Millipore GR470 (Figure 3e). The Whatman CF5 pad (thickness = 954 um) produced a flow that was more consistent than that using GR470 (thickness = 840 um) (Figure 3d). Running buffer also influences the flow of the sample and an antibody-latex probe across the membrane. It helps ensure the optimal time of antigen binding with the antibody. The types of buffers with varying ionic strengths and surfactants are known to affect the color development and exhibit inhibitory action of the test strips.[39] In the present study, a running buffer of 50 mM Tris-HCl and 1% Tween 20 gave a slightly higher signal than the other buffers (Figure 3g). Our findings are consistent with those of the previous study,[42] in which GNPs were used as a labeling detector.

The intensity of the signal was directly proportional to the concentration of the analyte in the samples (Figure 4a,b). This allows quantitative analysis by comparing the intensity of the test line to a standard curve generated using known concentrations of the analyte using strip reader devices. The linearity range of the developed LMs-LFS for NGAL detection was between 9.38 and 150 ng/mL and the LOD was 1.65 ng/mL. Even though the prior method, which used LFIA with fluorescent detection, including europium and upconverting nanoparticles (UCP), had a lower LOD and a wide linear range (Table 2), they required detection solely through a fluorescent reader. In contrast, the present study, based on LMs, allows detection using both a strip reader and naked eye observation.

The specificity test of LMs-LFS was conducted by examining its ability to detect NGAL in the presence of various substances commonly found in urine. Each substance was tested at high concentrations that can be found in human urine, including **10** mg/dL albumin, **10** mg/dL globulin, **40** mg/dL ascorbic acid, **4** mg/dL bilirubin, **500** mg/dL glucose, and **10** mg/dL hemoglobin. The results showed that these substances tested did not give black color at the test line of the LMs-LFS (Figure 5a,b). An interference test was also conducted to assess whether these substances in urine could interfere with NGAL detection by LMs-LFS. The results showed that the presence of albumin, globulin, ascorbic acid, bilirubin, glucose, and hemoglobin did not interfere with the detection of NGAL by LMs-LFS (Figure 5c,d). This finding supports the reliability of LMs-LFS to detect NGAL. An increase in signal intensity at the test line was observed during the specificity and interference tests when NGAL was mixed with other substances, as opposed to NGAL alone. This increase could be attributed to the presence of additional substances that alter the environment,[43] enhance the binding efficiency of NGAL, occupy nonspecific binding sites, reduce background noise, improve the flow dynamics of the sample, and facilitate more efficient detection of NGAL. These factors collectively contribute to the observed increase in signal intensity during these tests.The comparison of the measurement of NGAL in clinical urine samples by LMs-LFS and by ELISA method showed a very good correlation ($R^2 > 0.976$), as shown in Figure 6a. The median NGAL concentration was 117.03 (ranging from 4.59 to 1506.37) ng/mL in CKD individuals and 4.59 (ranging from 4.59 to 62.01) ng/mL in non-CKD individuals (Figure 6b). Another study using an ELISA kit showed the mean urinary NGAL concentrations of CKD patients and healthy controls were 378.28 ± 111.13 ng/mL and 7.38 ± 3.26 ng/mL, respectively.[44] Various studies have reported different thresholds of NGAL for identifying individuals who are positive or negative for CKD (Table S3). In our study, LMs-LFS determined a relatively low cutoff value of 26 ng/mL, while previous studies have reported values

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ranging from 2.2 ng/mL to 120 ng/mL (Table S4).[45−47] The thresholds for NGAL to classify CKD patients and healthy individuals can vary depending on factors such as the laboratory or healthcare institution, patient demographics, and methodologies used. Additionally, these findings suggest that our study's cutoff value of 26 ng/mL for urine NGAL level may be a more sensitive approach for the early screening of CKD. The present study demonstrate that the developed LMs-LFS is highly reliable and can provide a quick, sensitive, and optional method for onsite NGAL monitoring. Clinical testing with a large sample size is warranted for further validation of the usage of our LMs-LFS at POC.

5. CONCLUSION

In this study, an LMs-LFS based on a strip reader measurement was first developed and validated for the onsite detection of NGAL in urine samples.The vLODs for NGAL detection were 18.75 ng/mL and LODs were −1.65 ng/mL. The sample preparation was simple and quick and the results were available in 15 min. The LMs-LFS and ELISA methods showed a strong correlation to NGAL levels in blind urine samples. Additionally, the LMs-LFS, with an NGAL threshold value of 26 ng/mL, demonstrated a sensitivity of 95.2% and a specificity of 91.7%. According to the reported clinical levels of NGAL, the NGAL thresholds range from 2.2 to 120 ng/mL for identifying individuals with either positive or negative CKD. It is suggested that the developed LMs-LFS may serve as a more sensitive approach for the early screening of CKD. This LMs-LFS prototype has a great potential to determine NGAL in clinical urine samples, especially from CKD patients.

■ ASSOCIATED CONTENT

Data Availability Statement

The data sets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.4c04322.

Additional results including dynamic light scattering, zeta potential of the LMs before and after mAb conjugation; the recovery test and percent recovery of the LMs-LFS; *J*-index results; reproducibility and stability test of LMs-LFS and comparing NGAL thresholds to determine the presence or absence of CKD in different methodologies (PDF)

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