

Lateral Flow Assay for NGAL Detection Using Cysteamine-Modified Gold Nanoparticles

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KEYWORDS

NGAL detection;
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ABSTRACT

Neutrophil gelatinase-associated lipocalin (NGAL) is a promising biomarker of chronic kidney disease (CKD). A rapid and cost-effective method is required for CKD detection. This study aimed to develop and evaluate the performance characteristics of an antibody (Ab) and a method for NGAL detection in clinical samples and assess its potential as a point-of-care test. The developed assay was highly specific for NGAL and showed no cross-reactivity with other endogenous substances. The developed lateral flow immunoassay based on cysteamine-modified gold nanoparticles exhibited a sensitivity of 12.5 ng/mL. The results showed that the dual monoclonal Ab-based sandwich is ideal for screening patients with CKD and has the potential to provide rapid and cost-effective detection of NGAL as a point-of-care testing system.

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Introduction

Chronic kidney disease (CKD) is a widespread condition and was the 12th leading cause of death worldwide in 2017⁽¹⁾. Most patients with CKD die of cardiovascular disease rather than end-stage renal disease (ESRD). The United States Renal Data System reported that diabetic nephropathy is the leading cause of ESRD, accounting for 35-50% of cases⁽²⁾. The number of patients with CKD in stages 2-5 has continuously increased since 1988 due to diabetes and hypertension⁽³⁾. Disease progression is rapid in numerous patients due to poor hypertension and heavy proteinuria control⁽⁴⁾. Therefore, early detection can be helpful in the treatment of kidney disease and can prevent kidney disease progression to advanced or ESRD. Currently, the Kidney Disease Improving Global Outcomes (KDIGO) organization has summarized the stages of CKD based on the levels of dysfunction defined by the estimated glomerular filtration rate (stages G1-G5) and albuminuria (A1-A3)⁽⁵⁾. The disease is identified based on the symptoms presented by the patients for at least 3 months. However, the level of serum creatinine does not change until the loss of 50% of kidney function⁽⁶⁾. Therefore, new biomarkers that indicate early damage are required.

Neutrophil gelatinated associated lipocalin (NGAL) is a novel biomarker for early tubular damage detection⁽⁷⁾; NGAL increases within 3 h and reaches a peak at approximately 6-12 h, and is still present in the blood and urine for up to five days⁽⁸⁾. NGAL levels increase in the serum and urine of patients with CKD progression than those without progression. Therefore, NGAL levels can be used as a novel predictive biomarker for the progression of CKD⁽⁹⁾.

There are several methods for detecting NGAL. Enzyme-linked immunosorbent assays (ELISA) and chemiluminescence immunoassays are commonly used to evaluate NGAL levels⁽¹⁰⁾. However, these methods require reader equipment and specialists to interpret results. Moreover, the lateral flow strip (LFS) is an easy and rapid method

that can be used for NGAL detection. LFS has the advantage of being a one-step or on-site process for rapid clinical detection, owing to its rapid analysis and simple and user-friendly operation⁽¹¹⁾. In this study, we developed a rapid antigen test based on membrane-based immune-complex flow-through nitrocellulose principles for NGAL detection. There have been many studies on the use of LFS for NGAL detection^(12,13) using different detection labels. The common detection labels that are used to bind with the detection antibody (Ab) include gold nanoparticles (AuNPs), latex microparticles, carbon nanoparticles, luminescent particles (quantum dots and up-converting phosphor nanoparticles), and magnetic nanoparticles^(14,15). The use of luminescent colors as detection labels requires specialized equipment. Therefore, the colored particles are conjugated with the NGAL detection Ab, and the AuNPs and latex microparticles are observed in the interaction between the NGAL Ab and NGAL with the naked eye.

AuNPs are used as the detection label because of their unique optical properties that allow for distinct colorimetric changes that can be easily observed without specialized equipment⁽¹⁶⁾. Additionally, AuNPs exhibit high biocompatibility, low toxicity, and exceptional stability, making them ideal candidates for medical diagnostic applications⁽¹⁷⁾. The ease of functionalization further enhances the sensitivity and specificity of the assay⁽¹⁸⁾.

This study explored the use of cysteamine, a small molecule containing both an amino and a thiol group⁽¹⁹⁾, which was utilized as a modifying agent for AuNPs. Cysteamine offered unique benefits for nanoparticle stabilization and functionalization of LFS⁽²⁰⁾. Specifically, this study investigated the use of cysteamine-AuNPs as novel conjugates for NGAL detection in LFS. The distinctive properties of cysteamine enhance nanoparticle stability and promote effective conjugation with biomolecules, which can facilitate improved signal generation. This approach aimed

to leverage the advantages of cysteamine to develop a sensitive and reliable platform for NGAL detection in clinical diagnostics.

Materials and methods

Materials

C-terminal polyhistidine-tagged recombinant human lipocalin-2 (NGAL) was purchased from Sino Biological Inc. (Beijing, China). Mouse monoclonal anti-human NGAL Ab for use as a capture and detector Ab was purchased from Fitzgerald Industries International (North Acton, USA), and goat anti-mouse Ab as a control line (CL) was purchased from MyBioSource (San Diego, CA, USA). Globulin, bilirubin, hemoglobin, albumin, bovine serum albumin (BSA), chloroauric acid ($\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$), cysteamine hydrochloride, sodium borohydride, 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).

Methods

Ethics statement

All clinical samples were collected from the Clinical Chemistry Unit of the Srinagarind Hospital, Khon Kaen University. This study was approved by the Ethics Committee of Khon Kean University (HE641124).

Participant Selection and Urine Sample Collection

Urine samples were obtained from 10 individuals, comprising five individuals without CKD and five patients with CKD; all individuals were aged >18 years. The CKD group included patients diagnosed with CKD following the KDIGO clinical practice guidelines at Srinagarind Hospital, Khon Kaen University, Thailand, between May 2023 and June 2023. Blood creatinine levels and glomerular filtration rate (GFR) were assessed to confirm CKD, defined by a GFR of ≤ 60 mL/min. Patients with acute kidney injury were excluded from the study. The non-CKD group consisted of five participants without CKD. Patients with CKD

and non-CKD controls underwent detailed clinical evaluation and history recording. All participants were thoroughly informed of the study and provided written informed consent before enrollment. Random midstream urine samples from both groups were collected at the Clinical Chemistry Unit of Srinagarind Hospital, Khon Kaen University.

Preparation of Cysteamine-AuNPs and conjugation with detector Ab

We modified an established protocol to synthesize stable cationic AuNPs⁽²¹⁾, achieving a uniform size range of 10-20 nm. The synthesis started by placing 40 mL of an aqueous HAuCl_4 solution (1.4 mM) into a clean flask at room temperature. While stirring vigorously, 400 μL of an aqueous cysteamine hydrochloride solution (213 mM) was slowly added. After 20 minutes, 40 μL of freshly prepared sodium borohydride (NaBH_4 , 10 mM) was introduced into the mixture, while maintaining vigorous stirring. The next 8-10 minutes were critical for precise nanoparticle formation, as indicated by the development of a deep wine-red color. The resulting AuNPs exhibited a bright red hue in solution and adhered to the glass flask, likely owing to the interactions between anionic silicate ions and cationic particles.

Conjugation of cysteamine-AuNPs with the detector Ab was performed by first preparing a dispersion of cysteamine-AuNPs at an optical density of 1, measured across the wavelength range of 400-800 nm, adjusted with deionized water (DI). To this dispersion, 12 μL of detector Ab at a concentration of 0.5 mg/mL was added to 0.8 mL of the cysteamine-AuNP solution. The mixture was then incubated at room temperature on a rotator for 15 minutes to allow the Ab to effectively bind to the AuNPs. After this incubation, 90 μL of 10% BSA was added to the mixture to achieve a final concentration of 1% BSA, and the samples were incubated for an additional 10 minutes on a rotator to prevent non-specific binding. The samples were subsequently centrifuged at 6,000 g for 20 minutes to pellet the

detector Ab-AuNP conjugates. The supernatant was removed, and the pellets were resuspended in 200 μ L of a suspension solution containing 10 mM phosphate buffer, 5% sucrose, 1% BSA, and 0.25% Tween 20, and the pH was adjusted to 7.4. The cysteamine-AuNPs and conjugate solutions were then characterized by UV-Vis spectroscopy.

Assembly of LFS strip

Notably, 1 mg/mL and 2 mg/mL of test lines (TL) (monoclonal mouse anti-human NGAL Ab [capture Ab]) and 1 mg/mL of CL (goat anti-mouse Ab) were dispensed using KinBio Platform dispenser (Shanghai KinbioTech.Co., Ltd. (Shanghai, China)) with different conditions in these factors including speed, X-axis (vertical position), Y-axis (horizontal position), and Z-axis (position of

dispensing tip and platform height). The optimal conditions for dispensing were as follows: speed, 50 mm/s; X, 18; Y, 26, Z, 16 for nitrocellulose membrane (CN140). After dispensing, the membranes were dried at 37°C for 1 h.

For the conjugate pad, 5 μ L of the conjugate solution from each method was dropped onto the conjugate pad and dried at 37°C for 1 h. For LFS assembly, the strip test comprised five parts: conjugate pad (GF33 treated), nitrocellulose membrane (CN140, Sartorius Stedim Biotech SA, Goettingen, Germany), backing card, absorbent pad (CF5), and sample pad (GR470, obtained from Global Life Sciences Solutions USA LLC) as shown in figure 1.

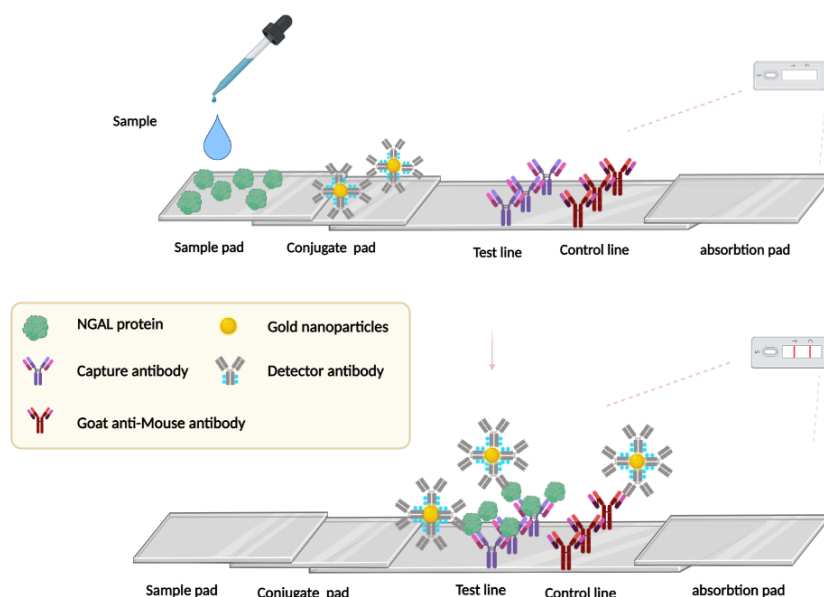


Figure 1 Schematic diagram of the lateral flow strip (LFS) for neutrophil gelatinated associated lipocalin (NGAL) detection (created with BioRender.com).

Note: LFS was divided into five parts: the sample pad, conjugate pad, nitrocellulose membrane (test, capture, control line (CL), and goat anti-mouse Ab), backing card, and absorbent pad.

Visual detection limit (vLOD) of the LFS

The different concentrations of the NGAL protein were diluted with phosphate buffer saline (pH 7.4) containing 0.5% BSA, and were tested with LFS from 0 to 100 ng/mL. Subsequently, 10 μ L of each sample was loaded onto the sample

pad. Subsequently, 110 μ L of the running buffer was added. If the sample contains NGAL, NGAL would bind with the Ab at the conjugate pad. After migration, the sandwich format would occur in this area because NGAL is captured with the capture Ab, after which the excess Ab binds with the goat

anti-mouse Ab. Consequently, a purple color is observed in the TL and CL, indicating a positive result. Color intensity varies with the amount of NGAL. If a purple color is not observed in the CL, the LFS is invalid.

Specificity of LFS

To determine the specificity of LFS, endogenous substrates were tested, 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 compared with standard NGAL protein 12.5 ng/mL as a positive control. Clinical sample testing and analysis

To evaluate the effectiveness of the developed LFS for NGAL detection, 10 μ L of urine from each of the five samples per group (CKD and non-CKD) was tested. After loading 10 μ L of each sample onto the sample pad, 110 μ L of running buffer was added.

Results

Characterization of cysteamine-AuNPs by UV-Vis spectrophotometry

The synthesized cysteamine-AuNPs and cysteamine-AuNP-conjugated Ab were characterized by UV-Vis spectrophotometry. The maximum absorption peaks of the cysteamine-AuNPs and the conjugates were at 516 nm and 526 nm, respectively. The results showed a peak shift from 522 to 525 nm, indicating conjugation (Figure 2).

vLOD of the LFS

Different concentrations of NGAL, ranging from 0 to 100 ng/mL, were spiked into urine samples and tested using the LFS. The vLOD, defined as the lowest concentration of NGAL that produced a visible TL, was 12.5 ng/mL (Figure 3).

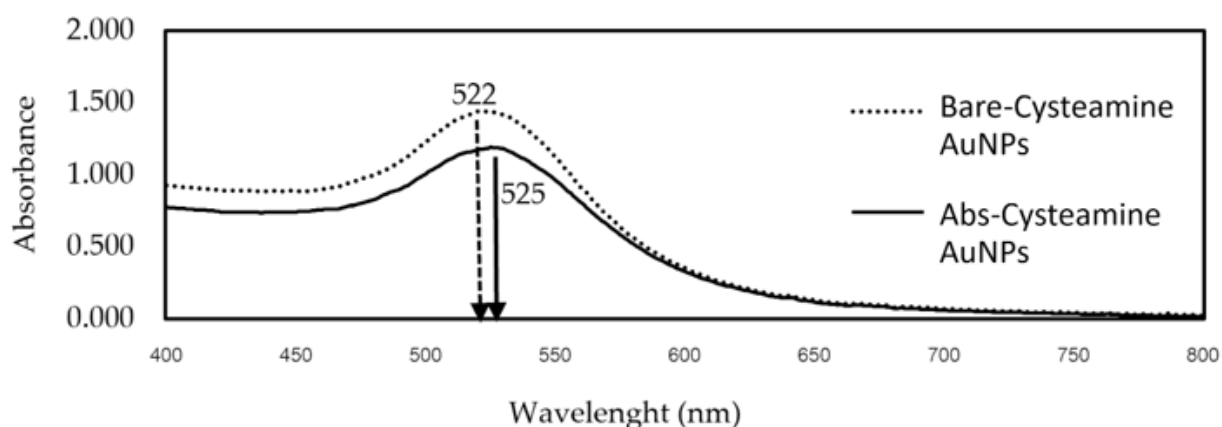


Figure 2 UV-Vis Spectrophotometry analysis of bare-cysteamine gold nanoparticles (Bare-Cysteamine AuNPs) and antibody-conjugated cysteamine AuNPs (Ab-Cysteamine AuNPs).

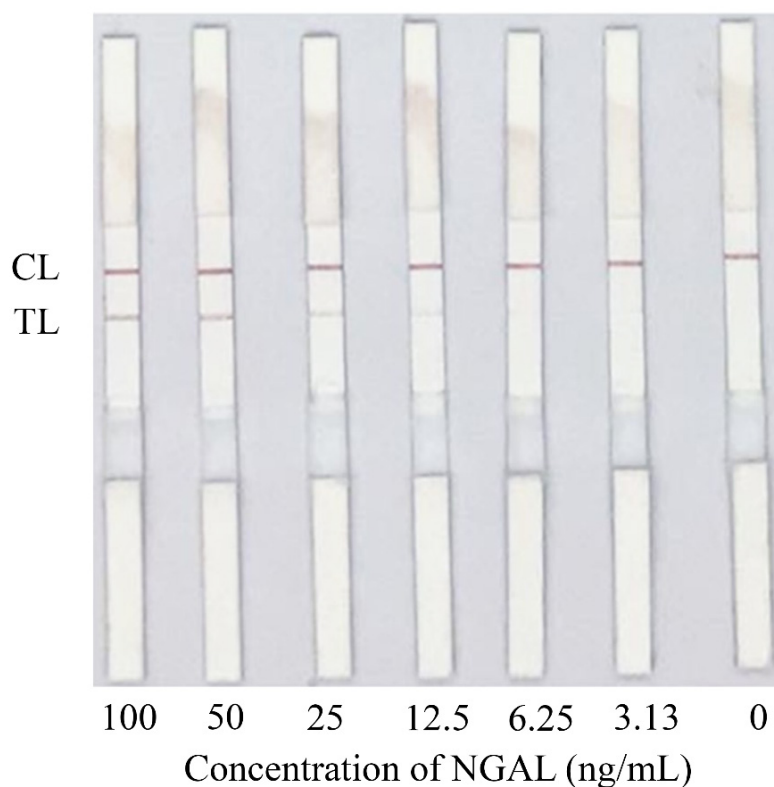


Figure 3 Visual limit of detection (vLOD) of the NGAL LFS.

Note: Representative images of LFS showing TL intensity at various NGAL concentrations (0-100 ng/mL) spiked into urine samples.

Specificity of LFS

To determine the specificity of the developed LFS for NGAL detection, a urine sample spiked with NGAL protein (12.5 ng/mL) along with ascorbic acid (40 mg/dL), bilirubin (4 mg/dL), hemoglobin (10 mg/dL), glucose (500 mg/dL),

albumin (10 mg/dL), and globulin (10 mg/dL) was tested. Only samples spiked with NGAL produced visible CL and TL. These findings indicated that the developed LFS was highly specific to NGAL (Figure 4).

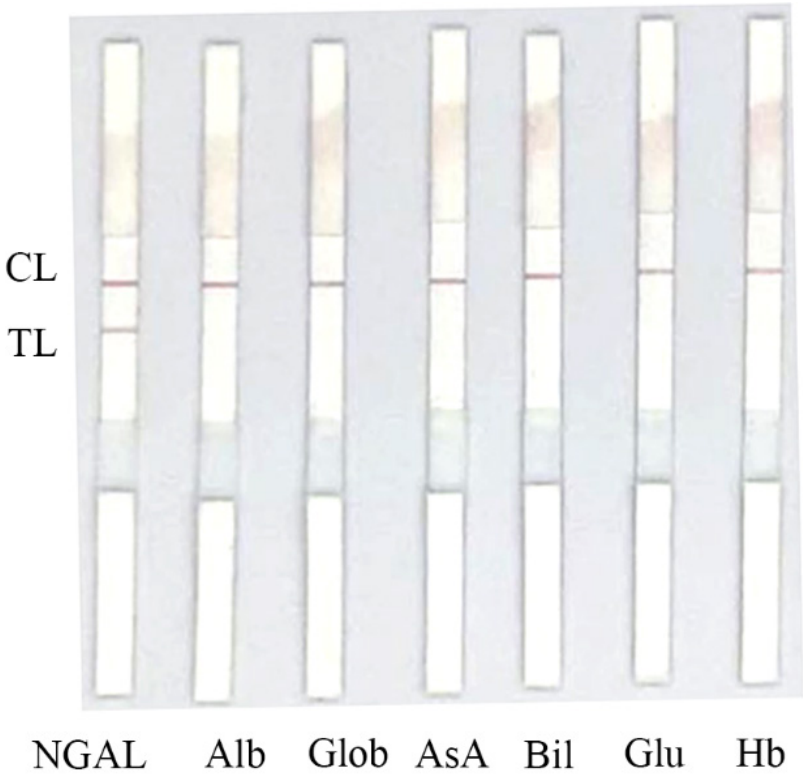


Figure 4 Specificity of the developed LFS.
Note: The specificity of the LFS was tested by exposing it to various substances, including NGAL (100 ng/mL), albumin (Alb, 10 mg/dL), globulin (Glob, 10 mg/dL), ascorbic acid (AsA, 40 mg/dL), bilirubin (Bil, 4 mg/dL), glucose (Glu, 500 mg/dL), and hemoglobin (Hb, 10 mg/dL).

Clinical sample testing and analysis

Five urine samples from each group (CKD and non-CKD) were tested to assess the performance of the developed LFS in detecting NGAL. The results were compared with those obtained using ELISA. The LFS accurately detected NGAL in all

five CKD samples, with a sensitivity of 100%, and correctly identified all five non-CKD samples as negative, resulting in a specificity of 100% (Figure 5). These results indicated that the Ab-based LFS was highly accurate for NGAL detection in clinical urine samples.

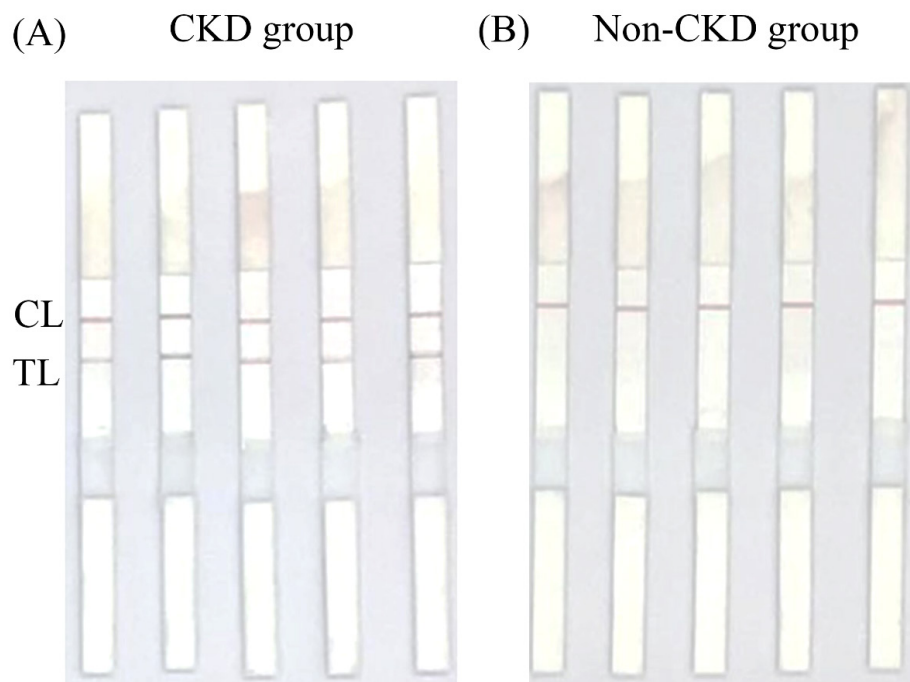


Figure 5 Clinical validation of the developed NGAL LFS using urine samples.

(A) Representative LFS results for CKD

(B) representative LFS results for non-CKD samples

Discussion

The development of a rapid and highly specific LFS for NGAL detection represents a significant advancement in the early diagnosis and management of CKD. This study demonstrated that the cysteamine-modified AuNPs used in our LFS assay provided a reliable and sensitive platform for NGAL detection, showing high sensitivity (12.5 ng/mL) and specificity without cross-reactivity with other endogenous substances, such as albumin, hemoglobin, glucose, and bilirubin. The ability of this assay to differentiate between CKD and non-CKD urine samples with 100% accuracy underscored its potential as a valuable tool for clinical diagnostics. Our findings suggested that the NGAL LFS assay could be a critical component of point-of-care testing for CKD. Early detection of kidney dysfunction allows for timely intervention, which can significantly slow the progression of CKD to ESRD. Traditional biomarkers

of kidney function, such as serum creatinine, do not indicate kidney damage until a substantial portion of renal function is lost⁽²²⁾. In contrast, NGAL is a sensitive biomarker that increases rapidly after kidney injury, making it a suitable candidate for early detection of CKD.

Although prior methods, such as LFS using fluorescent detection systems like europium and up-converting nanoparticles (UCP), demonstrated lower limits of detection of 0.36 ng/mL for europium⁽¹³⁾ and 7.68 ng/mL for UCP⁽¹²⁾, these methods necessitate the use of specialized fluorescent readers. However, the need for a fluorescent reader limits their practical applications in resource-limited settings. In contrast, the NGAL LFS assay developed in this study allowed for detection through naked-eye observation, eliminating the need for advanced equipment, thus enhancing its suitability for point-of-care testing.

The use of cysteamine-AuNPs in the assay improves the effectiveness of the nanoparticle conjugation with antibodies⁽²³⁾, which is crucial for achieving high sensitivity and specificity. Cysteamine allows for strong binding with antibodies and minimal non-specific interactions⁽²⁴⁾, which are crucial for accurate diagnostics. This innovative approach to nanoparticle functionalization can be applied to other biomarkers and diseases, thereby potentially broadening the scope and utility of LFS-based diagnostics. Given the global burden of CKD, particularly in low-resource settings, the NGAL LFS offers a promising solution for rapid on-site testing. Its ease of use, low cost, and rapid turnaround time make it suitable for deployment in remote or underserved areas where access to advanced laboratory facilities is limited. Furthermore, the assay does not require specialized equipment or extensive training⁽²⁵⁾, which can facilitate its widespread adoption and frequent monitoring of at-risk populations. Although the results are promising, further studies are necessary to validate the assay performance across diverse populations and clinical settings. Large-scale clinical trials could provide more comprehensive data on the diagnostic accuracy, reliability, and potential limitations of this assay. Additionally, investigating the long-term benefits of regular NGAL monitoring using the LFS in patients with CKD could provide valuable insights into its role in improving patient outcomes. Future research should explore the application of LFS technology to other biomarkers indicative of different diseases, thereby expanding its diagnostic utility. Enhancing the multiplexing capabilities of the assay could allow simultaneous detection of multiple biomarkers, further increasing its clinical relevance and application.

Conclusion

The developed NGAL LFS represents a promising point-of-care diagnostic tool for rapid and accurate detection of NGAL in urine samples. Its high sensitivity and specificity suggest that the

LFS could be an asset in the early detection and monitoring of kidney dysfunction, particularly in resource-limited settings, where rapid on-site testing is crucial. The ability of this assay to correctly differentiate between CKD and non-CKD samples demonstrates its potential clinical utility. Further large-scale clinical studies are warranted to establish its effectiveness in diverse patient populations and clinical settings.

Take home messages

We developed a simple and rapid urine test strip to accurately detect a kidney marker (NGAL). This test can help identify kidney problems early, potentially improving patient outcomes. These are particularly promising for use in areas with limited access to advanced medical facilities.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgments

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