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Distance-based lateral flow biosensor for the quantitative detection of bacterial endotoxin

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ABSTRACT

Bacterial endotoxin (a type of lipopolysaccharide, LPS) that acts as the strongest immune stimulant exhibits high toxicity to human health. The golden standard detection methods rely heavily on the use of a large amount of tachypleus amebocyte lysate (TAL) reagents, extracted from the unique blue blood of legally protected horseshoe crabs. Herein, a cost-effective distance-based lateral flow (D-LAF) sensor is demonstrated for the first time based on the coagulation cascade process of TAL induced by endotoxin, which causes the generation of gel-state TAL. The gelation process can increase the amount of trapped water molecules and shorten the lateral flow distance of the remaining free water on the pH paper. The water flow distance is directly correlated to the concentration of endotoxin. Noteworthy, the D-LAF sensor allows the detection of endotoxin with the reduced dosage of TAL reagents than the golden standard detection methods. The detection limit of endotoxin is calculated to be 0.0742 EU/mL. This method can be applied to the detection of endotoxin in real samples such as household water and clinical injection solution with excellent performance comparable to the commercial ELISA kit.

Lipopolysaccharide (LPS, 10–20 kDa) is a component embedding tightly in the cell wall of Gram-negative bacteria. It is also called bacterial endotoxin. The chemical structure of LPS typically contains the specific polysaccharide, non-specific core polysaccharide, and covalently bounded lipid A region. Generally, the lipid A moiety is responsible for immunotoxicity of LPS. The LPS endotoxins are ubiquitous in aqueous environments including groundwater, tap water, drinking water, and effluents of water treatment plants [1]. Even a trace amount of endotoxin (as low as pg/mL level) inhaled by contaminated air and ingested through potable reuse of the reclaimed wastewater may lead to fever, organ failure, and septic shock [2]. The detection and tracking of endotoxin as microbial by-products in environmental water quality monitoring is a major concern for public health and have attracted great attention.

Recently, many efforts have been devoted to develop alternative approaches for endotoxin detection such as colorimetric, electrochemical, and fluorescent assay [3,4]. Among them, the endotoxin quantification mostly relies on the affinity between endotoxin and the natural or artificial recognition molecules such as peptides [5], antibodies [6], aptamers [7], plasmonic nano-particles [8], and synthetic small molecules [9]. However, these methods are generally restricted in practice because of the high cost and complex operation. The golden standard for clinical detection of endotoxin, designated as the legal method by European and American Pharmacopoeia, is the tachypleus amebocyte lysate (TAL) assay. It inevitably requires the use of TAL reagents extracted from the unique blue blood of horseshoe crabs. The blood of horseshoe crabs contains a nucleated type of deformable cells, which have a large number of dense granules in the cytoplasm, containing coagulogen, C-factor, B-factor, and coagulated proteogen. In the presence of endotoxin, C-factor in TAL reagents can be activated to continually trigger the transformation of coagulated proteogen to coagulated protein, which results in the formation of a gel state. The coagulation process is shown in Fig. S1 (Supporting information). The TAL assay usually involves gel and spectroscopy methods dependent on the enzyme cascade agglutination reaction between endotoxin and TAL in the presence of proteinogen and activator C [10]. The gel method is a semi-quantitative test with the disadvantages of low sensitivity and difficulty to identify false positives. The spectrophotometry method is achieved by monitoring the turbidity variation induced by the gelation of TAL or absorbance change caused by the released *p*-nitroaniline from specific chromogenic substrate

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[11–13]. The spectrophotometry method exhibits the virtues of quantitative detection, and nevertheless often suffers from the requirement of specialized photometric instruments and high-price reagents [14]. In addition, all of the above methods are usually in demand for a large amount of TAL reagents (≥100 μL) for each test. Due to the factors such as marine environmental pollution and human overfishing, the number of horseshoe crabs has been declining sharply, so it is classified as protected animals, leading to the shortage of horseshoe crab blood supply. Therefore, it is highly demanded to develop alternative methods for rapid endotoxin detection using trace amounts of TAL reagents.

The current strategy is mainly focused on reducing the amount of TAL reagents in existing gel and spectroscopy methods. These methods still cannot avoid the subjective errors or require professional instruments, which greatly limits their applications in large-scale screening of environmental samples, especially in the resource-limited areas that are far away from the central laboratory. Hence, a portable quantitative detection method with high accuracy is highly demanded. The paper-based sensor is a most popular platform built on the economic and sustainable paper materials, which holds great promise for the development of a portable endotoxin detection method [15–17]. It is versatile for the detection of a variety of biomolecules (DNA, enzymes, and toxins) and contaminants (heavy metals, pesticides, and volatile organic compounds) based on different detection strategies, such as colorimetric, fluorescent, electrochemical, and SERS assay [18–20]. Among them, it is worth paying attention to the distance-based paper sensor based on the principle of viscosity change as a promising portable method with intuitive signal readout [21,22]. In fact, it is very attractive to develop a distance-based sensor that can avoid drawbacks of the existing methods for the detection of endotoxin and benefit for the environmental sample testing in real applications.

In this work, we aim to develop a simple, portable, and label-free approach for the detection of endotoxin with the reduced dosage of TAL reagents to alleviate the shortage of TAL reagents. A low-cost distance-based lateral flow (D-LAF) sensor for the quantitative detection of endotoxin is demonstrated in this study (Fig. 1). The detection principle is based on the coagulation of TAL triggered by endotoxin. The presence of endotoxin can trigger the cascade coagulation process in TAL for the gel formation, in which the water molecules are constrained in the gel matrix. The water lateral flow distance in paper device spontaneously may decrease due to the reduction of free remaining water. In this case, the water flow distance is directly correlated to the concentration of endotoxin. To the best of our knowledge, this is the first study on the detection of endotoxin using the distance-based sensor. It allows quantitative detection and significantly avoids subjective errors caused by visual interpretation in the existing gel method.

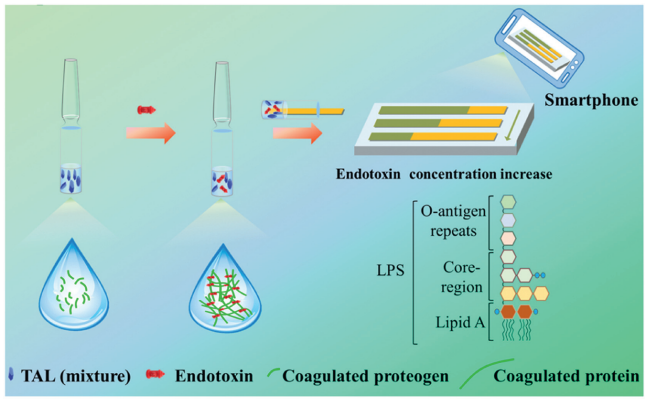

Fig. 1. Schematic illustration of the distance-based lateral flow (D-LAF) sensor towards endotoxin detection.

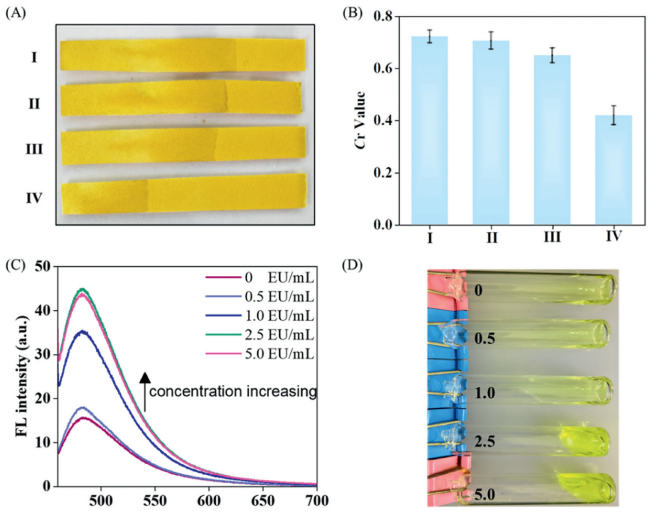

Fig. 2. Feasibility of the D-LAF sensor for endotoxin detection. (A) The photograph and (B) the Cr values of the sensor in BET water (I), endotoxin solution with 5 EU/mL (II), TAL solution (III), and the mixture of endotoxin and TAL after incubation for 1 h (IV), respectively. (C) The fluorescent intensity of thioflavin T in TAL reagents solutions with different concentrations of endotoxin (λ_{ex} = 440 nm). (D) The photograph of TAL reagents mixed with different concentrations of endotoxin.

Firstly, the feasibility of the D-LAF sensor for endotoxin detection was investigated. Fig. 2A shows the lateral flow distances of different samples including BET water, endotoxin solution (5 EU/mL), TAL solution, and the mixture of endotoxin and TAL after incubation for 1 h. The corresponding water coverage ratio (Cr) values were illustrated in Fig. 2B. The results show that TAL solution exhibits a slightly smaller Cr value than BET or endotoxin alone, which may be ascribed to its intrinsic viscosity of TAL solution. After incubation at 37 °C for 1 h, the Cr value of the mixture solution of endotoxin and TAL decreases obviously from 0.63 to 0.42. The results are consistent with the proposed mechanism, in which a series of specific agglutination reactions in TAL solution occurs in the presence of endotoxin, which makes the solution transformed to the gel state. The trap of water by the gel results in a significant decrease in the water coverage on pH test strips.

The viscosity of the TAL reagents change was then explored after co-incubation with endotoxin using a typical molecular rotor-based fluorophore thioflavin T (Fig. S4 in Supporting information), which shows stronger fluorescence intensity with the increase of viscosity [23,24]. In the solution with low viscosity, the molecule rotation between electron donor and acceptor is allowed, thioflavin T undergos twisted conformation wherein intramolecular charge-transfer rapidly occurs at excited state to effectively quench fluorescent emission. When rotation is restricted in the solution with higher viscosity, non-radiative decay is avoided, resulting in significant fluorescence emission. As illustrated in Fig. 2C, the fluorescence intensity of thioflavin T increases gradually accompanied by the endotoxin concentration increasing from 0–2.5 EU/mL and remains almost stable from 2.5–5.0 EU/mL, indicating the viscosity increase with the rise of endotoxin concentration. The TAL reagents completely agglutinated in the presence of 2.5 EU/mL endotoxin. The photograph of these solutions was shown in Fig. 2D. From the SEM images obtained by Zeiss Supra 55 (Fig. S5 in Supporting information), the appearances of the TAL reagents in the absence and presence of endotoxin are quite different, indicating the success of the endotoxin-triggered agglutination of TAL as well. All of the re-

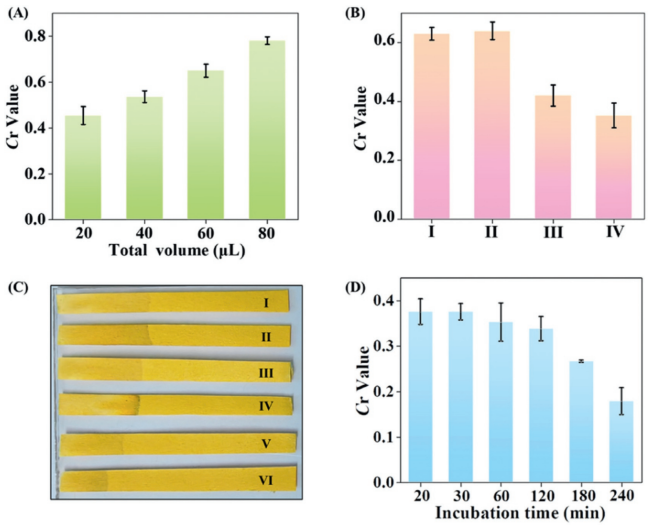

Fig. 3. Optimization of the experimental conditions of the D-LAF sensor. (A) The Cr values corresponding to different detection volumes. (B) The Cr values of the TAL solutions without (I) or with endotoxin (III) after incubation for 60 min at 37 °C, and those of the solutions incubated for additional 3 min at 4 °C (II, IV). (C) The photograph and (D) Cr values of sensor after co-incubation of the TAL reagents and endotoxin (5 EU/mL) for different time (from I to VI: 20 min, 30 min, 60 min, 120 min, 180 min, 240 min).

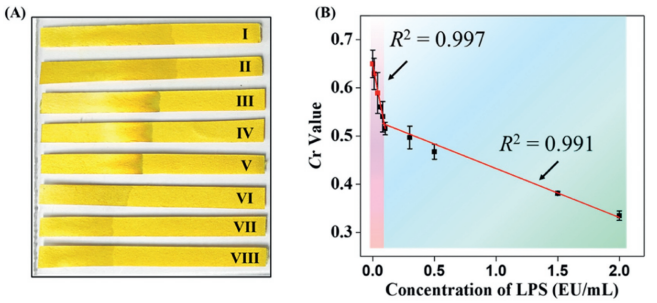

Fig. 4. The sensitivity of the D-LAF sensor. (A) The photograph of the sensor and (B) the function between the Cr value and the concentration of endotoxin (I, II, III, IV, V, VI, VII, VIII represent the responses of the sensor to endotoxin at 0, 0.01, 0.1, 0.3, 0.5, 1.5, 2.0, 5.0 EU/mL, respectively).

sults above demonstrate the feasibility of the constructed sensor for endotoxin detection.

The volumes of the final test solutions were optimized (Fig. 3A). Considering the viscosity of the TAL reagents itself, the concentration of endotoxin solution was fixed as 5 EU/mL in which the volume ratio between endotoxin and TAL was 1:1. It is obvious that the Cr value ascends with the volume increase from 20 μL to 80 μL (Fig. S6 in Supporting information). 60 μL of the final solution was selected for the following study to avoid the margin effect caused by a larger volume, giving higher detection accuracy. After incubation for 60 min at 37 °C, the Cr value decreased from 0.63 to 0.42. However, the subsequent incubation at 4 °C for additional 3 min resulted in a much more significant change of the Cr value from 0.64 to 0.35 (Fig. 3B). Thus, the incubation temperature at 4 °C was chosen as the optimal condition. The optimal reaction time was also investigated in Figs. 3C and D. The Cr value decreases with the increase of the incubation time from 0–4 h due to the agglutination reaction. It is worth noting that a prominent decrease of the Cr value can be observed just with incubation for 20 min. Even though the longer incubation time above 60 min may contribute to the further Cr value declination, the incubation time of 60 min was used in view of the time-efficient detection. In particular, the demanded dosage of TAL reagents is reduced from 100 μL to 30 μL while maintaining excellent detection performance.

The concentration-dependent response of endotoxin was evaluated for the D-LAF sensor. As shown in Fig. 4A, with the increase of endotoxin concentration from 0 to 5.0 EU/mL, the Cr value decreases gradually and remains almost invariable until the concentration of endotoxin is above 2.0 EU/mL. As discussed above, the decrease is ascribed to the capture of water *via* the coagulation of the TAL reagents. The linear relationship between Cr values and endotoxin concentrations was obtained over the range of 0–0.1 EU/mL ($R^2 = 0.997$) and 0.1–2.0 EU/mL ($R^2 = 0.991$) (Fig. 4B), respectively. The detection limit of endotoxin was calculated to be 0.0742 EU/mL ($3\sigma/k$, 7.42–14.84 pg/mL, 1 EU/mL = 0.1–0.2 ng/mL). Compared the improvement of the present work with other reported methods (Tables S1 and S2 in Supporting information), this work avoids the use of synthetic particles, screened recognition elements (*e.g.*, peptides and aptamers), and complex instrumentation. In addition, it reduces the amount of TAL reagents in each test from 100 μL to 30 μL. Therefore, it is a user-friendly and cost-effective method for the rapid and quantitative detection of endotoxin. More importantly, it possesses unique features of low-cost, and high portability to facilitate the accurate and quantitative endotoxin detection.

We chose inorganic salts ($H_2PO_4^-$, NaCl), small molecules such as amino acid (cysteine, proline), and glucose that is functional group in endotoxin. In addition, the substances with chemical structures similar to endotoxin such as POPG that is an anionic phospholipid from bacterial membrane as well as DPPC bearing two lipid chains (Fig. 5A) were also investigated. As illustrated in Figs. 5B and C, no obvious Cr value change is observed except for endotoxin indicating the D-LAF sensor is highly selective for discriminating endotoxin against potential interference substances. In addition, the anti-interference ability of the D-LAF sensor for endotoxin detection was also explored (Figs. 5D and E). In the co-existence of $H_2PO_4^-$, NaCl, cysteine, DPPC, POPG, proline, and glucose with endotoxin, respectively, the Cr values of the D-LAF sensor remain almost unchanged. Therefore, the D-LAF sensor is highly selective for the detection of endotoxin and can be a powerful candidate to overcome the matrix effect in real applications.

Encouraged by the above favorable results, we verified the reliability of D-LAF sensor for the endotoxin detection in real samples. Endotoxin contamination is common in household water due to the growth of microorganisms in water. Injectable clinical solution is also one of the main source of endotoxin infection and the absence of endotoxin in pharmaceutical manufacturing process need be verified for the medication safety. Therefore, tap water and clinical-grade NaCl intravenous injection were used to demonstrate the practicality of the sensor. The initial concentration of endotoxin in samples was firstly detected and then series of known amounts endotoxin were spiked into the samples. The photographs of the D-LAF sensor were shown in Figs. S7 and S8 (Supporting information), and the quantitative results were summarized in Table 1. No endotoxin was detected in the blank tap water and injection samples, indicating the safety of the tested samples. Endotoxin levels varied significantly in different water area. In addition, according to the requirement of the 2020 edition of the Pharmacopoeia of the People's Republic of China, the bacterial endotoxin of large-capacity (100 mL and above) water for injection is ≤0.50 EU/mL. As shown in Table 1, the satisfactory recoveries of endotoxin in tap water and NaCl injection solution range from 102.3% to 107.1% and 92.3% to 100.8%, respectively. These results are comparable to those of the ELISA kit, proving the high detection accuracy of the D-LAF sensor.

In summary, this work features the high portability, rapid quantification, and cost-effective distance-based lateral flow sensor for endotoxin detection. The mechanism is based on the viscosity vari-

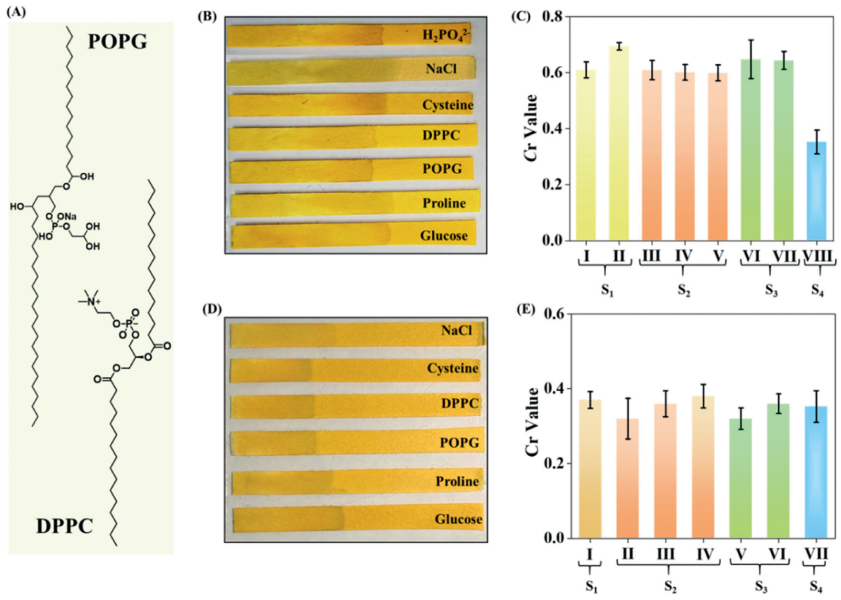

Fig. 5. The selectivity of the D-LAF sensor. (A) The chemical structures of POPG and DPPC. The photograph and corresponding Cr values (B, C) of the sensor in the presence of S_1 ($H_2PO_4^-$, NaCl), S_2 (cysteine, proline, glucose), S_3 (DPPC, POPG), and S_4 (endotoxin), respectively. The photograph and corresponding Cr values (D, E) of the sensor in the co-existence conditions of endotoxin with S_1 (NaCl), S_2 (cysteine, proline, glucose), S_3 (DPPC, POPG), and S_4 (endotoxin only), respectively.

Table 1
Endotoxin detection in tap water and NaCl injection solution by the D-LAF sensor and the ELISA kit.

Samples	Added (EU/mL)	D-LAF sensor Found (EU/mL)	Recovery (%)	RSD (%)	ELISA Kit Found (EU/mL)	Recovery (%)	RSD (%)
Tap water	0.00	0.09	–	1.04	n.d.	–	2.02
	0.60	0.56	107.1	2.07	0.62	102.1	13.2
	1.20	1.17	102.3	3.39	1.27	105.8	12.5
	1.80	1.71	104.6	8.72	1.72	95.9	6.67
NaCl	0.00	0.00	–	4.70	n.d.	–	4.16
injection	0.60	0.65	92.3	5.50	0.58	97.3	9.52
solution	1.00	0.99	100.4	4.13	1.12	112.0	16.2
	1.80	1.78	100.8	8.47	2.17	120.9	13.0

n.d. = not determined.

ation of the TAL reagents triggered by its agglutination in the presence of endotoxin. It is worthy to note that the D-LAF sensor can reduce a large usage amount of TAL reagents comparing to the current gold standard method, which is an effective alternative approach to alleviate the TAL shortage. This method has also been successfully applied for endotoxin detection in tap water and clinical injection solution, and exhibits the comparable accuracy and stability to the ELISA kit. Further directions will be focused on technical improvement to explore the feasibility of commercial production and the development of novel D-LAF sensor to expand its applications with the use of various stimuli-responsive polymers as well as the integration with different functional materials and biochemical techniques for the detection of other contaminants.

Declaration of competing interest

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

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.cclet.2024.109718.

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