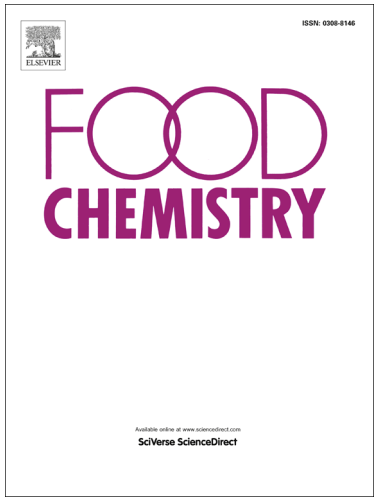

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PII: S0308-8146(18)31637-6
DOI: https://doi.org/10.1016/j.foodchem.2018.09.066
Reference: FOCH 23553

To appear in: *Food Chemistry*

Received Date: 3 January 2018
Revised Date: 8 August 2018
Accepted Date: 10 September 2018

Please cite this article as: Bu, T., Huang, Q., Yan, L., Zhang, W., Dou, L., Huang, L., Yang, Q., Zhao, B., Yang, B., Li, T., Wang, J., Zhang, D., Applicability of biological dye tracer in strip biosensor for ultrasensitive detection of pathogenic bacteria, *Food Chemistry* (2018), doi: https://doi.org/10.1016/j.foodchem.2018.09.066

Applicability of biological dye tracer in strip biosensor for ultrasensitive detection of pathogenic bacteria

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ABSTRACT

Here, a facile, label-free and sensitive lateral flow strip (LFS) biosensor for foodborne pathogens was established relying on the innovative introduction of Gram staining and the direct immunoreaction. Target bacteria can be directly marked with crystal violet (CV) by one-step staining which is superior to traditional signal marking techniques in LFS assay, and the method's selectivity can be guaranteed by high-specificity monoclonal antibody. With *Salmonella* Enteritidis (*S.* Enteritidis) as a model target, this protocol can selectively detect 80 CFU mL^{-1} *S.* Enteritidis within 11 min in the optimized conditions. Moreover, with *Listeria monocytogenes* as another model target, the biosensor shows a high universality for detections of both gram-negative and gram-positive bacteria. The unexpected applicability of biological dye tracer in strip biosensor reveals that the biological dye can be a potential tool serving as a universal signal tracer for pathogenic microorganisms in food safety monitoring and early clinical diagnosis.

Keywords: Label-free; Lateral flow strip biosensor; Crystal violet; Ultrasensitive detection; *Salmonella* Enteritidis

1. Introduction

Foodborne diseases, pose a great threat to public health worldwide. According to the U. S. Food and Drug Administration (Cho & Ku, 2017), there are about 48 million reported cases of foodborne diseases in the United States annually. Pathogenic bacteria are the main reason for the wide spread of these infectious diseases (Singh, Arutyunov, Szymanski, & Evoy, 2012). Bacteria at low concentrations are hard to detect; however, the low infectious dose of bacterial pathogens in water, food, and other biological samples not only pose a widespread threat to human health but also cause serious damage (Fauci & Morens, 2012). Moreover, it is well-known that the majority of bacteria can double their population in 30 min (Zhu et al., 2015). Therefore, the early identification of bacterial infections via point-of-care testing (POCT) is critical to the safety of food industry and clinical diagnosis (Wen et al., 2017). Up to now, a variety of methods, including conventional microbiological culture and colony counting (Wang et al., 2015), enzyme-linked immunosorbent assay (ELISA) (Wang et al., 2015), polymerase chain reaction (PCR) (Martin, Garriga, & Aymerich, 2012), electrochemical biosensor (Delibato et al., 2009) and LFS (Bu et al., 2017), have been well developed and applied to the detection of foodborne pathogens. Among them, the LFS has been widely noted due to its outstanding advantages such as fabrication, convenient operation, effective cost, disposable feature, short assay time and facile analytical procedure (Wang, Wang, Zhang, Su, & Luo, 2016), and become a well-established diagnostic tool for various POC testing and rapid detection.

Lateral flow strip biosensor is a classical on-site analytical device that can achieve

visual detection of foodborne pathogens based on antigen-antibody recognition and colored reaction (Yu, Li, Ding, & Zhang, 2017). Thus, antibodies and signal-marking materials are the key for reliable and sensitive target recognition in LFS assays. In general, the colorimetric signal is generated from aggregation of colored nanoparticles, such as gold nanoparticles (GNPs) (Lisa, Chouhan, Vinayaka, Manonmani, & Thakur, 2009; Liu, Tsao, Wang, & Yu, 2008), quantum dots (Medina-Sanchez, Miserere, Morales-Narvaez, & Merkoci, 2014; Zou et al., 2010), magnetic nanoparticles (Peterson, Chen, Cunningham, & Andrade, 2015; Zhang, Wang, Yang, Du, & Lin, 2013), carbon nanotubes (Qiu et al., 2015), graphene oxide (Lee, Joung, Kim, & Park, 2012; Yu, Li, Ding, & Zhang, 2017), and multifunctional nanocomposites including Pt-Au (Jiang et al., 2016), gold-silica nanorods (Xu et al., 2014) etc. Colored nanoparticles are widely accepted in LFS assays because they exhibit intrinsic colors and can be functionalized with antibodies. However, colored nanomaterials-based LFS biosensors are often restricted by either strict synthesis process, chemical instability or complicated cross-linking with antibodies of nanomaterials. (Zeng, Zhai, Xie, & Liu, 2017). On the other hand, the sandwich immunoreaction is frequently employed in detecting bacteria, in which two paired antibodies (Abs) are needed to recognize different antigen-binding sites and form a sandwich structure (Ngom, Guo, Wang, & Bi, 2016). In fact, the pairing of Abs is another obstacle due to the different properties and binding site of Abs, while not every antibody could be labeled or paired with another antibody through corresponding antigen (Zeng et al., 2016). Consequently, high-specificity direct immunoreactions seem to be promising

alternative for bacteria determination.

To address these issues, we firstly introduce the classic Gram staining technique and the direct immunoreaction to LFS assay for ultra-sensitive detection of microorganisms, in which bacteria would be stained by the biological dye (Chakraborty, Chowdhury, & Das Saha, 2011) and then captured specifically by the anti-bacteria monoclonal antibody (McAb). The combination of Gram staining and direct immunoassay not only got rid of a series of troublesome marking steps providing an unexpected alternative to traditional probes, but also avoid the use of paired McAb obtained by time-consuming screening process. To demonstrate the feasibility of the biosensor, a proof-of-concept assay for *S.* Enteritidis detection in drinking water, tomato and pork samples were performed with a low limit of detection (LOD) and good selectivity. To investigate the universal applicability of the biosensor in the pathogen bacteria detection, *Listeria monocytogenes* (*L. monocytogenes*), a gram-positive bacterial strain, was selected as another model target. The contribution pushed forward the progress of pathogenic bacteria's POC diagnosis or on-site monitoring by LFS biosensors, demonstrated the significant potential of biological dye-based biosensors in other microorganisms' detection.

2. Materials and methods

2.1 Materials and Instruments

Reagents. McAbs (2B4 and 6G9) against *S.* Enteritidis flagellin and *L. monocytogenes*, respectively, were prepared in our laboratory as previously reported (Zhang et al., 2011), after characterization by ELISA, the sensitivities of McAbs for *S.*

Enteritidis and *L. monocytogenes* detections were 10^3 and 10^6 CFU mL^{-1}, respectively (Fig. S1). And there were non-cross reactions of the McAbs with other interfering bacteria (Fig. S2). Glass fiber, sample pad, and absorbent pad were purchased from Shanghai Kingdiag-biotech CO., Ltd. (Shanghai, China). NC membrane was obtained from Millipore (Billerica, MA). Bovine serum albumin (BSA) and glycerol were ordered from Sigma-Aldrich (St. Louis, MO, USA). Luria-Bertani (LB) medium was bought from Beijing Land Bridge Technology Co. (Beijing, China). Crystal violet (CV), Rhodamine B, Comassie brilliant blue G-250, Erioglaucine disodium salt, Malachite green, Methyl orange, Methylene blue, Safranin O were purchased from Aladdin (Shanghai, China). All solvents and other chemicals used in this work were of analytical-reagent grade or better.

Equipments. Guillotine cutter (HGS-201) and Dispensing Platform (HGS510-2D) were purchased from Hangzhou Autokun Technology Co., Ltd. (Hangzhou, China). The high speed refrigerated centrifuge (HC-3018R) was obtained from Anhui USTC Zonkia Scientific Instruments Co., Ltd. (Anhui, China).

Bacterial Strains. S. Enteritidis and non-*S.* Enteritidis strains were kindly provided by professor Baowei Yang's research group in our college. The strains were transferred into LB liquid medium and cultured at 37 ℃ for 18–24 h (Ben Aissa et al., 2017). Then, the bacteria stock solution was diluted to approximately 10^8 CFU mL^{-1} as the testing concentration.

2.2 Preparation of the Novel Lateral Flow Strip

The principle of the CV-LFS was on account of a direct immunoassay. Briefly, the

strip included four parts of sample pad, conjugate pad, NC membrane and absorbent pad as usual which were all placed on a plastic scaleboard with about 2 mm overlapping. Then the assembled strip plate was cut into strips with 3 mm width and stored in a desiccator. In theory, it was unnecessary to retain the conjugate pad in the CV-LFS because no probe was dispensed on it as in traditional strips, but experiment results proved that it was likely to produced false-positive phenomenons without that pad. So, both the sample and conjugate pads had a contribution to the elimination of nonspecific adsorption. The difference was that only test (T) line was employed in test zone with capture McAb dispensed on, since the novel tracer could not bind with anti-Ab on the control line as the traditional strip tests (Fig. 1).

2.3 Assay Procedure

The novel LFS assay was processed by the following steps: Crystal violet (1 mg) was dissolved with deionized water (100 mL) to obtain the CV storage solution. Then, 90 μL of CV solution and 100 μL of bacteria (0 to 10^8 CFU mL^{-1}) were mixed thoroughly, and further incubated for about 1 min in a microtube. Afterward, 190 μL of the mixture solution was dipped onto the test strip to start the chromatography assay. Finally, the results could be observed by naked eyes after 10 min.

2.4 *Detection of S. Enteritidis in water, tomato and pork samples*

Drinking water, tomato, and pork were employed as sample matrices for the detection of pathogens in food. 5.0 g pork sliced into 5–7 mm cubes and crushed tomato samples were separately immersed in phosphate buffered solution (PBS) with a proportion of 1: 10 (w: v) under 4 ℃ for 12 h. Then, food matrices were clarified by

centrifuging at 5,000 rpm for 5 min, the supernatants were collected as sample solutions (Shukla, Leem, Lee, & Kim, 2014; Oh, et al., 2017). These sample solutions and drinking water were sterilized by high-temperature sterilization and filtrated through a 0.22 μm-membrane filter and artificially contaminated with *Salmonella* to obtain different final bacteria concentration of 0–10^8 CFU mL^{-1} (Bayrac, Eyidogan, & Oktem, 2017). The experiments were conducted in triplicate.

3. Results and discussion

3.1. Introduction of the principle and advantages of the CV- LFS Biosensor

Fig. 1 schematically illustrated the principle of the proposed LFS biosensor. By Gram staining, the bacteria were dyed with crystal violet. Then, the solution of the stained bacteria was dropped onto the sample pad, followed by the migration of bacteria cells to conjugate pad owing to the capillary action. When the sample solution reached the NC membrane, *S.* Enteritidis were combined with the pre-coated McAb because of the specific antibody-antigen interaction, resulting in the accumulation of stained bacteria on the zone. If there were target bacteria in the sample, a bluish-violet band would appear at the completion of the assay. On the other hand, no color would be observed on the T-line if no *S.* Enteritidis existed in the sample (Fig. 1). Moreover, we referred a great deal of related reports for correlation studies including *Salmonella* detections by immunoassays, PCR, ELISA and other methods. Two most important parameters of detection time and sensitivity (expressed as LOD) for *Salmonella* detection by these methods were compared to demonstrate the advantages of our method. Related reference data are listed in Table S1, which has

been added in the supplementary materials for meaningful interpretation. From Table S1, we can see that the assay time of our CV-LFS method is remarkably shorter than that of all other reports and the assay sensitivity is also obviously higher than that of most of other reports.

By this strategy, stained *S.* Enteritidis would be effectively immunocaptured by the McAb coated on T-line and no matched McAb was needed, which broke the conventional sandwich immunoassay format for bacterium analysis that was obliged to adopt detection and capture antibodies simultaneously. Compared to traditional sandwich immuno-strip biosensors, the CV-LFS biosensor system had six advantages at least: (1) the analysis procedure was simplified by the adoption of a direct immunoassay; (2) the analysis expense was reduced by the nonuse of another paired McAb and the second antibody (anti-mouse antibody); (3) the strip preparation was simplified by the omitting of pre-immobilization of signal probe and anti-mouse antibody on conjugate pad and C-line, respectively; (4) the McAb screening workload was well alleviated, because we just needed to screen out a satisfying McAb; (5) there was no requirement for early preparations of marking materials and signal probes; (6) the assay sensitivity was greatly improved compared to conventional sandwich immuno-strip assay for *S.* Enteritidis detection.

< Figure 1 >

3.2. Optimization of Experimental Parameters

Interference Investigation of Other Dyes to S. Enteritidis Staining. For the label-free and dye-based approach, a series of dyes including biological stains such as

crystal violet, rhodamine B, comassie brilliant blue G-250, malachite green, methylene blue and safranin O, and industrial dyes including erioglaucine disodium salt and methyl orange were evaluated to investigate the interference to *S.* Enteritidis staining. As shown in Fig. 2A, surprisingly, except for the strip with crystal violet, no color bands appeared on the T-line of strips with other dyes, revealing that the bacteria could not be stained by other dyes because the dyes could not accumulate on the T-line in company with bacteria due to the capture of coated antibody. Furthermore, compared to other dyes, the crystal violet did not generate background color interference on NC membrane, which was conducive to the observation of naked eyes. Therefore, other staining solutions had no interference to *S.* Enteritidis staining.

Dyeing Method and Conditions. To perfect the coloration procedure, the dyeing method and conditions were optimized by setting different concentrations and volumes of dyes, as well as incubation times of dyeing. The application concentration of CV to bacteria solution had prominent effect on the coloration efficiency. In this case, different CV concentrations of 0.005%, 0.001%, 0.0005% and 0.0001% (w/v) were studied at a fixed bacteria concentration of 10^8 CFU mL^{-1}. As seen from Fig. 2B, with the increase of CV concentration, the dyeing efficiency was enhanced and the color intensity of T-line was strengthened gradually. Nevertheless, when the CV concentration was up to 0.005%, an unsatisfactory shadow color would come along caused by the superfluous dye molecules. As a consequence, the optimum concentration of CV was set at 0.001%.

The volume of mixture of CV and bacteria solution played an important role in the

detection capability of the novel strip platform, which would affect the assay cost and sensitivity. In this step, different dye volumes from 10 to 120 μL mixed with 100 μL of 10^5 CFU mL^{-1} bacteria solution were incubated and tested, respectively. As shown in Fig. 2C, with the increase of CV volume from 110 to 190 μL, the color intensity on T-line deepened gradually, and thereafter kept at an almost constant level. Moreover, when the CV volume was higher than 190 μL, the result observation would be interfered by the bluish-violet CV remained on NC membrane. Thereby, 190 μL was considered to be the optimum CV volume.

The signal intensity on the T-line may also be affected by the duration of incubation. The mixture of CV and bacteria was allowed to incubate for different periods of time ranging from 10 to 180 s. As shown in Fig. 2D, the color intensity on T-line of incubation 10 s was visibly inferior to others incubated for 60–180 s which were almost identical in color intensities, revealed that the bacteria could be stained thoroughly after incubation with CV for at most 60 s (1 min). Therefore, in the subsequent experiment, the mixture of CV and bacteria was incubated for 1 min to shorten the assay time.

Selection of the Immunoreaction Time. The immunoreaction time was investigated for the influence on the T-line intensity. The results were recorded every 5 minutes within 15 minutes after the stained bacteria were added to the sample pad. As depicted in Fig. 2E, the T-line intensity was significantly enhanced with the extension of reaction time from 5 min to 10 min. Then, when the time was prolonged to 15 min, the T-line became brighter slightly, but at the same time, the background color on NC

membrane was obvious deeper than that of 10 min. Therefore, the reaction time was set at 10 min to save assay time, minimize background color and increase readability of the visualization results.

< Figure 2 >

3.3. Analytical Performance

Under optimal assay conditions, the sensitivity of the proposed platform was determined with *S.* Enteritidis at concentrations of 20–10^8 CFU mL^{-1} in 10 mM PBS (pH 7.4), with PBS as negative control. As shown in Fig. 3, on the test strips, color bands could be observed clearly and with the reduction of *S.* Enteritidis, the intensities of T-line decreased gradually and obviously. The naked-eye detectable bluish-violet band on T-line could be observed down to 80 CFU mL^{-1}. Therefore, the sensitivity of the CV-LFS for *S.* Enteritidis detection was 80 CFU mL^{-1} (inset image in Fig. 3). The corresponding optical densities were further confirmed by recording the optical density values of the bands on the test zone with Image J (Fig. 3). The resulting calibration curve showed that the gray value was proportional to the amount of *S.* Enteritidis in the range from 0 to 10^8 CFU mL^{-1} and good linear relationship was achieved in the range from 10^3 to 10^8 CFU mL^{-1} with a correlation coefficient of 0.9543 (Fig. 3). In addition, analytical sensitivities of a great deal of related reports for correlation studies including pathogen detections by LFSs were compared. As shown in Table 1, compared with other signal based LFSs for bacteria, the proposed staining biosensor had a much lower visual detection limit (80 CFU mL^{-1}) and a broader detection range. Furthermore, since *Salmonella* is a kind of gram-negative

bacteria, to investigate the universal applicability of the strategy to other bacteria, *L. monocytogenes*, a kind of gram-positive bacteria, was selected as another model target and detected using the proposed CV-LFS. The related results (Fig. S3) demonstrated that the developed CV-LFS could also be used for *L. monocytogenes* detection with a high sensitivity (10^4 CFU mL^{-1}) and selectivity.

< Figure 3 >

< Table 1 >

In order to evaluate the specificity of the biosensor, tests were carried out at the same concentration of 10^8 CFU mL^{-1} with four *Salmonella* strains including *Salmonella* London (*S.* London), *Salmonella* Typhimurium (*S.* Typhimurium), *Salmonella* Paratyphi B (*S.* Paratyphi B) and *Salmonella* Hadar (*S.* Hadar), and five non-*Salmonella* strains of *Staphylococcus aureus* (*S. aureus*), *Escherichia coli* (*E. coli*), *Candida albicans* (*C. albicans*), *Listeria monocytogenes* (*L. monocytogenes*) and *Campylobacter coli* (*C. coli*) as the interference pathogens, respectively, with PBS as negative control. As expected, except for *S.* Enteritidis, no color on T-line was observed for other bacteria (Fig. 4). The excellent results should be attributed to the *Salmonella* flagellin which tended to elicit specific antibodies in the animals (Fedirko et al., 2017). Such results indicated that this biosensor just responded to *S.* Enteritidis and thus, possessed remarkable selectivity.

< Figure 4 >

3.4. Application in Food Samples

With respect to the applicability of this strategy in the detection of real samples,

drinking water, tomato and pork samples that had been confirmed to be *S.* Enteritidis free beforehand were mixed with *S.* Enteritidis (0 to 10^8 CFU mL^{-1}). Clear and steady changes in the bluish-violet-colored area of the sensing platform were observed in relation to bacterial concentrations for the contaminated samples. The developed biosensor displayed the ability to detect *S.* Enteritidis contamination in different food matrices, with a lower detection limit of 80 CFU mL^{-1} in drinking water (Fig. 5A), which was almost identical with that of standard tests in PBS. And it was clear that the LODs for *S.* Enteritidis in tomato (B) and pork (C), were 10^2 and 10^3 CFU mL^{-1} respectively without incubation. By this technology, food matrices can be easily monitored on-site for the contamination of pathogenic *S.* Enteritidis.

< Figure 5 >

4. Conclusions

In the present study, a biological dye tracer based strip biosensor was developed for ultrasensitive, simple and cost-effective detection of *S.* Enteritidis. The proposed method was label-free with the crystal violet as a sensitive signal molecule, and was remarkably simple with a direct analysis model instead of conventional sandwich format. Under optimal conditions, the visual detection limit of 80 and 10^4 CFU mL^{-1} was achieved within 11 min (including incubation time), which was considerably lower than those of most conventional LFS biosensors. Besides, the biosensor could be well applied in the analysis of bacterial contamination in water, tomato and pork samples even under a lower pollution level, illuminating the evident feasibility. What' s more, by using *L. monocytogenes* as another model target, results demonstrated that

the developed CV-LFS possessed an universal applicability for detections of both gram-negative and gram-positive bacteria. The unexpected applicability of crystal violet in the immuno-strip biosensor brought excellent assay performance and outstanding advantages, and opens a way of using microbial staining technique to offer powerful label-free signals and designing simple and efficient biosensors thereon. Furthermore, this simple, sensitive, low-cost and small work-load optical biosensor will have a promising prospect in POC test of other foodborne pathogens and pathogenic microorganisms.

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Conflict of interest

The authors declare no competing financial interest.

Acknowledgments

We appreciate the kind support from the National Natural Science Foundation of China (No. 21675127), the New Century Excellent Talents in University (NCET-13-0483), and the Shaanxi Provincial Science Fund for Distinguished Young Scholars (2018JC-011).

References

Bayrac, C., Eyidogan, F., & Oktem, H. A. (2017). DNA aptamer-based colorimetric detection platform for *Salmonella* Enteritidis. *Biosens Bioelectron, 98*, 22-28.

Ben Aissa, A., Jara, J. J., Sebastian, R. M., Vallribera, A., Campoy, S., & Pividori, M.

I. (2017). Comparing nucleic acid lateral flow and electrochemical genosensing for the simultaneous detection of foodborne pathogens. *Biosens Bioelectron, 88*, 265-272.

Bu, T., Huang, Q., Yan, L., Huang, L., Zhang, M., Yang, Q., Yang, B., Wang, J., & Zhang, D. (2018). Ultra technically-simple and sensitive detection for *Salmonella* Enteritidis by immunochromatographic assay based on gold growth. *Food Control, 84*, 536-543

Chakraborty, S., Chowdhury, S., & Das Saha, P. (2011). Adsorption of Crystal Violet from aqueous solution onto NaOH-modified rice husk. *Carbohydrate Polymers, 86*(4), 1533-1541.

Cho, I. H., & Ku, S. (2017). Current technical approaches for the early detection of foodborne pathogens: challenges and opportunities. *International Journal of Molecular Sciences, 18*(10), 2078.

Delibato, E., Volpe, G., Romanazzo, D., De Medici, D., Toti, L., Moscone, D., & Palleschi, G. (2009). Development and application of an electrochemical plate coupled with immunomagnetic beads (ELIME) array for *Salmonella* enterica detection in meat samples. *Journal of Agricultural and Food Chemistry, 57*(16), 7200-7204.

Eltzov, E., & Marks, R. S. (2017). Colorimetric stack pad immunoassay for bacterial identification. *Biosens Bioelectron, 87*, 572-578.

Fauci, A. S., & Morens, D. M. (2012). The perpetual challenge of infectious diseases REPLY. *New England Journal of Medicine, 367*(1), 90-90.

Fedirko, V., Hao Quang, T., Gewirtz, A. T., Stepien, M., Trichopoulou, A., Aleksandrova, K., Olsen, A., Tjonneland, A., Overvad, K., Carbonnel, F., Boutron-Ruault, M.-C., Severi, G., Kuhn, T., Kaaks, R., Boeing, H., Bamia, C., Lagiou, P., Grioni, S., Panico, S., Palli, D., Tumino, R., Naccarati, A., Peeters, P. H., Bueno-de-Mesquita, H. B., Weiderpass, E., Castano, J. M. H., Barricarte, A., Sanchez, M.-J., Dorronsoro, M., Quiros, J. R., Agudo, A., Sjoberg, K., Ohlsson, B., Hemmingsson, O., Werner, M., Bradbury, K. E., Khaw, K.-T., Wareham, N., Tsilidis, K. K., Aune, D., Scalbert, A., Romieu, I., Riboli, E., & Jenab, M. (2017). Exposure to bacterial products lipopolysaccharide and flagellin and hepatocellular carcinoma: a nested case-control study. *Bmc Medicine*, *15*.

Jiang, T., Song, Y., Wei, T., Li, H., Du, D., Zhu, M.-J., & Lin, Y. (2016). Sensitive detection of *Escherichia coli* O157:H7 using Pt-Au bimetal nanoparticles with peroxidase-like amplification. *Biosens Bioelectron*, *77*, 687-694.

Lee, J. S., Joung, H.-A., Kim, M.-G., & Park, C. B. (2012). Graphene-based chemiluminescence resonance energy transfer for homogeneous immunoassay. *Acs Nano*, *6*(4), 2978-2983.

Leem, H., Shukla, S., Song, X., Heu, S., & Kim, M. (2014). An efficient liposome-based immunochromatographic strip assay for the sensitive detection of *Salmonella* Typhimurium in pure culture. *Journal of Food Safety*, *34*(3), 239-248.

Lisa, M., Chouhan, R. S., Vinayaka, A. C., Manonmani, H. K., & Thakur, M. S.

(2009). Gold nanoparticles based dipstick immunoassay for the rapid detection of dichlorodiphenyltrichloroethane: An organochlorine pesticide. *Biosens Bioelectron, 25*(1), 224-227.

Liu, B.-H., Tsao, Z.-J., Wang, J.-J., & Yu, F.-Y. (2008). Development of a monoclonal antibody against ochratoxin A and its application in enzyme-linked immunosorbent assay and gold nanoparticle immunochromatographic strip. *Anal Chem, 80*(18), 7029-7035.

Martin, B., Garriga, M., & Aymerich, T. (2012). Pre-PCR treatments as a key factor on the probability of detection of *Listeria monocytogenes* and *Salmonella* in ready to-eat meat products by real-time PCR. *Food Control, 27*(1), 163-169.

Medina-Sanchez, M., Miserere, S., Morales-Narvaez, E., & Merkoci, A. (2014). On-chip magneto-immunoassay for Alzheimer's biomarker electrochemical detection by using quantum dots as labels. *Biosens Bioelectron, 54*, 279-284.

Ngom, B., Guo, Y., Wang, X., & Bi, D. (2016). Development and application of lateral flow test strip technology for detection of infectious agents and chemical contaminants: a review (vol 397, pg 1113, 2010). *Analytical and bioanalytical chemistry, 408*(14), 3923-3923.

Oh, S. Y., Heo, N. Su., Shukla S., Cho H. J., Ezhil Vilian A. T., Kim J., Lee S. Y., Han Y.K., Yoo S. M., & Huh, Y. S. (2017). Development of gold nanoparticle-aptamer-based LSPR sensing chips for the rapid detection of *Salmonella* typhimurium in pork meat. *Scientific Reports, 7* (1) :10130.

Peterson, R. D., Chen, W., Cunningham, B. T., & Andrade, J. E. (2015). Enhanced

sandwich immunoassay using antibody-functionalized magnetic iron-oxide nanoparticles for extraction and detection of soluble transferrin receptor on a photonic crystal biosensor. *Biosens Bioelectron, 74*, 815-822.

Qiu, W., Xu, H., Takalkar, S., Gurung, A. S., Liu, B., Zheng, Y., Guo, Z., Baloda, M., Baryeh, K., & Liu, G. (2015). Carbon nanotube-based lateral flow biosensor for sensitive and rapid detection of DNA sequence. *Biosens Bioelectron, 64*, 367-372.

Shukla, S., Leem, H., Lee, J.-S., & Kim, M. (2014). Immunochromatographic strip assay for the rapid and sensitive detection of *Salmonella* Typhimurium in artificially contaminated tomato samples. *Canadian Journal of Microbiology, 60*(6), 399-406.

Singh, A., Arutyunov, D., Szymanski, C. M., & Evoy, S. (2012). Bacteriophage based probes for pathogen detection. *Analyst, 137*(15), 3405-3421.

Wang, H., Zhou, Y., Jiang, X., Sun, B., Zhu, Y., Wang, H., Su, Y., & He, Y. (2015). Simultaneous capture, detection, and inactivation of bacteria as enabled by a surface-enhanced raman scattering multifunctional chip. *Angewandte Chemie-International Edition, 54*(17), 5132-5136.

Wang, W., Liu, L., Song, S., Tang, L., Kuang, H., & Xu, C. (2015). A highly sensitive ELISA and immunochromatographic strip for the detection of *Salmonella typhimurium* in milk samples. *Sensors, 15*(3), 5281-5292.

Wang, P., Wang, R., Zhang, W., Su, X., & Luo, H. (2016). Novel fabrication of immunochromatographic assay based on up conversion phosphors for

sensitive detection of clenbuterol. *Biosens Bioelectron, 77*, 866-870.

Wen, C.-Y., Jiang, Y.-Z., Li, X.-Y., Tang, M., Wu, L.-L., Hu, J., Pang, D.-W., & Zeng, J.-B. (2017). Efficient enrichment and analyses of bacteria at ultralow concentration with quick-response magnetic nanospheres. *Acs Applied Materials & Interfaces, 9*(11), 9416-9425.

Xia, S., Yu, Z., Liu, D., Xu, C., & Lai, W. (2016). Developing a novel immunochromatographic test strip with gold magnetic bifunctional nanobeads (GMBN) for efficient detection of *Salmonella choleraesuis* in milk. *Food Control, 59*, 507-512.

Xu, H., Chen, J., Birrenkott, J., Zhao, J. X., Takalkar, S., Baryeh, K., & Liu, G. (2014). Gold-nanoparticle-decorated silica nanorods for sensitive visual detection of proteins. *Anal Chem, 86*(15), 7351-7359.

Yu, L., Li, P., Ding, X., & Zhang, Q. (2017). Graphene oxide and carboxylated graphene oxide: Viable two-dimensional nanolabels for lateral flow immunoassays. *Talanta, 165*, 167-175.

Zeng, H., Guo, W., Liang, B., Li, J., Zhai, X., Song, C., Zhao, W., Fan, E., and Liu, Q. (2016). Self-paired monoclonal antibody lateral flow immunoassay strip for rapid detection of Acidovorax avenaesubsp. *citrulli*. *Analytical and Bioanalytical Chemistry, 408*, 6071-6078.

Zeng, H., Zhai, X., Xie, M., & Liu, Q. (2017). Fluorescein isothiocyanate labeling antigen-based immunoassay strip for rapid detection of acidovorax citrulli. *Plant Disease*.

Zhang, D., Li, P., Yang, Y., Zhang, Q., Zhang, W., Xiao, Z., & Ding, X. (2011). A high selective immunochromatographic assay for rapid detection of aflatoxin B-1. *Talanta, 85*(1), 736-742.

Zhang, L., Huang, Y., Wang, J., Rong, Y., Lai, W., Zhang, J., & Chen, T. (2015). Hierarchical flowerlike gold nanoparticles labeled immunochromatography test strip for highly sensitive detection of *Escherichia coli* O157:H7. *Langmuir, 31*(19), 5537-5544.

Zhang, X., Wang, H., Yang, C., Du, D., & Lin, Y. (2013). Preparation, characterization of Fe_3O_4 at TiO_2 magnetic nanoparticles and their application for immunoassay of biomarker of exposure to organophosphorus pesticides. *Biosens Bioelectron, 41*, 669-674.

Zhang, X., Zhou, J., Zhang, C., Zhang, D., & Su, X. (2016). Rapid detection of *Enterobacter cloacae* by immunomagnetic separation and a colloidal gold-based immunochromatographic assay. *RSC Advances, 6*(2), 1279-1287.

Zhu, M., Liu, W., Liu, H., Liao, Y., Wei, J., Zhou, X., & Xing, D. (2015). Construction of Fe_3O_4/Vancomycin/PEG magnetic nanocarrier for highly efficient pathogen enrichment and gene sensing. *Acs Applied Materials & Interfaces, 7*(23), 12873-12881.

Zou, Z., Du, D., Wang, J., Smith, J. N., Timchalk, C., Li, Y., & Lin, Y. (2010). Quantum dot-based immunochromatographic fluorescent biosensor for biomonitoring trichloropyridinol, a biomarker of exposure to chlorpyrifos. *Anal Chem, 82*(12), 5125-5133.

Applicability of biological dye tracer in strip biosensor for ultrasensitive detection of pathogenic bacteria

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Figure captions

Fig. 1. Schematic principle of the CV-based LFS biosensor for *S.* Enteritidis detection.

Fig. 2. Optimization results of different experimental conditions. (A) Interference investigation of other dyes, under bacterial concentration of 10^8 CFU mL^{-1}. Nos. 1 to 8 stand for crystal violet, Methylene blue, Rhodamine B, Comassie brilliant blue G-250, Malachite green, Erioglaucine disodium salt, Safranin O and Methyl orange in turn. (B), (C), (D) and (E) are optimization results separately for the optimum CV concentration (%), the volume of mixture of CV and bacteria solution (μL), dyeing time (s) and immunoreaction time (min). *S.* Enteritidis of 10^5 CFU mL^{-1} were used in the strip tests of both groups (C) and (D). In (B) and (E), the bacteria concentrations used in left and right strips of each group were 10^8 and 0 CFU mL^{-1}, respectively. "T" stands for "test line".

Fig. 3. Calibration curve of *S.* Enteritidis detection with CV-based LFS biosensor (the inset image: the results for sensitivity test). "T" stands for "test line".

Fig. 4. Specificity results to different interfering bacteria strains at a concentration of 10^8 CFU mL^{-1}. Nos. 1–11 correspond to *S.* Enteritidis, *S.* London, *S.* Typhimurium, *S.* Paratyphi B, *S.* Hadar, *S. aureus*, *E. coli*, *C. albicans*, *L. monocytogenes*, *C. coli* and control group, separately. "T" stands for "test line".

Fig. 5. Detection of *S.* Enteritidis in (A) water, (B) tomato and (C) pork samples by the CV-based LFS biosensor. "T" stands for "test line".

Fig. 1

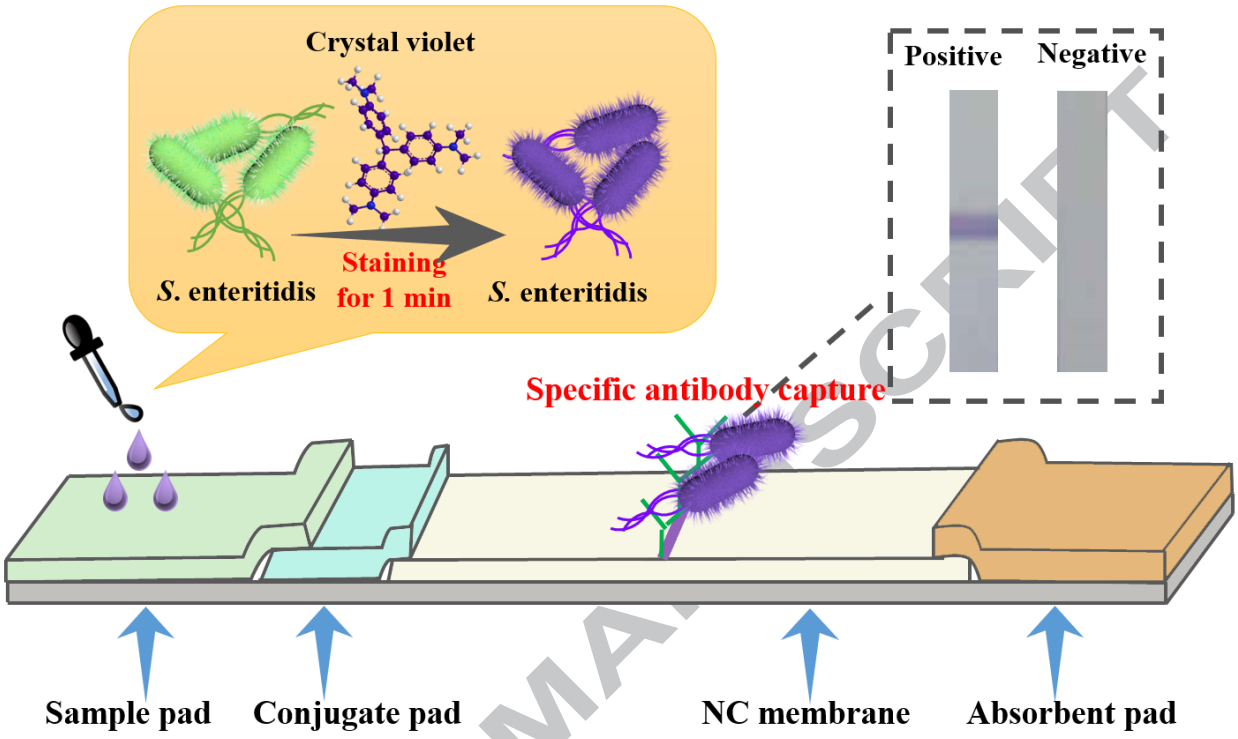

Fig. 2

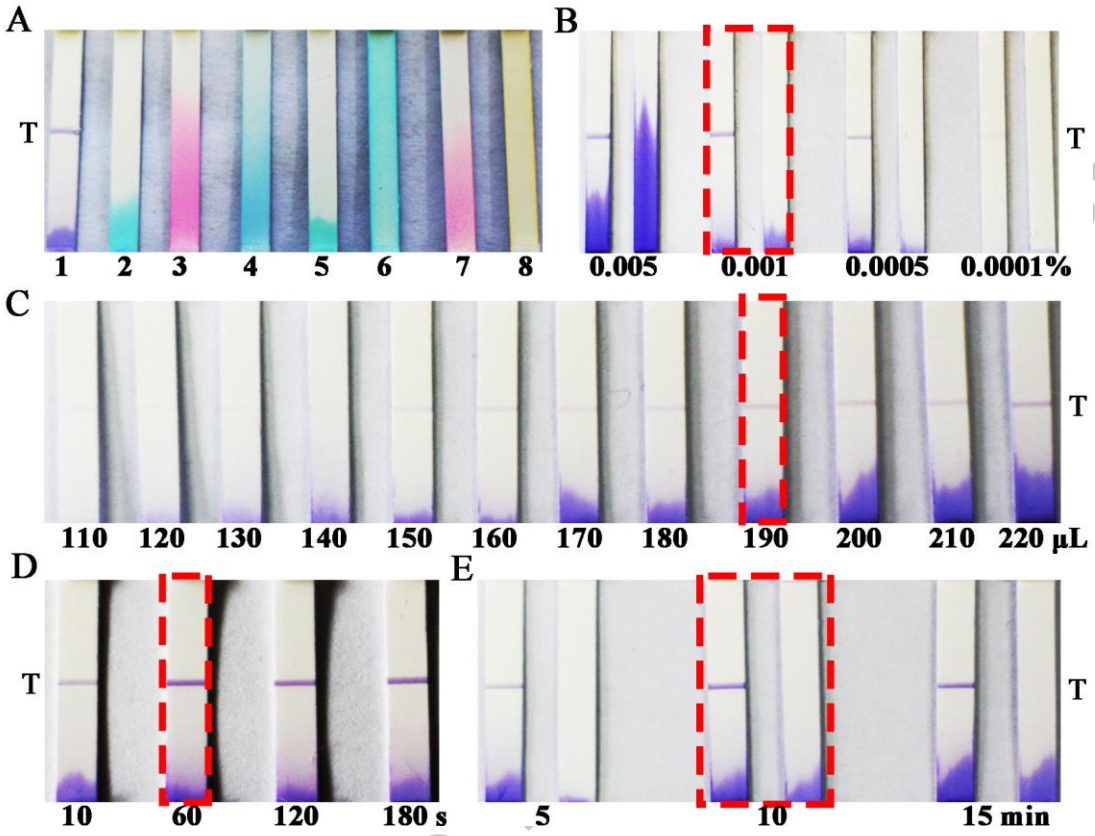

Fig. 3

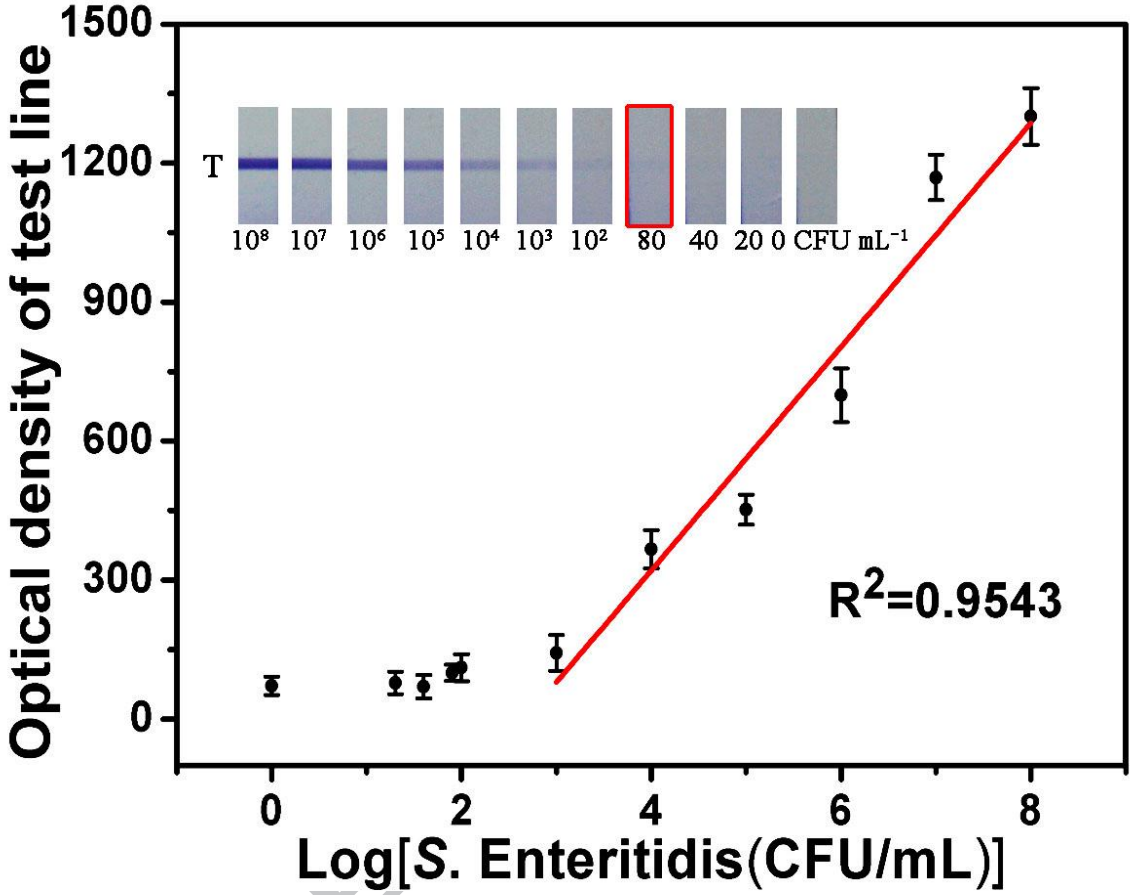

Fig. 4

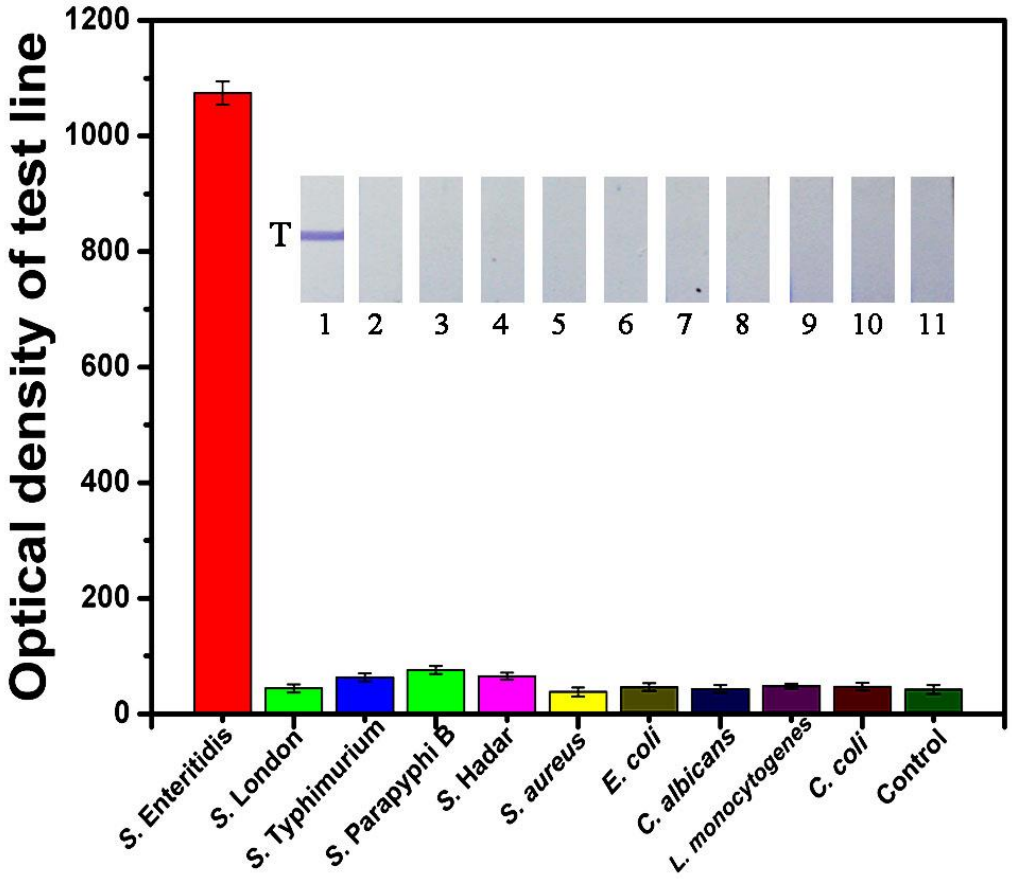

Fig. 5

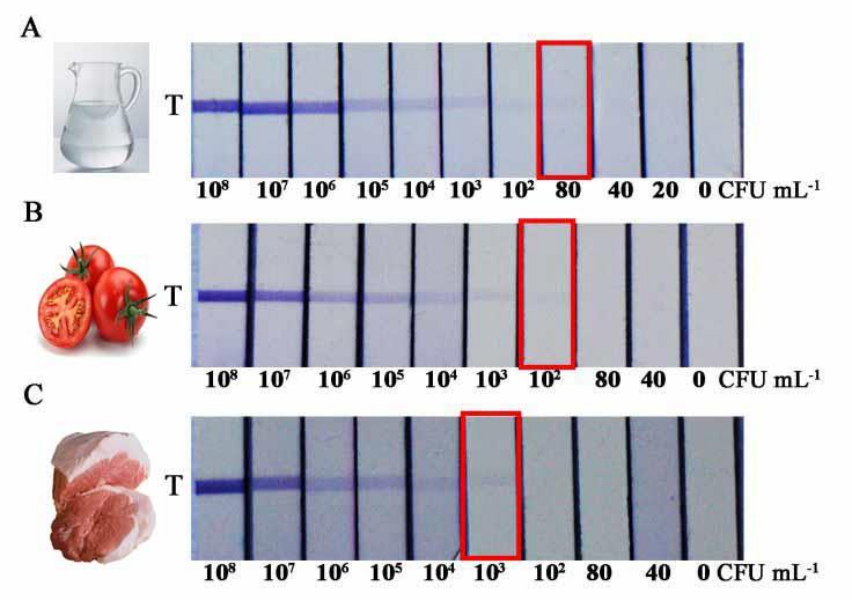

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Table 1 Comparison of sensitivity for pathogen detection by LFSs based on different signals in pure culture

Label	Target pathogen	LOD[a] (CFU mL^{-1})	Reference
Magnetic bifunctional nanobeads (GMBN)	*S. choleraesu*	5×10^5	(Xia, Yu, Liu, Xu, & Lai, 2016)
Hierarchical Flowerlike Gold Nanoparticles	*E.coli* O157:H7	10^3	(Zhang et al., 2015)
Colloidal gold	*E. cloacae*	10^2	(Zhang, Zhou, Zhang, Zhang, & Su, 2016)
Liposome	*S.* Typhimurium	10^6	(Leem, Shukla, Song, Heu, & Kim, 2014)
Colloidal gold	*S.* Typhimurium	1.25×10^5	(Wang et al.,2015)
Pt-Au bimetal nanoparticles	*E.coli* O157:H7	10^2	(Jiang et al., 2016)
Horseradish Peroxidase (HRP)	*Escherichia coli*	10^2	(Eltzov & Marks, 2017)
Crystal violet	*S.* Enteritidis	80	This work

[a] LOD, limit of detection.

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Highlights

- Gram staining and direct immunoassay were first adopted for *S.* Enteritidis detection.
- The marking signal was derived from one-step bacteria staining with crystal violet.
- The device exhibited excellent sensitivity, simplicity, selectivity and feasibility.
- Results could be clearly distinguished by naked eyes even under a lower analyte level.