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Highly sensitive detection of high-risk bacterial pathogens using SERS-based lateral flow assay strips

Rui Wang[a], Kihyun Kim[a], Namhyun Choi[a], Xiaokun Wang[a], Jiyoung Lee[a], Jun Ho Jeon[b], Gi-eun Rhie[b], Jaebum Choo[a,*]

[a] Department of Bionano Technology, Hanyang University, Ansan, 15588, South Korea
[b] Division of High-risk Pathogens, Laboratory Control of Infectious Diseases, Korea Centers for Disease Control and Prevention, Chungju, 28159, South Korea

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ABSTRACT

Bacterial pathogens such as *Yersinia pestis, Francisella tularensis,* and *Bacillus anthracis* are classified into the highest rank of potential bioterrorism agents. Colorimetric lateral flow assay (LFA) strips are commercially available but these conventional strips have drawbacks in terms of low sensitivity and limit of quantitative analysis. Therefore, there is an urgent need for a new sensing platform to detect these pathogens in the early contamination stage. In this study, a novel surface-enhanced Raman scattering (SERS)-based LFA strip was developed for sensitive detection of bacterial pathogens. Target-specific SERS nanotags (Raman reporter-labeled gold nanoparticles) were used as an alternative to the gold nanoparticles in conventional LFA strips. Using these SERS nanotags the presence of bacteria could be identified through a simple color change in the test line. Additionally, highly sensitive and accurate quantitative analysis could be performed by monitoring the characteristic Raman peak intensity of SERS nanotags that were captured in the test line. This highly sensitive method required a short assay time (15 min) and a tiny volume of pathogen sample (40 μL). We believe that the proposed SERS-based LFA technique has great potential as a valuable tool in the early detection of specific bacterial pathogens in the field due to its excellent analytical sensitivity.

1. Introduction

High-risk bacterial pathogens such as *Yersinia pestis, Francisella tularensis*, and *Bacillus anthracis* are categorized as lethal infectious agents that can be used as biological weapons for bioterrorism [1–3]. The Center for Disease Control and Prevention in the United States has classified them as "Category A" bio-threat agents, the highest rank among potential bioterrorism agents.

Y. pestis is a Gram-negative coccobacillus that causes an acute and lethal infection in humans and animals [4–6]. *Y. pestis* is notorious for its use as a biological weapon in World War II. The primary carriers of this pathogen are the Oriental rat flea and infected rodents [7]. *Tularemia*, also known as rabbit fever, is a serious infectious zoonotic disease caused by the bacterium *F. tularensis*, which is a non-motile, non-spore forming, Gram-negative, rod-shaped coccobacillus [8,9]. Inhalation of aerosolized bacteria and ingestion of contaminated food and water lead to pneumonic tularemia [10]. Anthrax is caused by *B. anthracis*, a Gram-positive and endospore forming bacterium. The spread of anthrax is closely associated with contact with bacterial spores, which are composed of dehydrated cells with thick walls and additional layers that form inside the cell membrane [11]. Since these spores are highly resilient, surviving in extreme temperatures, low-nutrient environments, and under harsh chemical treatment, *B. anthracis* has been considered one of the most dangerous bioterrorism agents [12,13]. Infection of humans with these highly contagious agents causes high morbidity and mortality and subsequently leads to widespread panic and social disruption, which is an extremely serious threat to national security. Therefore, it is critical to develop a rapid and sensitive detection technique for these high-risk bacterial pathogens.

Conventionally, bacteria colony counting has been considered the gold standard in the detection of *Y. pestis, F. tularensis,* and *B. anthracis* but it needs a long culture time up to several days because of the pre-enrichment and selective differential plating steps [14,15]. Real-time polymerase chain reaction (RT-PCR) is also extensively used as a microbiological identification technique, but it also needs sample pretreatment steps including DNA extraction and signal amplification by a sequential thermo-cycling process

* Corresponding author.
E-mail address: jbchoo@hanyang.ac.kr (J. Choo).

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[16,17]. In particular, any erroneous amplification of unrelated gene sequences leads to incorrect identification in RT-PCR. Therefore, there is still an urgent demand for a new detection platform that allows rapid and reliable detection of these bacterial pathogens [18,19].

Recently, a lateral flow assay (LFA) strip has attracted increasing attention as an alternative tool for the detection of high-risk pathogens because of its many advantages including a user-friendly format, fast detection time, and long-term stability [20–24]. LFA strips for three high-risk bacterial pathogens are commercially available but a more sensitive and accurate sensing platform is absolutely needed to reduce the risk posed by microorganisms as biological weapons [25,26] since the current commercial LFA strips possess major limitations in terms of detection sensitivity and limit of quantification capability. To improve these two factors, we developed a surface-enhanced Raman scattering (SERS)-based bacteria LFA sensor for the first time. We recently utilized the SERS-based LFA platform for sensitive analysis of staphylococcal enterotoxin B [27], HIV-1 DNA [28] and thyroid-stimulating hormone [29] but there has been no report about high-risk bacterial pathogens till now. Since the size of bacterial pathogen is much bigger than the size of protein, DNA or hormone, experimental conditions of running buffers and SERS nanotags should be carefully optimized. Here, we report the application of SERS-based LFA sensor for the highly accurate and sensitive analysis of low-abundance high-risk bacterial pathogens. This approach also provides new insights into early detection of specific bacterial pathogens in the field.

When reporter molecules are adsorbed onto the surface of gold nanoparticles, their Raman signals are greatly enhanced at SERS active sites known as “hot spots” as a result of electromagnetic and chemical enhancement effects [30–32]. In the SERS-based LFA assay platform, Raman reporter-labeled SERS nanotags were used as a detection probe and the presence of a target pathogen could be identified by the naked eye. In addition, highly sensitive quantitative analysis can be performed with the aid of a Raman reading system. In this study, a novel SERS-based LFA platform was proposed for highly sensitive detection of three high-risk bacterial pathogens. Target-specific SERS nanotags (Raman reporter-labeled gold nanoparticles) were used as an alternative to the gold nanoparticles utilized in conventional LFA strips. Highly sensitive quantitative evaluation of bacterial pathogens could be achieved by monitoring a characteristic Raman peak intensity change of SERS nanotags on the test line. This approach has strong potential to be a feasible method for on-site early detection of high-risk bacterial pathogens due to its excellent analytical sensitivity.

2. Experimental section

2.1. Reagents and materials

Gold (III) chloride trihydrate ($HAuCl_4{\cdot}3H_2O$), tris-sodium citrate (Na_3-citrate), dihydrolipoic acid (DHLA), 1-ethyl,3-(3-dimethylaminopropyl)carbodiimide (EDC), 4-(4-maleimidophenyl)butyric acid *N*-succinimidyl ester (NHS), bovine serum albumin (BSA), polyvinylpyrrolidone (PVP), Tween 20, Borax (pH 9.0), and Tris-EDTA (pH 8.0) were purchased from Sigma-Aldrich (MO, USA). Surfactant 10G was purchased from Fitzgerald (CA, U.S.A.). Malachite green isothiocyanate (MGITC) and phosphate-buffered saline (PBS, 10×, pH 7.4) were purchased from Invitrogen Corporation (CA, USA). Inactivated *Y. pestis, F. tularensis*, and *B. anthracis* were supplied by the Korea Center for Disease Control and Prevention (KCDCP). *Y. pestis* antibody sets were also provided by KCDCP. Mouse monoclonal antibody sets against *F. tularensis* (T14 and FB11) and against *B. anthracis* spores (SA26 and SA27) were purchased from HyTest (Turku, Finland). The nitrocellulose (NC) membrane attached to a backing card (HF090 MC100) was purchased from Millipore Corporation (MA, USA), and the absorbent pad (CF4) was purchased from Whatman-GE Healthcare (PA, USA).

2.2. Preparation of antibody-conjugated SERS nanotags

Gold nanoparticles (AuNPs) were prepared using the previously reported seeded-growth method [33]. All glassware was washed using aqua regia, rinsed with distilled water, and oven-dried prior to use. A 75-mL solution of 2.2 mM sodium citrate was heated to boiling, and 0.5 mL of 25 mM $HAuCl_4$ was added to the flask upon boiling. The color of the solution changed from light yellow to bluish gray and then to soft pink in 15 min. The resulting gold seed solution was cooled to 90 °C. To this solution, 0.5 mL of 60 mM sodium citrate and 0.5 mL of 25 mM $HAuCl_4$ solution were sequentially added 12 times at 2-min intervals to ensure complete mixing after each addition, and the color of the solution finally changed from pink to deep red. The solution was stirred for a further 30 min at 90 °C and then was cooled to room temperature. The shape and size distribution of AuNPs was characterized by dynamic light scattering (DLS) and transmission electron microscopy (TEM).

SERS nanotags were prepared using the method previously reported. Briefly, 1 μL of 10^{-4} M MGITC (Raman reporter) was added to 1.0 mL of AuNP solution and reacted for 30 min under vigorous shaking. For conjugation of antibodies on the surface of AuNPs, their colloid solution was controlled at pH 9 by addition of 100 μL of 0.1 M borax buffer solution. And then 4 μL of 1 mg/mL antibody was added to the MGITC-labeled AuNPs solution. After shaking for 2 h at room temperature, 20 μL of 10% BSA was added to block the unbound surfaces of AuNPs. The mixture was shaken for 30 min and centrifuged at 6000 rpm for 10 min to remove non-specific binding chemicals and antibodies. After discarding the supernatant, the pellets were re-dispersed in buffer solution.

2.3. Preparation of LFA strips

The typical LFA strip is composed of four compartments: a sample pad, a conjugate pad, a NC membrane with plastic backing card, and an absorbent pad. First, a test line and a control line were marked on the NC membrane by dispensing 0.5 mg/mL of capture antibody and 0.1 mg/mL of secondary antibody that binds the detection antibody on SERS nanotags, respectively. Each antibody was dispensed on the NC membrane at a rate of 0.8 μL/cm using a precision line dispensing system (Zeta Corporation, South Korea). The dispensed membranes were dried for 1 h at room temperature. The assembly membrane was cut into 3.8 mm-wide strips using an automatic paper-cutting instrument (Zeta Corporation). To simplify the operation procedure, the LFA strip marked with test and control lines was directly dipped into wells of a 96-well ELISA plate containing a sample solution and SERS nanotags as a dipping substrate.

2.4. Instrumentation

UV-vis absorption spectra were collected with a Cary 100 spectrometer (Varian, Salt Lake City, UT, USA). Dynamic light scattering (DLS) measurement was performed with a Nano-ZS90 instrument (Malvern, UK) and TEM images were acquired using a JEOL JEM 2100F instrument at an accelerating voltage of 200 kV. Enzyme-linked immunosorbent assay (ELISA) was performed using a microplate reader (Power Wave X340, Bio-Tek, Winooski, VT, USA) equipped with a 96-well plate. Raman spectra for the test line of the LFA strip were acquired using a Renishaw InVia Raman microscope system (Renishaw, New Mills, UK); a He–Ne laser with a power of 20 mW operating at λ = 633 nm was utilized as the exci-

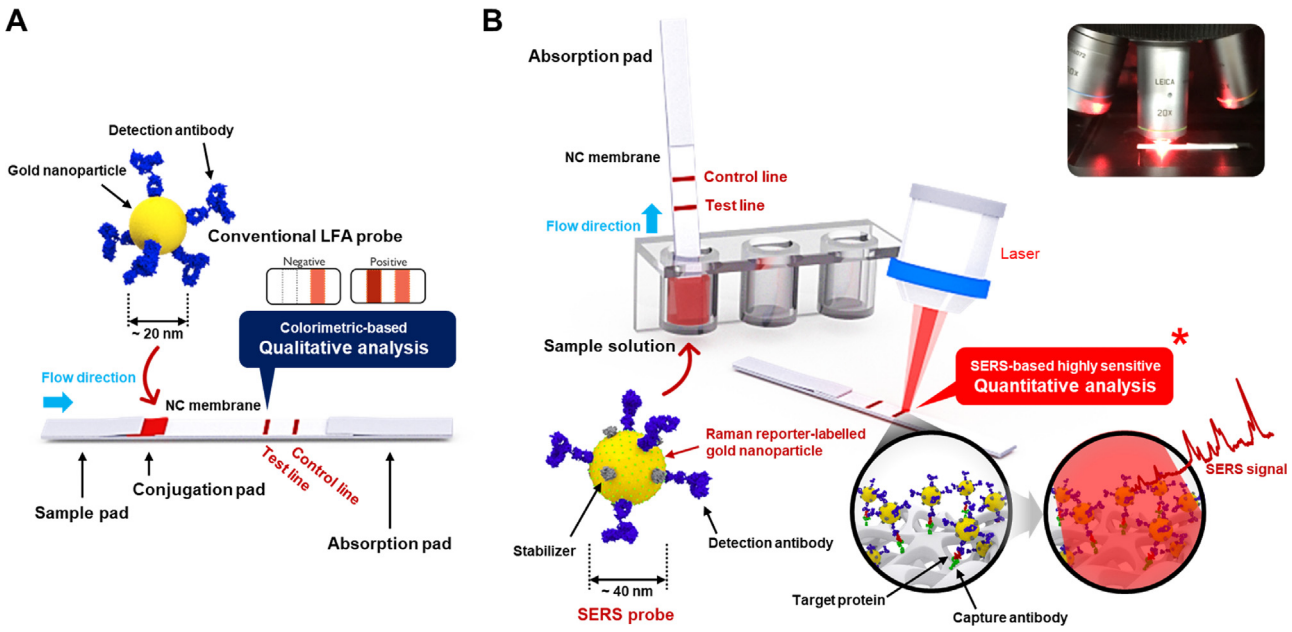

Fig. 1. Schematic illustration of (A) conventional LFA strip and (B) SERS-based LFA strip. In the conventional LFA strip, two color bands were observed ("on") in the presence of target whereas only one color band on the control line was observed in the absence of target ('off"). Using the SERS-based LFA strip, quantitative analysis of a specific target can be performed by monitoring the Raman intensity of a characteristic Raman peak of SERS nanotags captured in the test line.

tation source. The Rayleigh line was removed from the collected Raman data by placing a holographic notch filter in the collection path. A charge-coupled device (CCD) camera was coupled to a spectrograph to provide a combined spectral resolution of 1 cm^{-1}. SERS spectra were quantitatively analyzed with WiRE software (Renishaw, New Mills, UK).

3. Results and discussion

3.1. Principle of SERS-based lateral flow assay for the detection of bacterial pathogens

The operating principle of a conventional LFA strip is compared with that of a SERS-based LFA strip in Fig. 1. After the sample solution containing target is loaded onto a sample pad, the solution migrates toward the absorption pad through capillary force. When the migrating solution is flowing through the conjugate pad, immunoreactions between bacteria and antibody-conjugated gold nanoparticles occur under the flowing condition. These target–gold nanoparticle complexes continue to migrate along the pad and are captured by the antibodies that were pre-immobilized on the test line. The accumulation of gold nanoparticles induces a characteristic red band in the test line. Excess antibody-conjugated gold nanoparticles continue to migrate and are captured by the antibodies that were pre-immobilized on the control line by antibody–antibody interactions. In the presence of target, two red bands are observed ("on") but in the absence of target only one red band on the control line is observed ('off"). The red band on the control line also demonstrates that the LFA strip is working as intended. Fig. 1A demonstrates the operating principle of the conventional LFA strip. Although this strip is commercially available and has been extensively used in a large number of point-of-care tests, low sensitivity still limits its applications. In addition, it is impossible to perform quantitative analysis using this conventional LFA strip. To resolve these drawbacks, a SERS-based LFA strip was developed in this study. The integration of SERS with existing LFA platforms offers significant utility in biological sensing applications. The operating principle of a SERS-based LFA strip is displayed in Fig. 1B. Here, the presence of target could be identified through a color change in the test line just like a conventional LFA strip. Additionally, quantitative analysis of a specific target could be realized by monitoring the Raman intensity of a characteristic Raman peak during the assay.

To describe the operating principle of the SERS-based LFA sensing platform in more detail, its schematic is illustrated in Fig. 2. Three monoclonal antibody-immobilized LFA strips for *Y. pestis*, *F. tularensis*, and *B. anthracis* were dipped into different wells of a 96-well ELISA plate. In each well, mixtures of SERS nanotags and different concentrations of bacteria in buffer solution were prepared. The resultant immunocomplexes migrated by capillary action towards to the test line. Raman reporter-labeled AuNPs (SERS nanotags) were used instead of the AuNPs in the conventional LFA strip for the quantitative analysis of specific bacteria. AuNPs of 20 nm in size were used in the commercial LFA strips but 40 nm AuNPs were used in the SERS-based LFA strip to induce maximum localized surface plasmon effects.

One important issue in the SERS-based quantitative analysis of bacteria is keeping the reproducibility of Raman signals generated by SERS nanotags. Therefore, it is important to fabricate AuNPs with a good monodisperse shape for highly reproducible SERS measurements. In this work, AuNPs were prepared by the seeded-growth method and their morphological shapes and size distributions were analyzed using TEM and DLS, respectively as shown in Fig. S1. The surface of AuNPs was labeled with Raman reporter molecules, MGITC, and then detection antibodies were conjugated to the AuNPs. SERS nanotags were characterized through UV–vis spectroscopy, DLS, and SERS measurements as shown in Fig. S2.

3.2. Quantitative analysis of bacterial pathogens using SERS-based lateral flow assay

KCDCP provided three high-risk bacterial pathogens and some of the antibodies; other antibodies were purchased from HyTest. Detailed information about three bacterial pathogens and the corresponding antibodies used in this study is listed in Table 1.

Before the development of SERS-based LFAs for the three bacterial pathogens, ELISA was performed to validate the binding affinity between bacteria and antibodies. Here, a sandwich assay

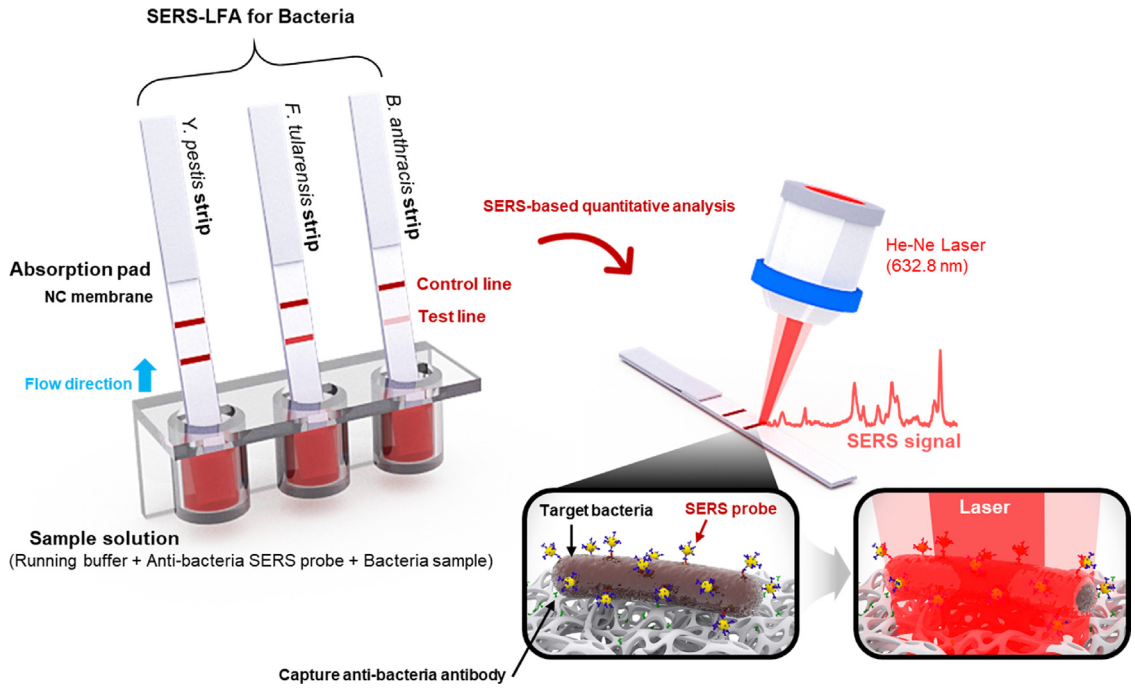

Fig. 2. Operating principle of the SERS-based LFA sensing platform. LFA strips for *Y. pestis, F. tularensis*, and *B. anthracis* were dipped into wells of a 96-well ELISA plate containing mixtures of SERS nanotags and different concentrations of bacteria in buffer solution. The immunocomplexes that formed migrated by capillary action toward to the test line, where their Raman signals were measured and analyzed.

Table 1
Bacterial pathogens and corresponding antibodies.

Target bacteria (Resource)	Role	Reagent	Origin	Resource
Yersinia pestis ([a]KCDC)	Capture	Anti-F1 protein monoclonal antibody (4A7)	Mouse	KCDC
	Detection	Anti-F1 protein polyclonal antibody	Rabbit	KCDC
Francisella tularensis (KCDC)	Capture	Anti-Francisella tularensis LPS monoclonal antibody (FB11)	Mouse	HyTest
	Detection	Anti-Francisella tularensis LPS monoclonal antibody (T14)	Mouse	HyTest
Bacillus anthracis (KCDC)	Capture	Anti-Bacillus anthracis spore antigen monoclonal antibody (SA26)	Mouse	HyTest
	Detection	Anti-Bacillus anthracis spore antigen monoclonal antibody (SA27)	Mouse	HyTest

[a] KCDC, the Korea Center for Disease Control and Prevention.

was performed for *Y. pestis* because polyclonal antibodies were commercially available. Indirect ELISA was performed for *F. tularensis* and *B. anthracis* because there were no commercially available polyclonal antibodies. In the sandwich ELISA, monoclonal capture antibodies (100 μL, 1 mg/mL) in Na_2CO_3-$NaHCO_3$ buffer (pH 9.6) were immobilized on the surface of a 96-well plate and incubated overnight at 4 °C. Each well was treated with 1% BSA to block non-specific binding sites. After washing three times with PBST (containing 0.05% (v/v) Tween 20, pH 7.4), various concentrations of *Y. pestis* bacteria in the 0–10^7 CFU/mL range were added to different wells. After washing with PBST, polyclonal detection antibodies and HRP-linked secondary antibodies were sequentially added to induce the color change. For indirect ELISA, different concentrations of bacteria (0–10^7 CFU/mL range) in Na_2CO_3-$NaHCO_3$ buffer (pH 9.6) were directly immobilized on the surface of a 96-well plate and incubated overnight at 4 °C. After washing three times with PBST, 1% BSA was used to block the remaining sites of the plate. After treatment with primary antibodies, HRP-linked secondary antibodies were added and further incubated. Finally, TMB substrate was added to observe a color change. Fig. S3 displays three calibration curves for *Y. pestis, F. tularensis,* and *B. anthracis* determined from ELISA. All the antibodies used in the experiments exhibited good binding affinities towards the corresponding bacterial pathogens. In the cases of *F. tularensis* and *B. anthracis*, two antibody sets were tested and both exhibited good binding affinities.

Next, the same bacteria targets and antibodies used in ELISA were employed for the SERS-based LFA. In the LFA, it is important to optimize the flowing conditions of running buffer and SERS nanotag solutions loaded on the strip. In the present work, tris-EDTA was selected as a dilution buffer because its divalent cation chelating agents reduce non-specific protein interactions. PVP and surfactant 10G were used to stabilize SERS nanotags and to optimize their flow conditions. After treatment with PVP, negative charges were generated on the surface of AuNPs. Consequently, the aggregation of SERS nanotags could be prevented because of electrostatic repulsion among AuNPs. In addition, surfactant 10G could improve the hydrophobic property of SERS nanotags and further improve their interaction with antibodies immobilized on the test and control lines.

Fig. 3 demonstrates the quantitative assay results using SERS-based LFA strips for three different bacterial pathogens, *Y. pestis, F. tularensis*, and *B. anthracis*. The assays were performed in the 0–10^7 CFU/mL range for all three bacteria. Fig. 3A shows photographic images of the LFA strips. With an increase in bacteria concentration, more immunocomplexes are formed on the test line and the intensity of the red color increases, as expected. The concentration that could be observed with the naked eye was estimated to be approximately 10^5 CFU/mL for all the three bacterial pathogens as shown in Fig. 3A. Fig. 3B indicates that Raman intensity gradually increases with increasing concentration of bacterial pathogens when more

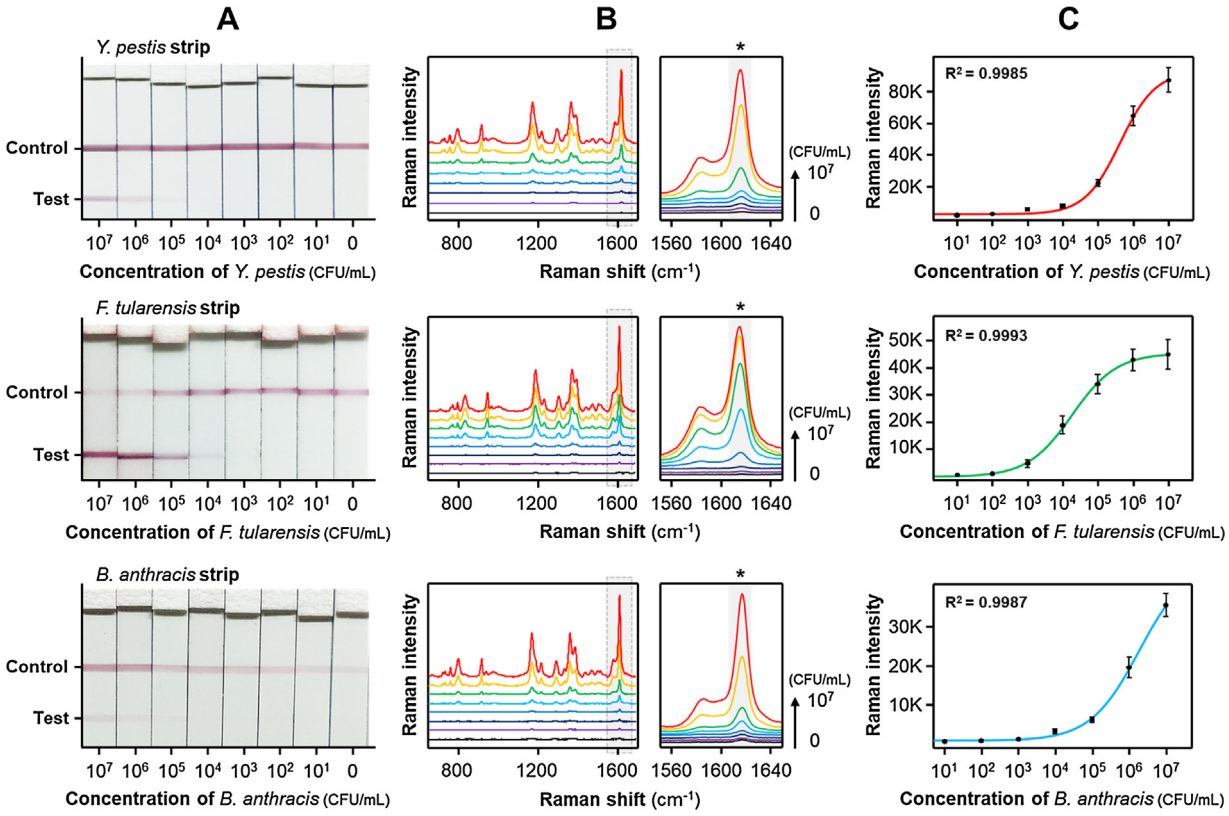

Fig. 3. Quantitative SERS-based assay results for three bacterial pathogens, *Y. pestis, F. tularensis*, and *B. anthracis*. The assays were performed in the 0–10^7 CFU/mL range for all three bacteria. (A) Photographic images of LFA strips (B) Raman spectra of the test line and (C) corresponding calibration curves for different concentrations (from 0 to 10^7 CFU/mL) of three bacterial pathogens. The error bars indicate the standard deviations calculated from five measurements.

bacteria-SERS nanotags complexes are captured on the test line. The characteristic Raman peak intensity of MGITC at 1616 cm^{-1} was used for the quantitative analysis of three bacterial pathogens. When the excitation laser's wavelength matches the absorption band of Raman reporter molecules, surface-enhanced resonance Raman scattering (SERRS) occurs, and the enhancement factor may be further enhanced approximately 100 times. Since the absorption maximum of MGITC (625 nm) coincides with the wavelength of He-Ne laser (633 nm), we could obtain the additional resonance Raman enhancement. Variations in the Raman peak intensity were used to construct the calibration curves for three bacterial pathogens. Here, a four-parameter logistic model was used to determine the fitting parameters. Fig. 3C shows the corresponding calibration curves for three bacteria determined by SERS-based assays. The error bars indicate standard deviations from five measurements. The limit of detection (LOD) determined from the standard deviations were 43.4, 45.8, and 357 CFU/mL for *Y. pestis, F. tularensis*, and *B. anthracis*, respectively. Such low values are approximately three to four orders of magnitude more sensitive than those determined from colorimetric LFA strips. The improvement of LODs was attributed to the significant enhancement provided by the antibody-conjugated SERS nanotags.

3.3. Reproducibility and selectivity tests of SERS-based lateral flow assay

To confirm the reproducibility of the assays, 10 repeated SERS-based LFA tests were performed for the same concentration (10^7 CFU/mL) of three different pathogenic bacteria. In addition, SERS signals for five different laser spots on the test line were averaged to achieve a reproducible intensity value. Fig. 4A–C show digital photographic images of LFA strips for *Y. pestis, F. tularensis*, and *B. anthracis*. Fig. 4D shows the intensity variations of the Raman peak at 1616 cm^{-1} of 10 different strips for each bacterial pathogen, and the small intensity variations indicate that the signals were obtained with good analytical reproducibility. In addition, the errors bars for the standard deviations of five measurements also indicate that each SERS-based strip possesses inherent high reproducibility.

To evaluate the selectivity of the SERS-based LFA strip, tests were performed for the same concentrations (10^7 CFU/mL) of three different pathogenic bacteria *Y. pestis, F. tularensis*, and *B. anthracis*. Photographs and SERS signal intensity variations of the detection are displayed in Fig. 5. As expected, the test line of a specific bacteria species only changed to red in its presence. For example, the test line of the *Y. pestis* strip only changed to red for the solution of 10^7 CFU/mL *Y. pestis* but no color change was observed for the *F. tularensis* or *B. anthracis* strip, as shown in Fig. 5A. Assay results for the other two bacterial pathogens (*F. tularensis* and *B. anthracis*) demonstrated the same results. In each case, only the corresponding bacteria induced the color change of the test line but no obvious color changes were observed for the other bacteria. SERS spectra yielded the same results. As shown in Fig. 5B, the Raman peak intensities at 1616 cm^{-1} demonstrate that our SERS strip only responds to the corresponding bacteria and thus possesses high selectivity.

4. Conclusion

In this study, we report a novel SERS-based LFA for the highly sensitive and rapid detection of three high-risk bacterial pathogens, *Y. pestis, F. tularensis*, and *B. anthracis*, for application in bioterrorism preparedness. Microbiological culture and colony counting,

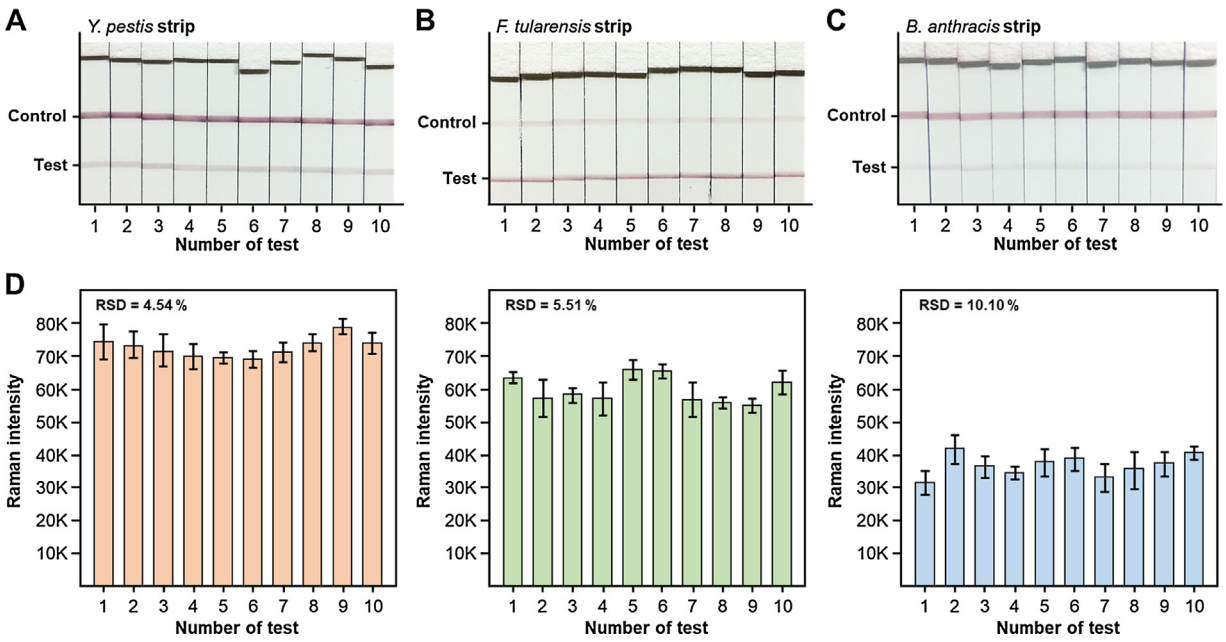

Fig. 4. Reproducibility test of SERS-based LFA strips. Digital photographic images of 10 strips for (A) *Y. pestis*, (B) *F. tularensis*, and (C) *B. anthracis*. Test concentration was 10^7 CFU/mL for all three bacteria. (D) Variations in corresponding Raman peak intensities at 1616 cm^{-1} for three bacteria. Error bars indicate the standard deviations of five measurements for each strip.

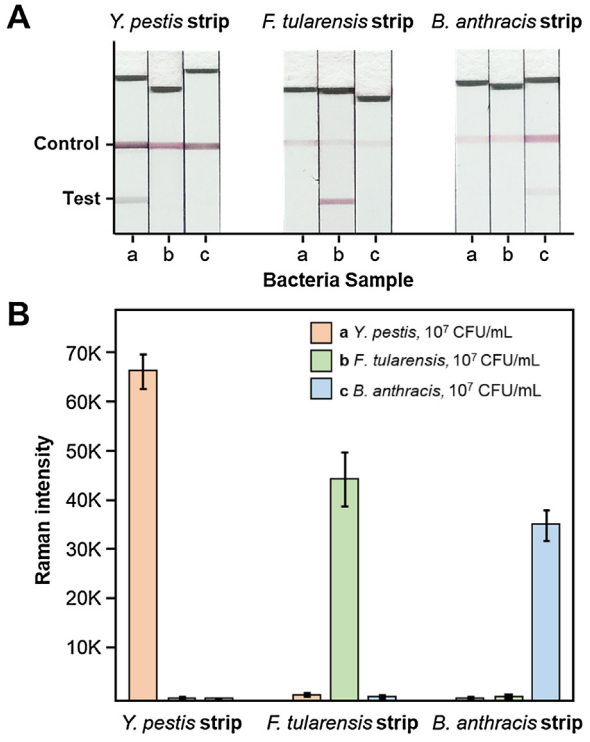

Fig. 5. Selectivity test of SERS-based LFA strips. (A) Digital photographic images of three strips and (B) their SERS signal intensity variations for *Y. pestis*, *F. tularensis*, and *B. anthracis*. The test concentration was 10^7 CFU/mL for all three bacteria. Error bars indicate the standard deviations of five measurements for each strip.

RT-PCR, and conventional LFA strips have been extensively used for detection of these bacteria but these methods cannot meet the requirements of a fast speed and low LOD because of the long sample preparation time and limited detection sensitivity. In the SERS-based LFA, the presence of bacteria could be identified through a simple color change in the test line. Additionally, highly sensitive and accurate quantitative analysis of bacteria was possible by monitoring the characteristic SERS signal intensity of Raman reporter-labeled AuNPs that accumulated in the test line of the SERS-based LFA strip. This method required a short assay time (15 min) and a small volume of pathogen sample (40 μL) and showed high sensitivity. The LODs for *Y. pestis*, *F. tularensis*, and *B. anthracis* were estimated to be 43.4 CFU/mL, 45.8 CFU/mL, and 357 CFU/mL, respectively, and these values are approximately three or four orders of magnitude more sensitive than those determined using commercial LFA rapid kits. We believe that the proposed SERS-based LFA technique has great potential to be a valuable tool in the early detection of specific pathogens due to its excellent analytical sensitivity.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.snb.2018.04.162.

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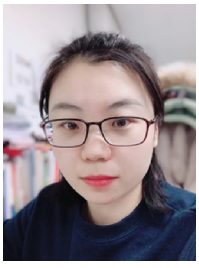
Biographies

Rui Wang is currently a doctoral candidate under the guidance of Prof. Jaebum Choo at Hanyang University in South Korea. He received his B.S. degree in 2009 from Department of Chemistry and Chemical Engineering at Shandong Normal University, China. His research interests include the development of highly sensitive surface-enhanced Raman scattering (SERS)-based immunoassay platforms.

Kihyun Kim is currently a doctoral candidate under the guidance of Prof. Jaebum Choo at Hanyang University in South Korea. He received his B.S. degree in 2017 from Department of Bionano Engineering at Hanyang University. His research interests include the development of a highly sensitive lateral flow immunoassay technique using metal nanoparticles and laser-induced optical systems.

Namhyun Choi is currently a doctoral candidate under the guidance of Prof. Jaebum Choo at Hanyang University in South Korea. He received his B.S. degree in 2010 from Department of Applied Chemistry at Hanyang University. His research interests include the development of droplet microfluidic-based nanobio sensors and molecular diagnostic systems.



Xiaokun Wang is currently a doctoral candidate under the guidance of Prof. Jaebum Choo at Hanyang University in South Korea. She received her M.S. degree in 2014 from Qufu Normal University; Joint-Supervision student of Yantai Institute of Coastal Zone Research, Chinese Academy of Sciences. Her research interests include the fabrication of SERS-based lateral flow biosensor, 3D substrate biosensor and their applications to biomedical diagnostics and mycotoxin detection.

Jiyoung Lee is currently a M.S. candidate under the guidance of Prof. Jaebum Choo at Hanyang University in South Korea. She received his B.S. degree in 2016 from Department of Applied Chemistry at Hanyang University. Her research interests include the development of surface-enhanced Raman scattering (SERS)-based early diagnostic methods for pathogenic diseases.

Jun Ho Jeon received his PhD degree from Chungnam National University in South Korea. He worked as a post doc. research associate in the Korea Research Institute of Bioscience and Biotechnology in 2005–2006. He also worked as a research professor at Sookmyung Women's University and Seoul National University in 2007–2011. He transferred to the Korea Centers for Disease Control and Prevention in Korea in 2011. He is currently a staff scientist at division of high-risk pathogens. His research interests include development of therapeutic antibodies for botulism and development of molecular diagnostic assays for high-risk pathogens and toxins.

Dr. Gi-eun Rhie, the director of the DHP in KCDC, received a Bachelor's degree from Seoul National University in 1987 and a Ph.D. in biochemistry from Brown University, USA in 1994. After Ph.D degree, she has continued her research in microbiology, biochemistry and vaccinology at several institutions both in Korea and USA including Brown University, Seoul National University Hospital and Harvard Medical School. She has published several excellent papers in peer-reviewed journals and now as the director of DHP in KCDC, she is conducting research focusing on; development of human anthrax vaccine, production of therapeutic antibodies against high-risk pathogens, development of diagnosis methods for high-risk pathogens.

Jaebum Choo has been in the faculty of the Bionano Engineering Department at Hanyang University since 1995. He obtained his PhD in laser-induced spectroscopy at Texas A&M University in 1994. In 2015, he became a Baik Nam Distinguished Professor due to his excellent academic achievements. He served as a President of Korea Biochip Society in 2015. His current research programs are centered on the development of ultra-sensitive optical detection systems for rapid and accurate *in vitro* diagnostics. He has given 120 invited lectures in the USA, Europe and Asia, and has published over 240 research papers in refereed journals and 5 book chapters.