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Capture and detection of urine bacteria using a microchannel silicon nanowire microfluidic chip coupled with MALDI-TOF MS†

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Fast bacterial identification in urine samples was achieved by capturing bacteria on a microchannel silicon nanowire microfluidic chip, followed by MALDI-TOF MS detection. Under the optimized conditions, bacteria with a concentration of 10^6 CFU mL⁻¹ in urine samples could be identified without culture. If cultured for 4 hours, bacteria with a concentration as low as 10^3 CFU mL⁻¹ were identified.

Bacterial infections are one of the leading causes of morbidity and mortality all over the world.¹ Urinary tract infections (UTIs), as one of the most common infectious diseases, affect an estimated 1 in 3 women before the age of 24 years.^{2,3} Early identification of pathogens is crucial for reducing inappropriate treatment and antibiotic abuse. However, traditional culture-based methods usually take 2 or more days to give results. Urine culture is time-consuming and up to 60–80% of urine cultures give negative results, leading to the high cost of unnecessary tests.⁴ Molecular methods such as polymerase chain reaction (PCR) enable quick analysis within 5–12 hours,^{5,6} but they require complex sample preparation steps and may produce misleading results due to sample contamination.

In recent years, matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) has been regarded as a fast and reliable method for bacterial identification.⁷ This method provides unique fingerprint mass spectra and identifies bacteria by comparing with the reference spectra in the database.^{8,9} MALDI-TOF MS requires fewer reagents, fewer steps, and less prior information compared to PCR or biochemical tests. Usually, a single bacterial colony is sufficient for MS analysis.^{10,11} However, for bacterial identification in liquid samples, culturing or selective bacterial enrichment is still needed to obtain sufficient bacterial density

and to avoid interference from the complex biological matrix.¹²

Nanostructured materials have a large surface/volume ratio and are easy to modify. Besides, nanoscale topographies have been reported to have a significant impact on the adhesions of bacteria.^{13,14} Various nanomaterials such as nanowires¹⁵ and nanoparticles^{16,17} have been developed for bacterial enrichment. Kwon *et al.* used a nanowire array with fishnet-like structure in a microfluidic channel to enrich bacteria for microscopic analysis.¹⁸ Jalali *et al.* developed a microfluidic device based on a hierarchical 3D nanostructured platform for bacterial capture followed by fluorescent detection.¹⁹ These works proved that bacterial enrichment could be significantly enhanced using specific nanostructure substrates. Besides, microfluidic devices offer the ability to manipulate a small volume of fluids, thus enhancing the interaction between the bacteria and capture platforms. To date, microfluidic devices have been coupled to surface-enhanced Raman spectroscopy (SERS)²⁰ and fluorescence microscopy¹⁹ for bacterial detection. However, these microfluidic devices are still not compatible with the MALDI-TOF MS platform for bacterial detection and identification.

Herein, we fabricated a microfluidic device that uses microchannel silicon nanowires (McSiNWs) as the substrate. To further improve the capture efficiency, concanavalin A (Con A) was attached on the surface of the McSiNW substrate because of its high affinity towards bacterial lipopolysaccharide components.^{15,21} Our previous work reported that a vertical nanowire array has the great ability to concentrate laser and thermal energy,²² thereby significantly enhancing the efficiency of laser desorption and ionization in MALDI-TOF MS detection. In combination with the capture and enrichment effect of the McSiNW substrate, bacteria in liquid samples can be directly detected and identified without a prolonged culture step. To evaluate the performance of this method, *Escherichia coli* (*E. coli*), the most common pathogen in UTIs,^{3,23} was chosen as the model bacterium. After enriching with the McSiNW microfluidic chip, *E. coli* with a concentration of 10^5

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CFU mL⁻¹ in PBS buffer and 10⁶ CFU mL⁻¹ in urine samples can be identified in a culture-free way. After culture for a short time, *E. coli* in urine samples can be identified at a concentration as low as 10³ CFU mL⁻¹. The approach established in this work is fast, cost effective, and convenient. Thus, it may have great potential in the quick diagnosis of infectious diseases, especially UTIs, in clinical samples.

The McSiNW substrate was fabricated as described in the ESI.† Briefly, the microchannel and silicon nanowires were prepared by wet etching^{24–26} and a two-step metal assisted chemical etching²⁷ (MACE) with a patterned SiO₂ layer as an etching mask (Fig. 1A). The dimensions of the microchannels (length, ~1.5 mm; width, ~200 μm; depth, ~40 μm) are shown in Fig. 1B. The total volume of the microfluidic channels was around 3 μL. Scanning electron microscopy (SEM) images of the substrate revealed that the nanowire structure only existed at the bottom and sidewalls of the channel since the presence of the SiO₂ layer hindered the deposition of silver particles and chemical etching on the top of the channel (Fig. 1C and D). The length of the nanowires was about 12 μm after MACE etching for 15 min. Then the prepared McSiNWs were functionalized with Con A through sequential chemical covalent coupling as described in the ESI.†

The structure of the McSiNW microfluidic device is presented in Scheme 1. The microchannel was formed by covering the McSiNW substrate with a PDMS film, which were clipped between two polymethylmethacrylate (PMMA) plates and compressed by tightening the screws to prevent leakage of the sample liquid. Quartz capillary tubes were inserted between the McSiNW substrate and PDMS film as the inlet and outlet for samples. Liquid samples were introduced using a syringe pump (PHD, 2000; Harvard Co.) and flowed over the McSiNW microfluidic channel at a constant flow rate. The detailed methods for capturing and enriching the bacteria with the McSiNW microfluidic chip are described in the ESI.† The bacteria captured on the McSiNW substrate were analyzed using an UltrafleXtreme MALDI-TOF MS instrument (Bruker Daltonics Corp.) in the linear positive mode in a mass range of 2–20 kDa. The instrumental parameters were set as follows: 80% laser intensity, laser attenuator with 32% offset and 30% range, accumulation from 500 laser shots, and 250 ns delayed extraction time. Mass calibrations were performed using a mixture of Protein Standards I and Peptide Standards II

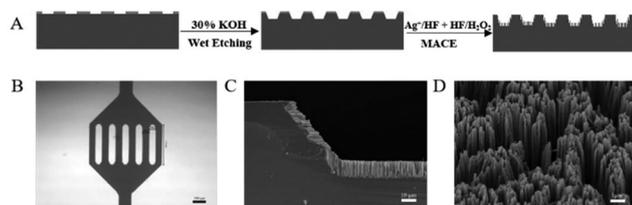
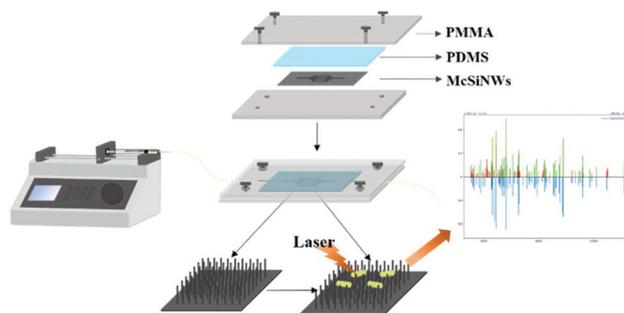


Fig. 1 (A) A schematic of the McSiNW substrate fabrication based on wet etching and two-step metal assisted chemical etching (MACE). (B) Stereoscopic image of the McSiNW substrate. SEM images: (C) cross-sectional view, (D) 45°-tilted view of the McSiNW substrate.



Scheme 1 Schematic illustration of bacteria captured by the Con A modified McSiNW chip from liquid samples and direct MALDI-TOF MS identification.

(Bruker Daltonics). Bacterial identification was conducted on an Autof ms1000 platform (Autobio Diagnostics Co., Ltd, China).

The bacterial capture ability of the McSiNW substrate was verified by fluorescence microscopy and SEM. After enriching *E. coli* from a 0.5 mL suspension (4×10^8 CFU mL⁻¹), the McSiNW substrate with the captured *E. coli* was incubated with 200 μL of DAPI staining solution (Beyotime, China) for 5 min. Then it was washed with PBS buffer three times and observed with fluorescence microscopy. Fig. 2A shows that the bacteria were mainly caught around the microchannels because the microstructures on the substrate hinder the flow of the liquid samples and force the mainstream flow around the microchannels. The fluorescence intensity shown in Fig. 2A was measured using ImageJ software. Compared to the substrate without surface modification (McSiNW), the substrate without microchannels (SiNWs-Con A) and the substrate without nanowires (McSi-Con A), the average fluorescence intensity of the Con A modified McSiNW substrate (McSiNWs-Con A) is about 10 times higher (Table S1†). This result verified that both the

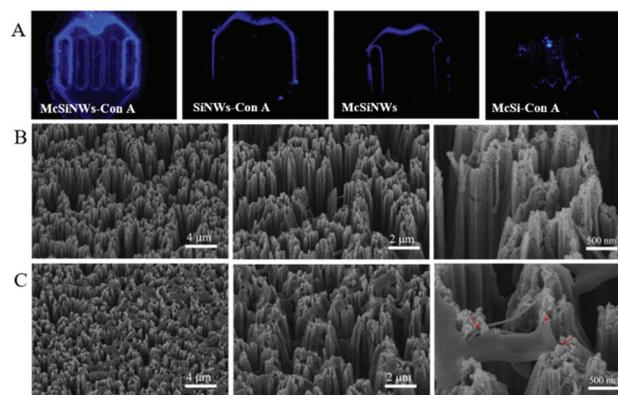


Fig. 2 (A) SEM image of the McSiNWs-Con A substrate at different magnifications. (B) SEM images of the McSiNWs-Con A substrate after capturing *E. coli* from the suspension (0.5 mL, 4×10^8 CFU mL⁻¹) at different magnifications. (C) Fluorescence microscopy images of four kinds of substrates namely McSiNWs-Con A, SiNWs-Con A, McSiNWs and McSi-Con A after *E. coli* capturing (0.5 mL, 4×10^8 CFU mL⁻¹).

micro-nano structure and surface modification synergistically enhanced the bacterial capture efficiency. In addition, SEM images (Fig. 2B and C) showed that the protrusions of the captured bacteria were attached to the tips of SiNWs (red arrows). The results inferred that the morphology of SiNWs may enhance the interactions between the bacteria and the substrate.

To effectively capture bacteria from liquid samples, the experiment parameters, including chip structures, surface modification, nanowire length, and flow rate, were optimized. The bacterial capture efficiencies on four kinds of substrate namely McSiNWs-Con A, SiNWs-Con A, McSiNWs, and McSi-Con A were compared and evaluated by MALDI-TOF MS detection (Fig. 3A). Among all types of substrates, McSiNWs-Con A produced ~71 MS peaks, which is far more than that obtained on other types of substrate (Fig. 3B). The results further proved the critical role of the nanowire topography, microchannel structure, and affinity moiety in the bacterial capture ability.¹⁵ In addition, both fluorescence and MS results indicated that nonspecific interactions between the bacteria and substrate were minimal.

The nanowire length also affects the capture efficiency. The MS peak numbers increased significantly as the nanowire

length increased from 5 μm to 12 μm (Fig. 3C and D). Longer nanowires provided a larger surface area for bacterial binding. Nevertheless, further increases in the nanowire length showed little influence on the MS signal. Meanwhile, longer nanowires needed more etching time. Thus, 12 μm long nanowires were used in the following experiments.

The flow rate of the suspension flowing through the micro-channel has a great influence on bacterial capture (Fig. 3E and F). We examined the capture efficiency by varying the flow rate of *E. coli* suspensions (4×10^8 CFU mL^{-1}) in the range between 5–20 $\mu\text{L min}^{-1}$ with a total volume of 0.5 mL. The results indicated that the capture efficiency gradually increased when the flow rate increased from 5 to 15 $\mu\text{L min}^{-1}$. The phenomenon might be caused by the increasing fluidic pressure in the microfluidic channels. Under high hydrodynamic pressure, bacteria would be forced into the space between the nanowires, thus increasing the binding possibility between the bacteria and nanowire substrate. However, once the flow rate exceeded 15 $\mu\text{L min}^{-1}$, the capture efficiency decreased significantly because the bacteria may not have sufficient time to interact with the nanowire substrate at the flow rate.²⁸ The results suggested that a flow rate of 15 $\mu\text{L min}^{-1}$ achieved the best performance.

To confirm the bacterial capture efficiency on a microfluidic device with the optimized McSiNW substrate, the MS spectra of bacteria in suspensions with or without enriching with the McSiNW microfluidic chip were compared. For the sample without the capture step, 1 μL of bacterial suspension was directly added to a steel target (MTP 384, Bruker Daltonics) and the SiNW substrate, respectively. Then the samples were overlaid with 1 μL of matrix solution (saturated CHCA in 50% acetonitrile and 2.5% trifluoroacetic acid) and dried at room temperature for subsequent MALDI-TOF MS analysis. As shown in Fig. 4, only 10 peaks could be detected even at a high bacterial concentration of 4×10^8 CFU mL^{-1} . When the bacterial concentration was lower than 4×10^7 CFU mL^{-1} , no signal appeared in the MS spectra (Fig. S1†). In contrast, peak numbers detected on the SiNW target doubled because the SiNWs could enhance the absorbance of laser energy and confine the thermal energy on the tip of SiNWs, thereby pro-

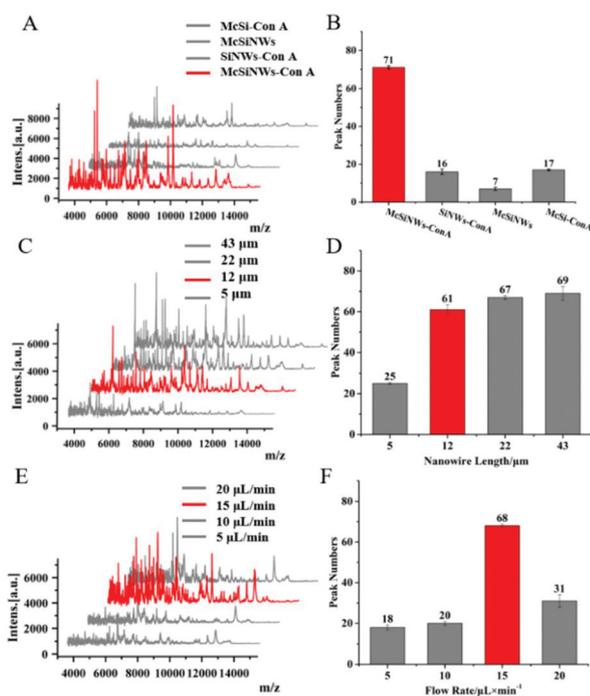


Fig. 3 (A) MALDI-TOF MS spectra of *E. coli* captured by four kinds of substrates namely McSiNWs-Con A, SiNWs-Con A, McSiNWs and McSi-Con A. (0.5 mL, 4×10^8 CFU mL^{-1}). (B) Average peak numbers of three corresponding MS spectra ranging from 3–15 kDa with S/N \geq 5. (C) MS spectra of *E. coli* captured by the McSiNWs-Con A substrate with different nanowire lengths. (D) Average peak numbers of three corresponding MS spectra ranging from 3–15 kDa with S/N \geq 5. (E) MS spectra of *E. coli* captured by McSiNWs-Con A substrates at different flow rates. (F) Average peak numbers of three corresponding MS spectra ranging from 3–15 kDa with S/N \geq 5.

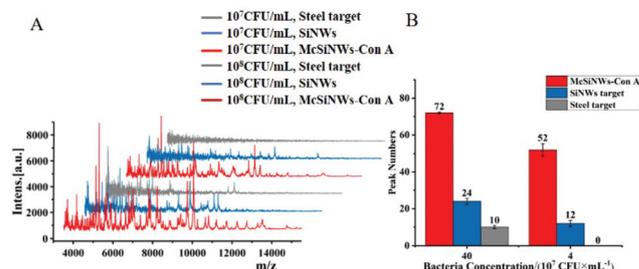


Fig. 4 (A) MALDI-TOF MS spectra of *E. coli* enriched with the McSiNWs-Con A substrate and spectra of a 1 μL *E. coli* suspension droplet on the steel target and SiNW target at a concentration of 4×10^8 and 4×10^7 CFU mL^{-1} . (B) Average peak numbers of three corresponding MS spectra ranging from 3–15 kDa with S/N \geq 5.

moting the desorption and ionization of proteins and peptides in the bacterial cells. After enriching with the McSiNW microfluidic chip, the peak number further increased to 72 at a bacterial concentration of 4×10^8 CFU mL⁻¹. The results confirmed that the McSiNW microfluidic chip is an effective platform to capture and enrich bacteria.

Under the optimized conditions, *E. coli* suspensions in PBS buffer with different concentrations were enriched with the McSiNWs-Con A substrates and analyzed by MALDI-TOF MS. Several characteristic MS peaks could be still detected even at a concentration as low as 10^4 CFU mL⁻¹ (Fig. 5A). Autof Analyzer software was used for bacterial identification. Several parameters such as sample preparation, culture conditions, sample storage, and MALDI target can affect the protein mass pattern of the same bacteria.^{10,11} To solve this problem, a library of reference spectra was built based on 15 spectra acquired from five McSiNW chips after *E. coli* enriching from suspensions in PBS buffer (0.5 mL, 4×10^8 CFU mL⁻¹). Only peaks in the range from 3–15 kDa with S/N ≥ 3 were included and the maximum number of peaks was set at 70. The peaks appearing in different spectra with $\Delta(m/z)/(m/z) \leq 1000$ ppm were regarded as identical peaks. As shown in Fig. 5, without a culture step, bacteria with a concentration $\geq 10^5$ CFU mL⁻¹ in PBS buffer can be directly identified by comparing with the reference spectra after enrichment.

In order to test the adaptability of the McSiNW microfluidic chip in the capture and identification of different types of bacteria, *Staphylococcus aureus* (*S. aureus*), a type of Gram-positive bacteria, was enriched with the microfluidic chip integrated with vancomycin-modified McSiNW substrates. After the enrichment step, around 40 MS peaks were obtained and *S. aureus* was identified with a score of 9.371 (Fig. S2†). In contrast, no reliable identification result was acquired without enrichment even when the bacterial concentration reached as high as 4×10^8 CFU mL⁻¹. In addition, the affinity moiety attached on the McSiNWs plays a vital role in the selective capture of bacteria. The capture efficiency for *E. coli* on vancomycin-modified McSiNWs is far lower than that on the Con A-modified substrate (Fig. S3†).

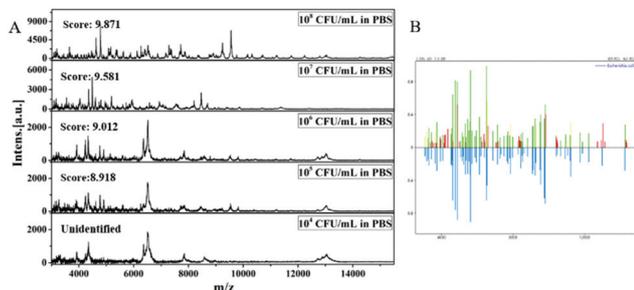


Fig. 5 (A) MALDI-TOF MS spectra of *E. coli* captured by the McSiNWs-Con A substrate with different concentrations in PBS (0.5 mL). (B) MALDI-TOF mass spectra of *E. coli* captured by the McSiNWs-Con A substrate in PBS buffer (0.5 mL, 4×10^8 CFU mL⁻¹), and comparison with the reference spectrum (in blue).

UTIs are among the most common bacterial infectious diseases, resulting in an annual global direct healthcare cost of about 6 billion dollars.²⁹ Initial assessment of UTI is mostly empirical, which may lead to inappropriate antibiotic treatment. Currently, urine culture is needed before the diagnosis of UTIs. However, this method is time consuming (~ 2 –5 days), and infections may further complicate during the diagnosis. Thus, patients would benefit greatly from the development of a rapid, accurate, and cost-effective method for bacterial identification in UTIs. To evaluate the performance of the present method for clinical usage, human urine samples spiked with various concentrations of *E. coli* were analyzed without a culture step. As shown in Fig. 6, bacteria in urine samples were correctly identified (score = 7.479) if the bacterial concentration was larger than 10^6 CFU mL⁻¹. The identification scores of the spectra obtained from different concentrations of bacteria in urine and PBS are compared in Table S2.† Overall, the identification score obtained in urine samples was not as high as that obtained in PBS buffers, probably owing to the interference from the complex components in urine samples.

Nevertheless, a short-duration culture before enrichment would greatly improve the identification efficiency (Table 1). After 4 hours of culture, bacteria in urine samples could be reliably identified at a concentration of 4×10^4 CFU mL⁻¹. On further increasing the culture time to 6 hours, the identification scores further improved (Fig. S4†). *E. coli* could be identified at a concentration as low as 4×10^3 CFU mL⁻¹. Currently, the methods for direct bacterial analysis reported in other literature can only identify bacteria with a concentration of $\geq 10^5$ CFU mL⁻¹.^{30,31} Our method shows better performance for bacterial identification at extremely low concentrations. Compared to nanoparticle-based methods,^{12,32} the present method does not need complex sample pre-treatment steps. Bacteria in urine samples could be reliably identified at a lower concentration after undergoing incubation for a short duration. Usually, the threshold of bacterial numbers was set

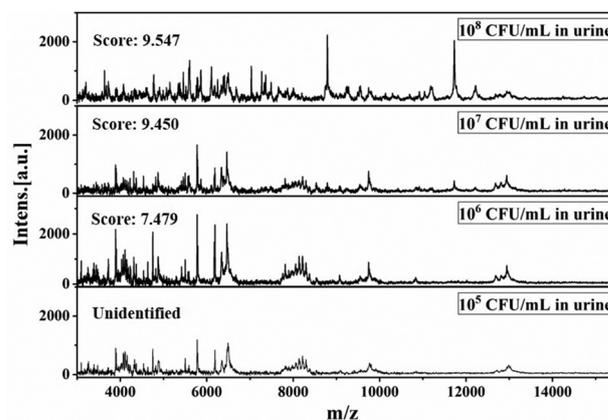


Fig. 6 MALDI-TOF MS spectra of *E. coli* captured by the McSiNWs-Con A substrate in urine samples spiked with different concentrations (0.5 mL).

Table 1 MALDI-TOF MS identification scores for *E. coli* at different counts in urine samples

| | 37 °C, 4 h | | | | 37 °C, 6 h | |
|---|---------------------|---------------------|---------------------|---------------------|---------------------|---------------------|
| Initial concentration in the urine sample (CFU mL ⁻¹) | 4 × 10 ⁶ | 4 × 10 ⁵ | 4 × 10 ⁴ | 4 × 10 ³ | 4 × 10 ⁴ | 4 × 10 ³ |
| Scores before culture | 7.479 | Unidentified | — | — | — | — |
| Scores after culture | 9.493 | 9.292 | 9.035 | 6.627 | 9.348 | 8.170 |

at 10⁴ CFU mL⁻¹ for uncomplicated UTIs and ≥10⁵ CFU mL⁻¹ for complicated UTIs.³³ Thus, the sensitivity of the present approach is sufficient for fast UTI diagnosis.

Conclusions

In summary, we established a method for fast bacterial identification using a McSiNW microfluidic chip coupled with MALDI-TOF MS detection. The synergistic effect of the surface modification and micro-nano structure of the McSiNW substrate greatly increased the bacterial capture efficiency. Together with the enhanced effect of SiNWs on laser desorption ionization efficiency, the mass fingerprint of bacteria can be sensitively acquired. Culture-free identification of low concentration bacteria in PBS could be achieved within 1 hour including 43 minutes for bacterial enrichment and 15 minutes for MALDI-TOF MS sample preparation and identification. As for the urine sample, bacteria at a concentration as low as 10³ CFU mL⁻¹ could be identified if the sample was cultured for 4–6 hours. Compared to the traditional urine culture methods, the time for bacterial identification is greatly shortened. Since Con A and vancomycin interact with a wide range of Gram-negative and Gram-positive bacteria, respectively, the application of this platform may not just be limited to the bacteria tested in this work. The potential use of the platform could be further expanded by developing new surface modifications (e.g., aptamer and antibody). Moreover, multiple bacteria might be identified simultaneously by loading urine samples sequentially through an array of McSiNW chips modified with different affinity moieties. Therefore, the McSiNW microfluidic chip coupled with MALDI-TOF MS might be a universal platform for fast bacterial identification in liquid samples.

Statement

Informed consent was obtained from each volunteer and the trial was performed in accordance with the guidelines of the Second Affiliated Hospital of Zhejiang University (Ethical review No. 2019-262).

Author contributions

Yuxin Li: conceptualization, data curation, formal analysis, methodology, software, validation, writing – original draft. Tao Wang: methodology, writing – review and editing. Jianmin Wu:

conceptualization, funding acquisition, project administration, resources, writing – review and editing.

Conflicts of interest

There are no conflicts to declare.

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