ABSTRACT: The shape and structure analyses capability of nanopore is powerful and complementary to mass spectrometry analysis. It is extremely attractive but challenging to integrate these two techniques. The feasibility of combining nanopore electrospray with mass spectrometry was explored in this study. A nanopore effect was observed during the nano-electrospray of single bacterium, through which the shape and dimension of a single bacterium could be obtained. Molecular information on these bacteria was then acquired by analyzing these bacteria deposited on the counter electrode through laser spray ionization mass spectrometry experiments. Proof-of-concept experiments were carried out for four types of bacteria. Results show that the combination of nanopore results with mass spectrum data could effectively improve the identification accuracy of these bacteria from 72.5% to 100%. Although initial experiments were demonstrated in this work, results showed that it is feasible and promising to integrate nanopore technology with mass spectrometry for large biomolecule studies in the near future.

INTRODUCTION

Mass spectrometry (MS) is a powerful analytical technique, which has been widely applied in various fields for the analysis of trace amounts of chemicals in complex matrices. In MS, the molecular composition of a sample is obtained by measuring the mass to charge (m/z) ratio of each ion, as well as a certain amount of ion structure information (mainly primary structure) from tandem MS. More recently, great efforts have been made to extend the mass range of MS instruments, so that direct analysis of large proteins, protein complexes, viruses, bacteria, or even cells could be achieved. Correspondingly, many new tandem MS methods have been developed, and the primary structure of biomolecules could be better resolved. Although biological functions of biomolecules heavily depend on their higher order structures, it is still a challenging problem to acquire this information conveniently. To solve this problem, attempts have been made to combine or integrate MS with structural analysis techniques, such as infrared photodissociation spectroscopy, ion mobility spectroscopy, and mobility capillary electrophoresis. Even though great advances have been made, it is still demanding to have alternative tools to resolve the complex stereostuctures of biomolecules.

On the other hand, nanopore technology is a versatile and label-free method for single-molecule detection, which has been successfully used to detect and characterize DNA and single proteins. Nanopore was first introduced as a new method for discerning the four bases of a single strand of DNA in the early 1990s. Over the past decade, significant advances have been achieved in terms of structural analysis of biomolecules. It has been demonstrated that nanopore can be used to characterize proteins and protein complexes, and it is sensitive enough to distinguish the folding state of a protein. Nanopore is actually an excellent confined space, and the electrochemically confined effect allows the wide application of nanopore structure in measurement science including the MS field. The nanoscale confined space provided by a nanopore enhances the electrostatic interaction between analytes and the sensing interface, which is suitable for chemical analysis at the single molecule level. Great efforts have been made to couple nanopore technology with MS. For instance, researchers have been utilizing nanopore to distinguish molecules with different masses/sizes, which resemble the function of MS.

Since the structure analysis capability of nanopore is complementary to the MS technique, it is highly attractive to combine nanopore technology with MS, so that three-dimensional (3D) structure information, the m/z ratio, and primary structure information on molecules in a sample could be obtained at the same time. However, great challenges are present. By monitoring the translocation event of a single analyte particle or molecule through a nanopore (typically located on a surface or a sharp tip), structure/size information on every individual particle/molecule can be obtained.
Nanopore experiments are conventionally performed in a liquid environment, and the translocation event is on the time scale of approximately millisecond. On the other side, MS is a gas phase technology, and samples/analytes need to be gasified and ionized before performing MS analysis, which is fast and on the time scale of a microsecond or less. In addition, sensitivity and mass range of conventional MS instruments may not be able to detect a single ion with heavy molecular mass. Therefore, in order to combine these two techniques, key parameters of these two technologies, such as detection speed of a nanopore device, sensitivity, and mass range of a MS instrument, might need to be improved.

In this study, a proof-of-concept design and workflow were proposed to combine the results of nanopore electrospray with MS. As a very initial exploration, a nanopore effect was observed at the emitter tip of a nano-electrospray ionization (nESI) source during the analysis of single bacterium. It is found that the passing event of a single bacterium through the emitter tip would induce a pulse (~4 nA) on the electrospray current, which lasts about 10–20 μs. Since a state-of-the-art current amplifier may still not satisfy both gain and bandwidth requirements, a data processing and signal reconstruction method was established to capture this transient pulse and neutralize signal distortions. With this method, the spheroid shape of each bacterium was extracted from their corresponding transient pulses. To acquire their molecular information, bacteria sprayed from the nESI source were collected on a metal plate and then analyzed by a home-built laser spray ionization (LSI) miniature mass spectrometer. Metabolites and some other small molecules on the cell wall were identified in the mass spectra. In this work, four types of bacteria were analyzed. By combining bacteria geometrical results with their molecular information, bacteria identification accuracy was improved from 72.5 to 100%, suggesting the feasibility and advantages of combining nanopore technology with MS.

### RESULTS AND DISCUSSION

Figure 1 shows a schematic diagram of the experimental setup. After samples will filled into a nESI capillary, an electrode was inserted into the capillary, and a negative high voltage (~ −0.7 kV) was applied onto it serving as the ionization voltage. The nESI capillary was then placed in front of a stainless-steel plate to collect the sprayed ion current, as well as the sprayed bacteria. The ion current signals were then amplified with a current amplifier (Keithley 428) and recorded with a Tektronix oscilloscope at a sampling frequency of 1 MHz. In conventional nESI experiments, a constant electrospray current would be observed. Similarly, a constant electrospray current was also observed in our setup when a blank solution was filled in the nESI capillary. However, since a bacterium has a comparable size with that of the emitter orifice (~1.5 μm), a bacterium would partially block the electrospray current path when it migrates through the tip of the nESI emitter. Therefore, when diluted bacteria solutions were filled in the capillary, an ion current with inverted peaks would be observed as shown in Figure 1a. Each peak corresponds to the event that a bacterium is passing through the emitter tip.

After mixing with matrix (3-NBN), bacteria deposited on the stainless-steel plate then underwent analysis using a home-developed laser-spray ionization miniature mass spectrometer in the positive ion mode (Figure 1b). Details about the MS instrument setup could be found in our previous literature studies. Briefly, bacteria were quickly heated and fragmented by the pulsed laser. Small molecules from bacteria together with matrix molecules were then desorbed from the surface of the metal plate. It is believed that ionizations happened during the molecule transfer process from the atmosphere environment into the vacuum region. In this study, pulse frequency and energy of the laser were set as 10 Hz and 1.2 mJ/pulse, respectively. The mass spectrometer was scanned over a range of 200–900 Th. To minimize experimental errors, at least 20 mass spectra were collected for each bacterium for multivariate statistical analysis.

Nanopore has been utilized to measure the geometrical shape of individual protein molecules, which is highly complementary to MS results. It would be beneficial and attractive to merge nanopore and MS techniques. However, great challenges are present. In conventional nanopore experiments, liquid environments present on both sides of the nanopore membrane. Driven by a small voltage difference, molecules could migrate from one side to the other side of the membrane through a nanopore. When size of the molecule is on the same scale of the nanopore, this passing event would change equivalent impedance of the nanopore, which induces a current variation. The geometrical shape of the molecule was then calculated by analyzing this current variation. In the case of nESI, the opening of the emitter tip...
shown in Figure 2a top, the electrospray current would be partially blocked when a bacterium is passing through the emitter tip, which results in an inverted current pulse on top of the stable electrospray current. Duration of this current pulse is related to the length of the bacterium \( (a) \), while its relative amplitude \( (\Delta I/I_0) \) correlates with the bacterium radius \( (b) \). Specifically, the shape of this current pulse could be described as,

\[
\frac{\Delta I}{I_0} = k \frac{\Delta S}{S} = k \frac{b^2 \left( 1 - \left(\frac{z_0}{r_0}\right)^2 \right)}{r_0^2}, \quad 0 \leq t \leq \frac{2a}{v}
\]

in which \( S \) is the area of the ESI tip cross-section and \( r_0 \) is its radius, \( k \) is a proportional constant, \( v \) is the migrating speed of the bacterium when it is passing the ESI tip, and \( t \) represents time. In this work, \( S \approx 1.77 \, \mu m^2 \), \( r_0 \approx 0.75 \, \mu m \), \( k \approx 1.0 \). The flow rate of the nESI was measured as \( 1.5 \, \mu L/h \), so \( v \) is estimated to be \( 0.24 \, m/s \). Derivation of this equation could be found in the Supporting Information.

Equation 1 describes the theoretical peak shape induced by the passing event of a spheroid through the ESI tip. Under the electrophoretic force, a charged protein passes through a nanopore at a constant drift velocity. This velocity is determined by the nanopore dimensions and the translocation time, which is usually considered as \( mm^{-1} \). However, in the case of nESI, the migration speed of a target particle (bacterium) would be much faster (>100 times) than that of a protein through a conventional nanopore, which requires an amplifier with a bandwidth >236 kHz. Up to now, there is no low noise current amplifier available that could cover such a broad bandwidth. In order to have enough gain (10^7) for small current detection, the Keithley current amplifier only has a bandwidth of 39.42 kHz. With limited bandwidth, a distorted peak would be observed, which is labeled as the expected signal in Figure 2a bottom. Theoretical expression of this distorted peak is essential to characterize the shape of a bacterium, which could be obtained by performing convolution between input signal \( (eq 1) \) and transfer function of the current amplifier \( (H(t)) \) in time domain. The voltage-frequency characteristic curve of the current amplifier was first measured (Figure 2a, middle), and transfer function of the amplifier was then acquired by fitting this voltage-frequency characteristic.
curve with the mathematic expression of a second-order amplifier (please refer to Support Information for details).

\[
\Delta I = k \times \text{Conv}
\left[ \frac{1}{1 - \left( \frac{-a + b}{a} \right)^2} \right] H(t), \ t \geq 0
\]

(2)

Figure 2b top shows a typical nESI current chart collected for bacteria, in which each inverted pulse corresponds to the event that a bacterium passing through the emitter tip. The detection circuit has a sampling rate of 10 M/s, and a signal-to-noise ratio (SNR) of \(\sim 12\) was achieved. As shown in Figure 2b, a data processing procedure was developed to determine the spheroid dimension of each individual bacterium. Each peak was first inverted and then normalized by the constant current amplitude \(I_0\). After this normalized peak was fitted using eq 2, length and radius of each bacterium could be obtained.

Figure 3a plots the measured spheroid dimensions of four types of bacteria with this nanopore nESI, in which each dot represents an individual bacterium. The scatter distributions of experimental results calculated from current pulses were centered around the theoretical values within the margin of error. For instance, the measured dimensions of Escherichia coli MC-5 (0.6–0.7 by 1.2–2.0 \(\mu\)m, Figure 3a-I), Pseudomonas aeruginosa (0.6–0.7 by 1.4–2.3 \(\mu\)m, Figure 3a-II), Staphylococcus aureus (0.7–0.8 by 0.7–1.0 \(\mu\)m, Figure 3a-III) are in good agreement with their theoretical dimensions, which are (0.5 by 1.0–3.0 \(\mu\)m), (0.5–0.8 by 1.5–3.0 \(\mu\)m), and (0.8 by 0.8 \(\mu\)m), respectively. However, the measured sizes of Bacillus cereus using the nanopore nESI (0.9–1.0 by 0.7–1.0 \(\mu\)m, Figure 3a-IV) are much smaller than their theoretical sizes, which are (1.0–1.2 by 3.0–5.0 \(\mu\)m). The Bacillus cereus sample was then examined under a microscope. It is found that a large number of spores presents in the sample, and most of the mature bacteria aggregate into bacteria chains, preventing them from translocating through the nESI emitter. Therefore, the measured dimensions in Figure 3a IV are the dimensions of Bacillus cereus spores. In this case, the measured results (0.9–1.0 by 0.7–1.0 \(\mu\)m) agree well with the theoretical dimensions of Bacillus cereus spores (1.0 by 1.0 \(\mu\)m).

Morphology is an important feature of microorganism, and dimensions are one of the most direct indications for bacteria differentiation. Differentiation of these four types of bacteria was first performed using their size data shown in Figure 3a. All data were first classified to determine the category centers using the canopy algorithm, and then further classified by the K-means algorithm. The K-means uses iterative refinement to get a final clustering result, which has a risk of instability.65 To overcome this limitation, a precluster step based on the canopy clustering was carried out before the K-means algorithm.66 The prepartition results from canopy clustering algorithm could pass the appropriate center points to K-means, which improve the stability of K-means. Detailed program procedure is found in the Supporting Information. As shown in Figure 3b, data points are divided into four categories by the algorithm: Escherichia coli MC-5 (blue), Pseudomonas aeruginosa (green), Staphylococcus aureus (red), and Bacillus cereus (black). In particular, misidentified data points are plotted in gray. Classification accuracy of the algorithm is \(\sim 72.5\%\), suggesting that morphology is an important parameter for the differentiation of bacteria at the genus level. However, it is hard to differentiate bacteria with similar shape and dimensions, such as Escherichia coli MC-5 and Pseudomonas aeruginosa.

Conventionally, nESI is an ionization method for a mass spectrometer, through which molecular components within a liquid sample could be analyzed. The data obtained by nanopore ESI and MS would be complementary to each other, and it is highly desired to combine these two techniques together. Although a particle mass spectrometer exists, there is no mass spectrometer available to us that could couple with a nanopore nESI source for the direct analysis of bacteria. As a proof-of-concept experiment, bacteria deposited on the metal plate were further analyzed by LSI mini MS. Figure 4a displays representative mass spectra of four bacteria in the positive ion mode. Small metabolite and lipid ions were observed in the mass spectra. Generally, most of the peaks observed in the 690–800 Th range are related to lipids, which exist in the cell wall and membrane of bacteria. In accordance with previous reports, the peaks at \(m/z\) 690 Th refer to phosphatidylethanolamine PE (16:0/16:1).71 Furthermore, metabolites of bacteria could also be observed in the spectra, which also play important roles in bacterial differentiation. Specifically, two peaks at \(m/z\) 248 and 278 Th observed in the mass spectra of Pseudomonas aeruginosa and Escherichia coli MC-5 are teichoic acid and compositions of peptidoglycan on bacteria cell walls.72 The mass peak at \(m/z\) 308 Th observed in the mass spectra of Staphylococcus aureus and Bacillus cereus (spore) refers to glutathione.71

The combination of bacteria shape results obtained from nanopore experiments with their molecular information obtained from MS analysis would greatly improve identification accuracy of bacteria. To differentiate these bacteria based on their shape and mass spectrum data, principal component analysis (PCA) was first applied to reduce the high
order data set to a two-dimensional data set. Then, the same cluster algorithm/procedure was used to process the two-dimensional data for clustering, which is using the canopy algorithm to determine category centers and the K-means algorithm for further data classification. As shown in Figure 4b, these four types of bacteria could be well separated in the plane of PC1 and PC2, and the accuracy of classification becomes 100%. Figure S6 also plots the classification results using the MS data only, in which only small differences were observed when MS data solely or shape plus MS data were used. Several reasons would contribute to this particular phenomenon. (1) The chemical information obtained from MS is rich and typically sufficient to classify the type of bacteria. As a result, although the shape information is different and complementary to the chemical information from MS, MS data are overwhelming over the shape information in bacteria identification. The shape information obtained here is a two-dimensional data, length, and radius. On the other hand, many metabolite and small molecules within a bacterium were detected by the MS, which becomes a high-order multidimensional data (~800 variables within the mass range 200–900 Th at a unit mass resolution). (2) Intrinsically, shape of a bacterium is not critical for bacteria differentiation, especially when compared with the molecule composition information. As we know, genetic differences are the key for bacteria differentiation. Therefore, gene sequencing is the gold standard for bacteria identification, and this genetic information could translate into proteins (thus metabolites and the corresponding small molecules), which enable the use of MS for bacteria identification. However, different bacteria could have similar shapes and dimensions, and even the same type of bacteria may have different dimensions at different growth stages.

In many other cases, the coupling of nanopore electro spray and MS would be important, and sometimes essential in biomolecule analyses. For example, the primary sequence of a protein could be well measured by a mass spectrometer. However, by keeping the same primary sequence, a protein could have different conformations under different environments or disease conditions. In such cases, the capability of measuring its shape and geometric dimension would be critical in differentiating protein conformations, which becomes essential in understanding its biological function or in disease diagnosis. Therefore, we believe the coupling of nanopore electro spray and MS would greatly enhance protein and protein complex analyses, and continuous efforts need to be placed to extend this technique for biomolecule analyses. To extend this technique to protein complex or protein analyses, we need to develop more sensitive current detection circuits with broader bandwidth and preferably a MS instrument with single ion detection capability.

## EXPERIMENTAL SECTION

### Chemicals and Materials.

NESI ionization emitters are made from borosilicate capillaries (1.5 mm o.d./0.8 mm i.d.) by pulling their tips to i.d. of 1.5 ± 0.1 μm using a Flaming/Brown micropipette style puller (model P-1000, Sutter Instrument Inc., USA). A scanning electron microscopy (SEM) image of the emitter tip was also shown in Figure S1 in the Supporting Information. Ammonia was purchased from Sigma-Aldrich (St. Louis, MO). Deionized water was purchased from Wahaha (Hangzhou, China). All samples were diluted in NH₃H₂O (pH = 8.7). 3-Nitrobenzonitrile (3-NBN) was purchased from Acros Organics (Geel, Belgium). The matrix (0.1 mg/μL) was prepared by dissolving 3-NBN into acetonitrile.

### Microorganism Culturing.

In this work, four kinds of bacteria (Escherichia coli MC-5, Pseudomonas aeruginosa, Staphylococcus aureus, Bacillus cereus) were studied. Bacteria samples, stored at −80 °C in magnetic bead preservation tubes, were activated for culturing and subculturing. All microorganisms were cultured on MRS agar plates and incubated at 36 °C for 24 h. Bacteria culturing experiments were carried out in biological safety cabinets. Bacteria samples cultured on MRS agar plates were collected by a sterile inoculation loop in NH₄H₂O solution (pH = 8.7) to make a suspension with a final concentration of about 4.0 × 10⁴ cfu/mL. NH₄H₂O solution ensures that bacteria are negatively charged, and the Coulomb force would repel bacteria away from each other. Bacteria dilution in a NH₄H₂O solution could minimize bacteria aggregation and adhesion to the channel wall, which helps preventing bacteria chains blocking the emitter tip.

![Figure 4. (a) The mass spectra of four types of bacteria in the positive ion mode. Species are indicated by color and Roman numerals: (I) Escherichia coli MC-5, (II) Pseudomonas aeruginosa, (III) Staphylococcus aureus, (IV) Bacillus cereus (spore). (b) Bacteria differentiation based on their shape and mass spectrum data; 2D PCA score plots of PC1 against PC2.](https://dx.doi.org/10.1021/acssentsci.0c00622)
In this work, it has been demonstrated that nanopore effects do exist in nESI sources, and geometrical results from nanopore nESI measurement are complementary to the $m/z$ ratio information from MS analysis. As a proof-of-concept exploration, four types of bacteria were first analyzed by the nanopore nESI to acquire their spheroid dimensions. Deposited bacteria on a metal plate then underwent LSI-MS analysis, in which molecular information (mainly metabolites) and their spheroid dimensions could be obtained by measuring the current perturbation induced by the passing event of a single particle, the passing speed of a particle through a nESI emitter is $>10^3$ times faster than that in a conventional nanopore experiment. This poses a great challenge for the current detection circuit in terms of sensitivity and bandwidth. As shown in this work, a state-of-the-art current amplifier could be used to track the passing event of a bacterium through a nESI emitter. A current amplifier with $\sim >100$ times broader bandwidth is required to track the passing event of a protein ion. Either alternative detection methods or advanced current detection chips would enable the direct coupling of nanopore ESI with MS instruments, and their coupling would greatly enhance protein complex and protein analyses in the near future.

**ASSOCIATED CONTENT**

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acscentsci.0c00622.

Theoretical modeling and finite elementary modeling (FEM) simulations of the passing event of a bacterium through a nESI tip, transfer function of the amplifier, the clustering algorithms (PDF)

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Notes

The authors declare no competing financial interest.

**ACKNOWLEDGMENTS**

This work was supported by NNSFC (Nos. 21922401, 21827810, 61635003), MYHT (No. D030207).

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