Published in final edited form as:

Nanomedicine. 2019 April ; 17: 246–253. doi:10.1016/j.nano.2019.01.015.

Nanotube Assisted Microwave Electroporation for Single Cell Pathogen Identification and Antimicrobial Susceptibility Testing

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Abstract

A nanotube assisted microwave electroporation (NAME) technique is demonstrated for delivering molecular biosensors into viable bacteria for multiplex single cell pathogen identification to advance rapid diagnostics in clinical microbiology. Due to the small volume of a bacterial cell (~femtoliter), the intracellular concentration of the target molecule is high, which results in a strong signal for single cell detection without amplification. The NAME procedure can be completed in as little as 30 minutes and can achieve over 90% transformation efficiency. We demonstrate the feasibility of NAME for identifying clinical isolates of bloodborne and uropathogenic pathogens and detecting bacterial pathogens directly from patient's samples. In conjunction with a microfluidic single cell trapping technique, NAME allows single cell pathogen identification and antimicrobial susceptibility testing concurrently. Using this approach, the time for microbiological analysis reduces from days to hours, which will have a significant impact on the clinical management of bacterial infections.

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Graphic abstract

A nanotube assisted microwave electroporation (NAME) technique is demonstrated for rapid microbiological analysis of pathogenic bacteria. NAME delivers molecular biosensors into viable bacteria for multiplex single cell pathogen identification and antimicrobial susceptibility testing. The technique determines the species in as little as thirty minutes and perform antimicrobial susceptibility testing in less than three hours.

Keywords

infection; antibiotic resistance; bacteria; urinary tract infection; microfluidic

Background

The emergence of multidrug-resistant pathogens is a major healthcare threat and the annual associated healthcare cost is over \$20 billion.¹ For example, several Gram-negative pathogens, including *E. coli*, *P. aeruginosa* and *K. pneumoniae*, are common causes of healthcare-associated and community-acquired infections (e.g., pneumonia, urinary tract infection and bloodstream infections). Increasingly, these bacteria are found to be resistant to first-line and second-line antibiotics. The conventional culture-based assays in clinical microbiology require at least 2-3 days and can be even longer for slow-growing bacteria. This significant delay in microbiological analysis leads to empiric broad-spectrum antibiotic usage by healthcare providers resulting in unnecessary treatment, ineffective antibiotic selection, and the potential for poor clinical outcome. Furthermore, the very use of these drugs creates a selective pressure that leads to the emergence of multidrug-resistant pathogens. For these reasons, novel technologies for rapidly identifying bacterial pathogens and their antibiotic resistance profiles will have a significant impact on patient care and antimicrobial stewardship.²

In a typical clinical microbiology workflow, the first step involves receipt and processing of a specimen for culture. Culture based methods, which can include colony morphology (form, elevation, and appearance), Gram stain, and biochemical or mass spectrometry

phenotyping, take days to finalize the identification of the bacteria. The culture procedure represents a major time limiting step for the microbiology workflow. To perform antimicrobial susceptibility testing (AST), additional broth microdilution cultures phenotypically determine the growth of the culture isolate in the presence of serial dilutions of antibiotics. These AST procedures typically require an additional 1-2 days and a laboratory setting. Molecular approaches, such as multiplex PCR, can either be performed directly from specimens or bacterial isolates. Compared with culture-based methods, molecular analysis is capable of identifying pathogens rapidly because the target gene can be amplified much faster than natural bacterial growth. However, amplification techniques typically do not quantify a clinically relevant concentration of viable bacteria or provide robust AST results. They also require moderate to high complexity testing. These issues limit their practicality in point-of-care diagnostic applications. Recently, molecular probes, droplet microfluidics, digital LAMP quantification, and electrochemical biosensors have enabled culture-free detection of bacteria in physiological samples with minimal processing.³⁻⁹ Integrated pathogen identification and AST systems have also been demonstrated by high-resolution melt-curve analysis and electrochemical biosensors.^{10, 11} Nevertheless, the cost, complex procedures, bulky instrumentation and the intensive labor requirements present challenges for rapid microbiological analysis. A broad-based technique for comprehensive microbiological analysis in resource-limited settings remains an elusive goal.^{2, 12}

In this study, we investigate an approach for single cell pathogen identification and AST by transforming (i.e., delivering) molecular biosensors into viable bacteria using a nanotube assisted microwave electroporation (NAME) technique (Figure 1A). The carbon nanotube serves as an antenna for coupling microwave energy to generate a highly localized electric field for microwave electroporation.¹³⁻¹⁵ Single cell pathogen identification is achieved by intracellular delivery of double-stranded nucleic acid probes that target species-specific regions of the bacterial 16S rRNA (Figure 1B).^{3, 16, 17} In this homogeneous sensing scheme, hybridization with the target 16S rRNA displaces the quencher probe from the donor probe allowing the fluorophore to fluoresce. Fluorescence is detected only when the specific probe is hybridized to the target in the bacterium (Figure 1C). In particular, we investigate the NAME technique for identifying bacterial pathogens at the single cell level. We evaluate the NAME protocol for detecting bacterial pathogens isolated from clinical urine and blood culture. We also study direct detection of bacteria in patients' urine samples. Furthermore, we apply NAME for single cell pathogen identification and AST by incorporating a microfluidic single cell trapping device.¹⁸

Methods

Molecular probe design and preparation: Four double-stranded probes were used in this study (Supplementary Table S1). Each probe consisted of two DNA strands. The donor strands were 22-24 nucleotides long and complementary to the target RNA of interest.^{19, 20} The fluorophores, 6-FAM (Fluorescein) or 6-TAMRA (NHS Ester), were labeled at the 3' end of the donor strands. The quencher strands were 11-12 nucleotides in length. The dark quenchers, Iowa Black FQ for 6-FAM or Iowa Black RQ for 6-TAMRA, were labeled at the 5' end of the quencher strands immediately adjacent to the fluorophores on the donor

strands. The probes were synthesized by Integrated DNA Technologies (Coralville, IA). Other reagents were purchased from Sigma Inc. (St. Louis, MO) unless otherwise specified. To conduct the assay, the probes were made by mixing the fluorophore and quencher strands in the buffer solution, which contains 10 mM Tris-EDTA with 100 mM NaCl at a 1:3 molar ratio, at 95°C for 5 minutes. The mixtures were then allowed to cool down to room temperature slowly. The final concentration of the probes in the transformation experiment was 1.5×10^3 nM.

Clinical samples:

Unidentified positive urine samples and clinical isolates including three *Escherichia coli* (urine), *Escherichia coli* (blood culture), *Pseudomonas aeruginosa* (urine), and *Klebsiella pneumoniae* (urine) were obtained from the clinical microbiology laboratory at the Veterans Affairs Palo Alto Health Care System (VAPAHCS) or the clinical microbiology laboratory at the Penn State Hershey Medical Center (Supplementary Table S2). The procedure was approved by the Pennsylvania State University Institutional Review Board. Bacterial transformation by NAME: Bacteria were grown in Mueller Hinton Broth. To transform the double-stranded probe into bacteria, 100 µl of bacterial sample was centrifuged at 4500 rpm for 5 minutes and washed twice with phosphate buffer (PBS 1X). The multiwall carbon nanotubes that are functionalized with a carboxyl group (COOH) have a diameter of 30 ± 15 nm and a length from 1 to 5 µm (NanoLab, Inc.). The nanotube solution (30 mg/ml) was filtered with a 1 µm microfilter. The electric field enhancement can be estimated using the following equation: $E/E_0 = \alpha \cdot L/D$, where E is the electric field near the nanotube tip, E_0 is the uniform electric field in the oven, $\alpha=3$ is a constant, L and D are the length and diameter of the nanotube.¹³⁻¹⁵ The aspect ratio of the nanotube was ~30 in this study. The local electric field was enhanced by ~90 times at the tip of the nanotube. The pellet was resuspended into 100 µl filtered nanotube solution and incubated for 10 minutes at room temperature. Then, 10 µl molecular probe was added and incubated at room temperature for 10 minutes. The sample was in a conical centrifuge tube. The material of the tube was polypropylene and the volume was 0.65 ml. The sample was incubated in ice for 5 minutes and then put into a microwave oven (General Electric JES738WJ 02, 2.45 GHz) for 10 seconds. The microwave treated bacteria was incubated at room temperature for 30 minutes and washed 3 times with PBS (1X) to remove the extra probes and resuspended in PBS for imaging.

Bacterial counting and AST testing on Microfluidic Device: The bacteria suspension (1 µl) was loaded onto a microscope slide and covered with a cover glass for imaging and counting using a fluorescence microscope (Leica DMI 4000B, objective 40X). The transformation efficiency was estimated by the number of bacteria with an observable fluorescence intensity over the total number of bacteria (bright-field). To conduct AST, the transformed bacteria were loaded onto the microfluidic device by capillary force. The microfluidic device was mounted onto a heated stage for microscopic observation.

Statistical analysis: Statistical analyses were performed with GraphPad Prism 5 software. The data were analyzed using one-way analysis of variance and Tukey's post-hoc test. Data represent mean $\pm$ s.e.m.

Results

We developed a microwave electroporation approach for transforming molecular probes into viable bacteria for intracellular sensing and pathogen identification (Supplementary Figure S1). Multiwall carbon nanotubes, which couple and localize the microwave energy, can enhance the electric field in a manner similar to a lightning rod for microwave electroporation with a minimal effect on the cell viability.^{14, 15} To evaluate the feasibility of multiplex single cell pathogen identification using NAME, two molecular probes targeting the species-specific regions of bacterial 16S rRNA of *E. coli* (EC probe) and *P. aeruginosa* (PA probe) were designed and transformed into the bacteria (Supplementary Tables S1-2). The performance of the double-stranded nucleic acid probe was first calibrated for use in homogeneous sensing (Supplementary Figure S2). For multiplex detection, the PA probe was labeled with 6-carboxyfluorescein (6-FAM) and the EC probe was labeled with 6-carboxytetramethylrhodamine (6-TAMRA). Uropathogenic *E. coli* and *P. aeruginosa* were transformed with both probes for pathogen identification (Figure 1D). For samples with *E. coli* only, the fluorescence signal from the EC probe (red) was clearly observed in the bacteria while emission from the PA probe (green) was not observable. In contrast, *P. aeruginosa* displayed a high fluorescence signal from the PA probe (green) but not from the EC probe (red). Multiplex detection of both *E. coli* and *P. aeruginosa* was demonstrated by mixing the bacteria together. These results demonstrate the feasibility of NAME for multiplex pathogen identification, including polymicrobial samples.

A high transformation efficiency (i.e., percentage of bacteria that are transformed with probes) for single cell pathogen identification using intracellular probes will facilitate quantification of the bacteria concentration, which is important for detecting flora contaminations and polymicrobial infections.²¹ To study the influence of nanotubes on the transformation efficiency, the microwave electroporation procedure was performed with and without multiwall carbon nanotubes (Figure 2A). Fluorescence intensity from bacteria was only observed in samples with nanotubes, supporting the notion that nanotubes enhance the performance of microwave electroporation. The effect of the duration of microwave treatment on the transformation efficiency was investigated by counting the portion of bacteria with an observable fluorescence intensity (Figure 2B). Examining the results revealed that the transformation efficiency gradually increased with time and reached a high transformation efficiency (>90%) at 10 seconds. Further increases in the microwave duration did not improve the transformation efficiency. An experiment was performed using a thermal couple to measure temperature rise due to the microwave treatment (Supplementary Figure S3). The temperature rise was 9.7°C after 10 seconds of microwave treatment. We also tested the influence of the transformation solution (buffer and nanotube concentration) on the transformation efficiency and measured the ability of the bacteria to grow by agar plate culture after undergoing the NAME procedure (Figure 2C and Supplementary Table S3). We observed a low transformation efficiency ($4.35 \pm 2.5\%$) of NAME with Mueller Hinton broth and a high efficiency with deionized water ($89.3 \pm 16.2\%$). Nevertheless, NAME treatment in deionized water inhibited the growth of the bacteria in agar plates, despite the bacteria remaining motile in culture media. PBS 1X also exhibited a low transformation efficiency. Diluting PBS to 0.5X allowed a high transformation efficiency (~80-90%) while maintaining

the bacteria growth to $49.48 \pm 5.49\%$. Similarly, a high nanotube concentration enhanced the transformation efficiency and reduced the bacteria growth. We also observed that the incubation time (incubation after microwave treatment) had a significant effect on the transformation efficiency (Figure 2D). Fluorescence intensity in bacteria could be observed in as little as one minute (with a transformation efficiency of 4.4%). The transformation efficiency gradually increased with the incubation time to as high as 99.3%. Unless otherwise specified, 10 seconds of microwave treatment in 0.5X PBS with an incubation time of 30 minutes was applied for the rest of the study.

We applied NAME to identify bacteria isolated from clinical blood and urine samples. EC and PA probes were used in this experiment. In addition, a universal (UNI) probe that targets the conserved region of the bacterial 16S rRNA was designed for detecting all bacteria (Supplementary Figure S4). The performance of these probes was evaluated with a panel of Gram-negative bacteria (*E. coli*, *K. pneumoniae*, and *P. aeruginosa*), which are common causes of bacterial infections (Figure 3A).²² *E. coli* and *P. aeruginosa* were specifically identified at the single cell level with the EC and PA probes, respectively. The universal probes detected all three species successfully. In addition to clinical isolates, we also evaluated the ability of NAME to detect bacteria from clinical samples directly. Figure 3B shows culture-free identification of bacteria from a patient's urine sample. Figure 3C shows multiplex detection of *E. coli* and *P. aeruginosa* with EC, PA and universal probes. These results collectively suggested that NAME is capable of rapid diagnosis of bacterial infections.

The viability of the bacteria was preserved after NAME pathogen identification (Supplementary Movie 1), which allows phenotypic AST in the same assay. We integrated pathogen identification and single cell AST by applying a microfluidic confinement technique (Figure 4A).¹⁸ In particular, the channel height was 0.7 μm and the width was 1.5 μm , which effectively captures bacteria in the sample. In this integrated identification and AST assay, the bacteria were transformed, loaded into the microchannels via capillary flow, and trapped in the channels for phenotypic AST. Pathogen identification with subsequent growth monitoring was demonstrated based on the fluorescence and bright-field images (Figure 4B). A delay in the bacterial growth was observed after microwave electroporation compared to the untreated control. After a recovery time of 1.5 hours, the growth of transformed bacteria could be observed. AST was implemented by monitoring the bacterial growth in the channels with and without the presence of antibiotics. Uropathogenic *E. coli*, EC137 and EC132, which are susceptible and resistant to ciprofloxacin respectively, were tested to evaluate the integrated assay (Figure 4C and Supplementary Figure S5). The growth of EC137 was inhibited in the presence of ciprofloxacin (Figure 4D). The bacteria did not display any observable growth in the channels. On the other hand, EC132 grew at a rate similar to the control group under the same condition (Figure 4E). These data suggested that EC137 was susceptible to ciprofloxacin and EC132 was resistant to ciprofloxacin. The results were consistent with clinical microbiology, broth dilution, and agar pad data (Supplementary Figure S6). These data collectively demonstrate the feasibility of performing pathogen identification and AST in a single assay with a total assay time of approximately 3 hours, compared to days in standard approaches.

Discussion

In this study, we demonstrate intracellular delivery of molecular biosensors for identifying bacterial pathogens at the single cell level. Conventional transformation methods are often limited to competent cells and have a low efficiency and cell viability.^{23, 24} We address these issues by performing microwave electroporation enhanced by multiwall carbon nanotubes. The nanotube-based delivery can be understood by the lightning rod effect, which creates a strong, localized field enhancement to induce temporary membrane disruptions to increase permeability.^{14, 15} In this study, we perform pathogen identification by transforming molecular biosensors into clinically relevant bacterial pathogens in as little as 30 minutes. This method allows culture-free, amplification-free pathogen identification at the single cell level. Unlike typical molecular biosensors that lyse the bacteria and dilute the intracellular content, our approach detects species-specific regions of the 16S rRNA inside the cells. The small volume of a bacterium (~femtoliter) leads to a high target concentration for single cell detection. In our experiment, the DNA probes were stable in the bacteria during the measurement timeframe (within one hour). Interestingly, delivery of single-stranded DNA probes (without the quencher probe) resulted in rapid degradation of non-specific probes, suggesting the hybridization of the probe with the 16S rRNA stabilizes the probes and further enhances the specificity of the assay. For applications that require intracellular detection for an extended period of time, modified nucleic acids, such as locked nucleic acids and peptide nucleic acids, should be incorporated into the design of the molecular biosensors.²⁵ These characteristics of NAME collectively enable specific pathogen identification at the single cell level.

An important aspect of the single cell analysis approach is the ability to quantify the bacteria, even in clinical samples containing multiple types of bacteria. This quantification capability is essential for distinguishing commensal flora from pathogens and identifying polymicrobial infections. Unlike normally sterile biological fluids (e.g., blood), microbiological analysis of urine, stool and respiratory secretions can often be complicated by the presence of commensal flora. Quantitative bacterial culture for urine and bronchoscopic samples determines the clinical significance of pathogenic and commensal bacteria isolated from these samples. Unlike molecular amplification approaches (e.g., PCR), NAME can estimate the bacterial concentration by counting from microscopic images. In the future, miniaturized imaging systems and automated imaging analysis can be incorporated into our platform for simplifying the imaging procedure.^{26, 27} The NAME technique will potentially eliminate the long time delay in conventional culture-based approaches and will avoid the uncertainty in the enumeration of viable bacteria in amplification-based approaches.

Intracellular detection of bacterial 16S rRNA in viable cells facilitates subsequent AST in the same assay. The data presented demonstrate the feasibility of integrated identification-AST to improve the microbiology workflow using a microfluidic single cell confinement device, which completes AST in a time scale comparable to the doubling time of the bacteria.¹⁸ Despite the recovery time (~1.5 hours), which are also reported in other transformation techniques,²⁸ integration of the microfluidic device and the NAME technique allows comprehensive microbiological analysis in approximately 3 hours. This capability

can potentially support antimicrobial stewardship and will improve clinical management of bacterial infections by reducing unnecessary treatment, accelerating de-escalation to narrow-spectrum antibiotics, and avoiding undertreatment of multidrug-resistant bacteria. Compared to other diagnostic platforms that perform both pathogen identification and AST,^{10, 11} our approach is rapid, cost-effective, and requires only a small amount of patient sample and a small number of bacteria. Further instrument development and pre-clinical studies with an extended panel of diverse bacterial pathogens and additional clinical matrices should be performed for evaluating the clinical utility of this integrated identification-AST approach.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This work is supported by NIH NIAID (R01AI117032) and Penn State Convergence of Materials and Life Sciences grant.

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Fig 1. Nanotube assisted microwave electroporation (NAME) for single cell pathogen identification.

(A) Schematic of microwave electroporation enhanced by multiwall carbon nanotubes for delivering molecular probes into viable bacteria. (B) Multicolor double-stranded probes for multiplex 16S rRNA detection. Hybridization of the target 16S rRNA with the donor probe displaces the quencher probe allowing the fluorophore to fluoresce. Fluorescence is detected only when a specific probe is transformed into the bacteria for pathogen identification. (C) Intracellular detection of bacterial 16S rRNA in viable cells enables pathogen identification and subsequent antimicrobial susceptibility testing at the single cell level. (D) Multiplex detection of *E. coli* and *P. aeruginosa* by NAME. Multicolor double-stranded probes targeting *E. coli* (EC probe, red) and *P. aeruginosa* (PA probe, green) were transformed into samples with *E. coli*, *P. aeruginosa*, or a mixture of both bacteria at 1:1 ratio. Fluorescence images with merged red and green channels (top) and bright-field images (bottom) demonstrate pathogen identification at the single cell level. Images are representative of three experiments. Scale bars, 25 μ m.

Fig 2. Transformation of double-stranded probes into viable bacteria.

(A) Overlay images of *E. coli* clinical isolates (EC137) transformed with or without multiwall carbon nanotubes. Fluorescence was observed only in samples with nanotubes. *E. coli* treated with the same microwave duration (i.e., no probe) was applied as control. Scale bars, 50 μ m. (B) Overlay images illustrating the effect of the microwave time on the transformation efficiency. Scale bars, 50 μ m. Images are representative of three experiments. (C) Effects of the transformation solution on the transformation efficiency and ability of the bacteria to grow. (D) The effect of the incubation time on the transformation efficiency (n=3).

Fig 3. Single cell pathogen identification of clinical specimens.

(A) Overlay images demonstrating detection of *E. coli*, *P. aeruginosa* and *K. pneumoniae* clinical isolates from patient urine and blood samples. Scale bars, 25 μ m. (B) Culture-free pathogen identification of patient urine samples. Scale bar, 25 μ m. (C) Multiplex detection of *E. coli* and *P. aeruginosa* with EC, PA, and UNI probes (n=3).

Figure 4. Microfluidic single cell pathogen identification and antimicrobial susceptibility testing (AST).

(A) Schematic of the microfluidic single cell AST device. Bacteria are loaded into the channels by capillary force. Physical trapping of the bacteria allows rapid phenotypic AST by monitoring the bacterial growth as the single cell level. (B) Overlay images showing pathogen identification and growth monitoring of a single bacterium (EC 137). Scale bars, 5 μm . (C) For single cell pathogen identification and AST, fluorescence detection was first performed at the beginning of the experiment. Time-lapse bright-field microscopy is performed to monitoring the growth kinetics of the bacteria in the microfluidic channel. Time-lapse images illustrate the growth of the two uropathogenic clinical isolates EC137 (ciprofloxacin susceptible) and EC132 (ciprofloxacin resistant). Yellow boxes indicate bacteria trapped in the channel. Scale bars, 5 μm . (D-E) Representative growth curves of bacteria with and without antibiotics. Each curve represents the growth of a single bacterium.