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Optimized surface acoustic wave nebulization facilitates bacterial phenotyping

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ABSTRACT

We report on the characterization of three different Surface Acoustic Wave Nebulization (SAWN) chip designs for use in mass spectrometric (MS) analysis of the bacterial glycolipid known as lipid A. We used three different statistical methods to objectively calculate MS noise level and, subsequently, signal-to-noise ratio for the purpose of choosing the optimum SAWN chip between three different designs. The best performing standing wave SAWN chip enabled MS detection of 125 fmol of the commercial standard monophosphoryl lipid A. All three chips allowed detection of lipid A extracted from 9×10^4 CFU of *Francisella novicida*. Finally, we show that SAWN-MS could be used to distinguish between different Gram-negative bacterial species based on their lipid A MS profiles, which has implications in the field of bacterial phenotyping.

1. Introduction

Mass spectrometry (MS) has been extensively used in the development of bioassays and is popular due to its high sensitivity, specificity, and speed of analysis [1,2]. Detection of charge-neutral molecules in the gas phase has long been a primary challenge in the field of MS [3] because only charged molecules can be directly detected by a mass spectrometer. Over the past three decades, two primary soft ionization technologies have fundamentally changed the field of biomolecular analysis: electrospray ionization (ESI) [4] and matrix-assisted laser desorption/ionization (MALDI) [5,6]. Although both technologies offer increased capabilities for detection of biomolecules and provide softer ionization than prior methods, each has some drawbacks, which has spurred continued development of new methods. Specifically, ESI requires a capillary for operation that is problematic for some samples due to the risk of contamination or clogging (*e.g.*, lipids and glycolipids [7], and insoluble particulates [3]). Additionally, high voltages applied on the ESI emitter pose a potential safety hazard to the user and provide an opportunity for unwanted electrochemical oxidation of sample components [8]. While MALDI is known for its salt tolerance, it can produce ions with higher energetics than ESI resulting in fragmentation of labile bonds in some molecules prior to MS analysis. Matrix ions can obscure analyte signals of interest in the low m/z range that are typically below 500 m/z depending on the matrix.

Over the years, MALDI and ESI have proven to be very useful tools in clinical and research settings. In addition, other technologies have been developed that aim at ambient ionization and to produce ions of lower energy [9–12]. The Goodlett laboratory contributed to this trend with the development of surface acoustic wave (SAW) technology for nebulization, which has applications in other related fields such as microfluidics [13] and biosensors [14]. SAW nebulization (SAWN) has proven to be a simple method for transferring analytes in solution at atmospheric pressure to the gas phase for ionization [15]. SAWN relies on radio frequency signal that are applied to piezoelectric substrates patterned with thin metalized interdigitated transducers (IDTs). The resulting high frequency SAWs travel along the surface of the piezoelectric substrate

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Table 1
Summary of MPL and *F. novicida* lipid A LODs determined by three different methods of SNR calculation.

SAWN Chip	SNR (I/N_{SD})		SNR ($I/N_{Mean+SD}$)		SNR (I/N_{RMS})	
	MPL (fmol)	*F. novicida* lipid A (CFU)	MPL (fmol)	*F. novicida* lipid A (CFU)	MPL (fmol)	*F. novicida* lipid A (CFU)
Chip 1	50	3.6×10^3	1250	1.8×10^5	500	9.0×10^4
Chip 2	50	3.6×10^3	1250	1.8×10^5	500	9.0×10^4
Chip 3	50	3.6×10^3	250	1.8×10^5	125	9.0×10^4

and transfer energy to a liquid droplet placed in their travel path. The energy transfer from surface wave to liquid results in surface rippling of the sample containing the analyte(s) and subsequent nebulization [16,17]. Several studies have reported applications of SAWN-MS ranging from peptide detection [15], lipid structural analysis [7,18], protein detection [19], and drug analysis in complex mixtures [20]. Other studies combined SAWN with microfluidics [21] and digital microfluidics for hydrogen/deuterium exchange [22]. Huang et al. have shown that SAWN produces ions with lower internal energies than ESI, highlighting SAWN as a 'soft' ionization method [23]. Another report demonstrated the coupling of SAWN with liquid chromatography separation for proteomics analysis [24]. Moreover, SAWN circumvents the use of matrix for charged particle formation from a planar surface, making it easier to optimize than MALDI.

MALDI-TOF-MS is increasingly used in clinical settings and plays an important role in microbial diagnostics [25]. This approach, embodied in the Bruker MALDI Biotyper and Biomerieux/Shimadzu VITEK MS systems, uses organism-specific mass spectral libraries of bacterial protein patterns to identify bacteria. These methods are relatively simple in their implementation, but are time intensive due to their requirements for overnight bacterial culture. This drawback resulted in continued development toward methods that can provide same day identification [22,23]. Recently, we developed a MALDI-TOF-MS method [27] that uses bacterial glycolipids for identification, *e.g.* lipid A from Gram-negative bacteria, in place of the more traditional identification based on bacterial protein patterns. Our approach requires only small amounts of Lipid A extracts directly from the sample specimen (*i.e.*, no need for enrichment through overnight culture) and allows same day identification [26]. Lipid A is the endotoxic component and membrane anchor of lipopolysaccharide in the outer leaflet of Gram-negative bacterial outer membranes and is directly involved in microbial pathogenesis and innate immune system recognition [28]. Rapid same-day detection of Gram-negative bacterial infections is of great importance in clinical settings. Patients infected with Gram-negative bacteria may develop life-threatening inflammatory complications, especially sepsis [29], through host innate immune system detection of lipid A and a consequent cytokine storm [30,31] that produces high mortality of patients even after antibiotics have cleared the infection [32].

Lipid A occurs as a mixture of molecules of related structures. Recent reports have shown that SAWN-MS facilitates the detection and analysis of lipid A by allowing facile generation of tandem mass spectra for structural characterization [7,18]. Here we provide results showing that SAWN-MS, similar to MALDI-TOF-MS [27], can be used to identify bacteria *via* their species-specific lipid A ions. We report our results with an optimized design of a standing wave SAWN chip that was previously shown to produce a 100-fold improvement in signal-to-noise ratio (SNR) compared to the first generation progressive wave SAWN chip design [19]. We validate our findings by statistical analysis. Specifically, we present our results by defining the LOD for SAWN-MS detection of lipid A for a relatively pure lipid A commercial standard and for lipid A extracted from *Francisella novicida* (*F. novicida*).

2. Materials and methods

2.1. Materials

Commercially available monophosphoryl lipid A (MPL; Avanti Polar Lipids, Inc., Alabaster, AL, USA) was used as an internal standard throughout this study. A stock solution of MPL was prepared in a 1:2 mixture of methanol/chloroform at a concentration of 1 mg/mL. *F. novicida* (U112), and the four Gram-negative species that are part of the clinically significant set of six ESKAPE pathogens, which include *Enterococcus faecium* (Gram +), *Staphylococcus aureus* (Gram +), *Klebsiella pneumoniae* (Gram −, TBE 824), *Acinetobacter baumannii* (Gram −, TBE 1022), *Pseudomonas aeruginosa* (Gram −, BE175), and *Enterobacter cloacae* (Gram −, FN2543) were grown at 37 °C overnight with shaking after picked pure colonies from agar plates. Lipid A was extracted using a rapid ammonium isobutyrate lipid micro-extraction method [33]. Bacterial cells were estimated by colony forming units (CFU) from liquid culture using standard plating methods. Serial dilutions of MPL were prepared from a MPL stock solution and diluted in a mixture of chloroform/methanol/water (12:6:1, v/v/v) followed by vortexing. *F. novicida* lipid A extracts were reconstituted in the same solvent mixture, serially diluted, and spiked with MPL in each dilution as a reference before mass spectrometric analysis. The final concentration of spiked MPL was 5 μM for each sample.

2.2. SAWN chip design and fabrication

All chips were designed to have an operating frequency of 9.56 MHz as the major criteria which was a requirement of the SAWN controller designed and supplied by our collaborator. The modifications to the design followed a multi-parameter assessment based on our original standing wave chip (Chip 1) [23]. All chips retained an interdigital spacing of 100 μm and the parameters governing the changes in the designs were focused on the number of interdigitated transducer (IDT) pairs, the width of the IDTs, and the delay region (the area between IDT pairs). The SAW generated by any IDT is a function of the IDT spacing (kept constant in the chips presented in this study), the number of IDT pairs (varied between chips), the width of the IDT (varied), and the delay region (varied between chips). The latter parameter is crucial as it defines the overlap of counter-propagating SAWs on the chip, based on the SAW wavelength. The resulting SAW has a maximum operating power that is defined by resistance the SAWN chip creates on the circuitry of the SAWN control box. The operating power presented here is the optimal power that could generate a fine mist and not exceeds the maximum operating power. The changes in the parameters are summarized in Table 1 of the Supplemental Information and briefly stated here. All chips had 100 μm wide interdigitated electrodes, spaced 100 μm apart (20 pairs for Chips 1 and 3, 10 pairs for Chip 2), and with varying apertures (the width of overlapped fingers of IDT pairs), W_{IDT} (Chips 1 and 2 = 9.9 mm, Chip 3 = 5.0 mm). The delay region was varied to be 6.2 mm (Chip 1), 4.1 mm (Chip 2), and 8.1 mm (Chip 3). All modifications to the original SAWN chip presented here (Chip 1) aimed at reducing the chip's lateral dimensions and to allow positioning the chip closer to the source inlet. The aim in the design of chip 2 was a reduction of the chip's foot-

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