Analysis of mixed biofilm (Staphylococcus aureus and Pseudomonas aeruginosa) by laser ablation electrospray ionization mass spectrometry

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Pseudomonas aeruginosa and Staphylococcus aureus are ubiquitous pathogens often found together in polymicrobial, biofilm-associated infections. This study is the first to use laser ablation electrospray ionization mass spectrometry (LAESI-MS) to rapidly study bacteria within a mixed biofilm. Fast, direct, non-invasive LAESI-MS analysis of biofilm could significantly accelerate biofilm studies and provide previously unavailable information on both biofilm composition and the effects of antibiofilm treatment. LAESI-MS was applied directly to a polymicrobial biofilm and analyzed with respect to whether P. aeruginosa and S. aureus were co-localized or self-segregated within the mixed biofilm. LAESI-MS was also used to analyze ions following LL-37 antimicrobial peptide treatment of the biofilm. This ambient ionization method holds promise for future biofilm studies. The use of this innovative technique has profound implications for the study of biofilms, as LAESI-MS eliminates the need for lengthy and disruptive sample preparation while permitting rapid analysis of unfixed and wet biofilms.

**Keywords:** laser ablation electrospray ionization mass spectrometry; biofilm; mass spectrometry; bacteria; antimicrobial peptide; anti-biofilm

Introduction

Imaging mass spectroscopy (IMS) methods allow for two-dimensional visualization of biological samples (Cornett et al. 2007; Watrous & Dorrestein 2011). IMS, when performed with ambient ionization techniques, has enabled a new approach to the efficient, rapid identification of bacterial species from intact biological samples (Hettick et al. 2006). Such methods are bringing about a new paradigm of microbiology (Vertes et al. 2012), where spatial mapping of molecules can correlate to phenotypes, allowing for chemical hypotheses to be tested (Lanni et al. 2014). The two-dimensional nature of IMS lends itself well to the analysis of biofilm.

Currently there are several different IMS techniques available for biofilm analysis, including secondary ion mass spectrometry (SIMS), matrix assisted laser desorption/ionization (MALDI), laser desorption with postionization (LDPI), desorption electrospray ionization (DESI), and laser ablation electrospray ionization (LAESI). Among the ways by which IMS methods can be compared are spatial resolution and molecular mass limitations. Higher spatial resolution allows for more detailed images of biofilm structures; mass limitation describes the highest molecular weight of biofilm molecules that can be detected. Ion beams can be focused finely to enable subcellular resolution for SIMS MS (for MALDI and LDPI it is < ~1 μm). However, because of matrix crystal sizes and sample preparation, MALDI imaging is usually performed at a resolution of ~100 μm (LDPI-MS is normally ~300 μm, LAESI spatial resolution of imaging is generally ~200 μm, and DESI is ~100–1,000 μm). The molecular mass limitations and resolution for IMS are generally anticorrelated. For SIMS, the upper limit is 0.3 kDa. LDPI is limited to molecular masses up to ~1 kDa. DESI extends the high mass limit for detection to ~5 kDa, whereas MALDI and LAESI can produce ions up to ~100 kDa.

The development of new atmospheric pressure (ambient) MS ion sources such as DESI and LAESI enables the ability to study biofilm natively, where the other techniques mentioned above fall short. The drawbacks of MALDI, LDPI, SIMS, and other ionization methods include specific requirements such as a conductive or insulating surface on which the sample must be placed, the application of an organic matrix to the sample, a lack of sensitivity below m/z 300, narrow molecule specificity, and use of an ultra-high vacuum for sample analysis (Watrous & Dorrestein 2011; Vertes et al. 2012).

LAESI-MS does not require extensive sample processing or fixation prior to analysis of biological samples (Watrous et al. 2013). Therefore it produces cleaner spectra, increasing sensitivity for small molecules such as lipids to below m/z 300 and can be performed on a variety of substrata, including glass, paper, or plastic.

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(Nemes & Vertes 2007). This recently developed technique can identify molecular ions from native bacterial samples (Nemes & Vertes 2010). Because of these attributes, LAESI-MS was tested to determine whether it would enable quick and efficient analysis of a polymicrobial biofilm, a recent topic of interest in microbiology, on a relevant surface and without disruption of the biofilm by sample preparation.

The prevalence of coinfection with *Pseudomonas aeruginosa* and *Staphylococcus aureus*, two ubiquitous opportunistic pathogens, has led to studies of polymicrobial biofilm infections and their use in model mixed biofilms (Dowd et al. 2008). *In vitro* biofilm models can be valuable tools for studying polymicrobial biofilm communities and the effect of potential therapies, such as antimicrobial peptides (AMPs). Studies have recently demonstrated that AMPs such as the human helical cathelicidin peptide LL-37 can inhibit biofilm formation by *S. aureus* or *P. aeruginosa* individually (Overhage et al. 2008; Dean et al. 2011a) and can disperse a pre-formed biofilm produced by *P. aeruginosa* (Dean et al. 2011b). This study is part of a follow-on investigation of the mechanism of this action of LL-37 on *S. aureus* and *P. aeruginosa* cocultured in biofilms.

An *in vitro* model was generated to study mixed biofilms prepared on polycarbonate filters for use in future wound model studies (Zhao et al. 2010; Dalton et al. 2011; Yang et al. 2011; Pastar et al. 2013). For this study, polycarbonate filter and glass slides were chosen as the substrata for LAESI-MS analysis.

The use of LAESI technology for the visualization of *S. aureus* and *P. aeruginosa* was investigated in a mixed biofilm. Unique mass spectral data were identified revealing signatures from each organism grown alone, as well as generating 2D images of the location of each organism within the polymicrobial biofilm. The LL-37 peptide was used as an example therapeutic treatment, showing that LAESI-MS results correlate well with traditional microbiology techniques but also produce insights not attained by conventional approaches.

**Materials and methods**

**Strains and materials**

*P. aeruginosa* green fluorescence protein (GFP) (PAO1 pTDK-GFP) and *S. aureus* red fluorescence protein (RFP) (SH1000 pAH9) were used for all experiments in this study (Flickinger et al. 2011; Schwartz et al. 2012). Bacteria were grown from frozen enumerated aliquots in trypticase soy broth with 0.25% glucose (TSBg) (Difco Laboratories, Detroit, MI, USA) at 37°C for 24 h with shaking at 200 rpm. Mannitol salt agar (MSA) for *S. aureus*, and tryptic soy agar (TSA) with triclosan or centrimide agar plates for *P. aeruginosa*, were used as selective media.

**Static biofilm characterization**

Static biofilms on 6 mm polycarbonate filters were created as previously described (Zhao et al. 2010; Dalton et al. 2011; Yang et al. 2011; Pastar et al. 2013). Briefly, 6-mm disks were cut out of polycarbonate filters (0.2 μm, GE), UV-sterilized for 30 min on each side, and placed on TSAg plates. Stocks of *S. aureus* and *P. aeruginosa* were inoculated on top of the filters in a 1,000:1 ratio at ~10⁵ CFU filter⁻¹. The filter-based biofilms were transferred every 24 h to fresh TSAg plates with forceps, with care taken to maintain the integrity of the biofilm on top of the filter. The biofilms were treated with LL-37 (10 μg in 2 μl) at 24, 48, and 72 h by applying the peptide on top of the filter-based biofilm. The minimum inhibitory concentration (MIC) of LL-37 against many common pathogens is between 32 and 96 μg ml⁻¹ (Overhage et al. 2008). The filters were then incubated for another 24 h and then sampled at 24-h intervals throughout the experiment to analyze biofilm composition. The sampled filter-grown biofilms were placed into 1 ml of 1× PBS, sonicated for 60 s, and plated in serial dilutions on selective TSA and MSA plates and the colonies were counted. Each dilution was plated three times.

Production of static biofilms in 96-well plates and the effect of LL-37 on preformed biofilm were measured as previously described (Dean et al. 2011a). Briefly, *S. aureus* and *P. aeruginosa* in a ratio of 1,000:1 CFU and totaling ~10⁵ CFU well⁻¹ were added to 200 μl of sterile TSBg. Following the formation of biofilm at 16 h, the biofilms were treated with LL-37 for 8 h at 37°C. The OD₆₀₀ nm of the cultures at 24 h was determined to measure bacterial growth prior to the crystal violet (CV) staining procedure. Biofilm production was determined using the CV staining technique (Durham-Colleran et al. 2010) with modifications, as follows. Following several washes with tap water, biofilms were fixed with Bouin’s solution (Sigma) and stained with 1% CV. Absorbance was measured at OD₆₀₀ nm, with n = 6 per concentration. Each experiment was performed in triplicate.

**Flow cell biofilm analysis**

*P. aeruginosa* and *S. aureus* mixed biofilms were grown on 0.17-mm-thick rectangular coverslips (VWR, Radnor, PA, USA) in a flow cell apparatus as previously described (Boles & Horswill 2008). Briefly, using a dual-chamber flow cell (BioSurface, Bozeman, MT, USA) and a peristaltic variable-flow mini-pump (VWR), coverslips were inoculated at a 1,000:1 ratio of *S. aureus: P. aeruginosa* for 1 h without flow, after which flow of TSBg was maintained at ~300 μl min⁻¹ at 37°C. Following 24-h biofilm formation, the coverslips were treated with either 2 μM LL-37 in 10 mM sodium phosphate buffer or 1× PBS without flow for 1 h, after which flow of TSBg was...
continued for 8 h. Flow cell analysis was performed in triplicate. Z-stacks were captured in triplicate, using confocal laser scanning microscopy (CLSM) as described below.

Confocal laser scanning microscopy
In preparation for CLSM, the biofilms were washed with 1× PBS and then fixed with 4% paraformaldehyde in 1× PBS. The paraformaldehyde solution was then rinsed with PBS, and ProLong Gold Antifade Mountant with DAPI was added and allowed to harden overnight (Invitrogen, Carlsbad, CA, USA). The biofilms were observed using a CLS microscope (Eclipse TE2000 Nikon, Tokyo, Japan) and z-stacks were obtained using a CLS microscope (Eclipse TE2000 Nikon, Tokyo, Japan) and z-stacks were obtained using EZ-C1 (Nikon). Z-stacks were obtained in triplicate from each sample and were analyzed for biomass and thickness using COMSTAT2 (Heydorn et al. 2000).

LAESI analysis
A LAESI DP-1000 Direct Ionization System (Protea Biosciences, Morgantown, WV, USA) was used for LAESI imaging of the biofilms with electrospray at 3,700 to 3,900 V. The flow rate was set to 0.75 to 1 μl min⁻¹. A 50% methanol/0.1% acetic acid electrospray ionization (ESI) solution was used for positive ion mode and a 50% methanol/0.1% ammonium hydroxide ESI solution was used for negative ion mode. Laser ablation was performed with 10–20 pulses/location at 800–900 μl with a center-to-center resolution of 200 μm to 540 μm. An LTQ Velos mass spectrometer (Thermo Scientific, Waltham, MA, USA) was used in full-scan and tandem modes. The parameters of the LAESI instrument during each run are listed in Table S1. Biofilms analyzed using LAESI were formed using S. aureus and P. aeruginosa from overnight cultures separately or together (1,000:1), on 6 mm polycarbonate filters and placed on TSAg plates (as described above). LAESI analyses were performed at least three times per condition.

A noise value was calculated for the background electrospray for each ion in each run, and the threshold for the lowest color bin was adjusted so that all representations corresponded to a signal:noise ratio of 3. All ion maps represent the summed signal intensity for a given ion at each pixel.

Ions unique to P. aeruginosa and S. aureus in the LAESI spectra from monocultures were chosen. Following the scan of the mixed biofilm filters, matrices were generated for each ion. Matrices from each condition (+/− LL-37) and ion were input into MATLAB, and filled two-dimensional contour plots were made where the ion intensity values were interpreted as the z height with regard to the xy plane. The color bar scale limits were set to 0 and the highest intensity value for each ion group ± LL-37. The correlation coefficient between matrices was determined in MATLAB, where two matrices A and B were compared with a two-dimensional Pearson’s correlation coefficient:

\[
r = \frac{\sum_m \sum_n (A_{mn} - \bar{A})(B_{mn} - \bar{B})}{\sqrt{(\sum_m \sum_n (A_{mn} - \bar{A})^2)(\sum_m \sum_n (B_{mn} - \bar{B})^2)}}
\]

where \(\bar{A}\) is the mean of A and \(\bar{B}\) is the mean of B, and \(A_{mn}\) is the value at \(A(m, n)\).

Statistics
Statistical tests, other calculations, and graphing were performed using GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA), MATLAB R2014a (The MathWorks, Inc., Natick, MA, USA), and Microsoft Excel 2011 (Microsoft Corporation, Redmond, WA, USA). Each experiment was performed in triplicate.

Results
Characterization of polymicrobial biofilm composition
Mixed biofilms of S. aureus and P. aeruginosa were grown on either a glass substratum or 6-mm polycarbonate filters. To ensure that the biofilm contained a 1:1 ratio of S. aureus: P. aeruginosa, serial dilutions of the biofilm were plated on selective media and the colonies were counted. From an initial inoculum containing 1,000:1 of S. aureus: P. aeruginosa, over the course of 96 h the S. aureus: P. aeruginosa ratio was found to decrease steadily in the filter biofilm (Figure 1). However, the number of S. aureus cells remaining after 24 h incubation was still significant (>10³ CFU filter⁻¹). By the 96-h time point there were ~10⁷ CFU filter⁻¹ of S. aureus, while for P. aeruginosa there were 10³ CFU filter⁻¹ (Figure 1). This result led to the conclusion that 24–48 h was sufficient to form mixed biofilm on polycarbonate filters at the desired ratio (~ 1:1). Knowing this, it was possible to reproducibly create mixed biofilms containing these two organisms for detailed study and characterization. Biofilms with this ratio of S. aureus to P. aeruginosa were used in subsequent experiments.

The effect of a sublethal concentration of LL-37 on mixed biofilm
Since the MIC of LL-37 against many common pathogens is between 32 and 96 μg ml⁻¹ (Overhage et al. 2008), it was expected that LL-37 would be able to disperse a mixed biofilm of these two bacteria cultured together. In assessing the effects of LL-37 peptide on an established S. aureus and P. aeruginosa mixed biofilm a significant decrease in the amount of biofilm remaining under both static and flow conditions was observed. On the filter,
with a preformed mixed biofilm under static conditions, no decrease in the total number of bacteria per filter (CFU filter\(^{-1}\)) for either \(S.\) \textit{aureus} or \(P.\) \textit{aeruginosa} was seen when treated with LL-37, confirming that peptide applied at the low concentration of 10 \(\mu\)g filter\(^{-1}\) (in 2 \(\mu\)l) was not acting by killing the bacteria (data not shown), in agreement with previous findings (Dean et al. 2011a, 2011b).

In TSBg liquid medium in a 96-well plate, LL-37 dispersed the preformed mixed biofilm in a dose-dependent manner; 2 \(\mu\)M of peptide caused a 50\% \((p < 0.05)\) decrease in CV-stained biofilm (Figure 2). Monocultured \(P.\) \textit{aeruginosa} and \(S.\) \textit{aureus} biofilms displayed similar and significant decreases, as expected \((p < 0.05)\). Interestingly, the mixed biofilm had a small but significantly larger amount of remaining biofilm with 0.5, 1.5, and 2 \(\mu\)M LL-37 \((p < 0.05)\), possibly indicating a decreased susceptibility to dispersal by the peptide (Figure 2A).

Flow-cell experiments were run under \(~\)300 \(\mu\)l min\(^{-1}\) constant flow of medium. In the flow cell experiments, the overall biomass and biofilm thickness calculated using COMSTAT2 decreased significantly \((p < 0.01)\) following treatment with 2 \(\mu\)M LL-37 (Figure 2). This \(~\)80\% decrease appeared to be a larger effect than was observed in the static biofilm experiments, potentially due to an increased capacity for the LL-37-treated biofilm to disperse and be removed under the shear forces of the flow-cell conditions. The removal of biomass from the glass substratum following treatment with LL-37 was observable as the loss of the three-dimensional structure that was present on the untreated slide (Figure 2). At the 48-h time point when the biofilm was imaged, there was still a significant amount of \(S.\) \textit{aureus} visible, which was calculated using COMSTAT2 as constituting \(~\)20\% of the total biomass (Figure 2). These results indicate the capacity of LL-37 to induce the dispersal of preformed monoculture and mixed \(P.\) \textit{aeruginosa} and \(S.\) \textit{aureus} biofilm under multiple conditions.

**LAESI**

Using LAESI analysis, the hypothesis that \(S.\) \textit{aureus} and \(P.\) \textit{aeruginosa} would self-segregate into clusters within the mixed biofilm was tested, given that microcolony formation is one of the first steps in biofilm formation, especially for \textit{Pseudomonas}. The alternative hypothesis was that the two bacteria would be fully interspersed with each other (co-localized) within a mixed biofilm.

Recently, MALDI-MS has been applied to the identification of bacteria based on their signature ionization patterns from lipids and other small molecules (Hettick et al. 2006). Similar bacterial identification was attempted using LAESI. In the monoculture biofilms, imaging experiments using negative ion mode were performed on filter biofilms, as very low signal intensity was observed for \(S.\) \textit{aureus} in positive ion mode (data not shown). Distinct ions were identified from each bacterial species, enabling spectral fragments to be clearly distinguished from one another (Table 1). Representative spectra for each species are shown in Figure 3 (and Figure S1). The ions \(m/z\) 241.4 and 941.6 were chosen to represent the distribution of \(S.\) \textit{aureus} in the mixed biofilm, and ions \(m/z\) 716.6, 281.4, and 255.3 were distinct ions from \(P.\) \textit{aeruginosa}.

Three independent LAESI runs, at distinct locations from a single filter for each treatment, were performed on the filter biofilms. The parameters of the LAESI instrument during each run are listed in Table S1. Each run consisted of analysis of mixed biofilm with or without LL-37 treatment. Ion maps were created from each run and representative maps are shown in Figure 4. With these data, mixed biofilm composition and architecture were investigated. The correlation between the intensity of \(S.\) \textit{aureus}-specific ions at every position \((x, y)\) and \(P.\) \textit{aeruginosa} ion intensity at the same positions was compared using a two-dimensional correlation coefficient. (Since ion intensity does not necessarily correlate with biofilm thickness, conclusions concerning thickness or biomass cannot be made, but only for the presence and location of the ion.) In agreement with the CLSM images obtained under flow conditions, the LAESI images show co-localization of \(S.\) \textit{aureus} with \(P.\) \textit{aeruginosa} within the mixed biofilm (Table 2). Specifically, \(S.\) \textit{aureus}\(\ m/z\) 241.4 and \(P.\) \textit{aeruginosa}\(\ m/z\) 255.3 have a correlation coefficient and standard deviation (SD) of 0.75 ± 0.16, suggesting a high level of positive correlation in the mixed
Figure 2. LL-37 activity against *S. aureus*, *P. aeruginosa*, and mixed biofilm. (A) 1.5 and 2 μM LL-37 significantly decreased both mono- and coculture preformed biofilms on microtiter plates (*p < 0.05*). (B) Mixed *P. aeruginosa* (GFP) and *S. aureus* (RFP) flow cell biofilm untreated (left) or treated with 2 μM LL-37 (right). Yellow indicates co-localization; blue indicates representative LAESI-MS spectrum from monoculture filter biofilms DAPI. (C and D) COMSTAT analysis of the flow cell imaging demonstrated that the biomass and maximum thickness of the biofilm decreased significantly following treatment with 2 μM LL-37 (*p < 0.01*). * and ** indicate *p < 0.05* and *p < 0.01*, respectively.
biofilm. Both *S. aureus* m/z 241.4–*P. aeruginosa* m/z 281.4 and *S. aureus* m/z 941.6–*P. aeruginosa* m/z 716.6 comparisons also resulted in positive correlation coefficients, while other comparisons did not. The CLSM images (Figure 2B) also revealed that *S. aureus* and *P. aeruginosa* in a mixed biofilm were significantly co-localized, indicated by a yellow color; red indicates *S. aureus*, green indicates *P. aeruginosa*, and blue is DAPI. However, in contrast to the LAESI experiments, the CLSM experiment required fixation, staining, and microscope visualization. The LAESI data thus supported the CLSM finding that *S. aureus* and *P. aeruginosa* co-localize in the mixed biofilm.

Interestingly, when the preformed mixed biofilms were treated with 2 μM LL-37, the correlation coefficient decreased in each ion–ion comparison that showed positive correlation without treatment. Overall, the correlation between the ion intensities of *S. aureus* and *P. aeruginosa* (among those with an initial positive correlation) decreased ~75% with LL-37 treatment (Table 2). For example, the correlation between the ion intensities of *S. aureus* m/z 241.4 and *P. aeruginosa* m/z 281.4 dropped from 0.72 ± 0.06 to 0.37 ± 0.46. Similarly, the correlation between ion intensities of *S. aureus* m/z 241.4 and *P. aeruginosa* m/z 255.3 dropped from 0.75 ± 0.16 to 0.54 ± 0.42 (Table 2). This noticeable difference could be caused by an asymmetrical physiological response to LL-37 treatment, among other possibilities. This is supported by the different sensitivity of the monoculture biofilms to LL-37. Figure 2A and recent studies have shown that *P. aeruginosa* biofilm is more sensitive than *S. aureus* to inhibition and dispersal by LL-37 (Dean et al. 2011a, 2011b).

An unexpected finding was the increase in the overall intensity of small species-specific ions with LL-37 treatment. *S. aureus* m/z 241.4 and *P. aeruginosa* m/z 255.3 increased their overall ion intensity significantly with LL-37 treatment (*p < 0.05*) (Figure 5). The fact that LL-37 significantly decreases the thickness and biomass of preformed biofilm, under static conditions or under flow was previously established. Thus only the remaining bacteria in the biofilm following peptide treatment are likely to be responsible for the ions produced, perhaps suggesting a physiological adaptation in those bacteria that are left.

Because of the increase in the overall intensity of certain ions upon treatment with LL-37, control experiments were performed to determine whether LL-37 itself would contribute to the ions in the biofilm spectra. LL-37 was analyzed using LAESI in negative ion mode, but there was no discernible signal intensity detected within the range of m/z 100–2000 using the same electrospray conditions as for the mixed biofilm analysis (data not shown). The result was expected, as this peptide is positively charged and the pH of the electrospray (pH 5) did not favor deprotonization of basic residues. Therefore, there was no discernible spectral contribution of LL-37 in the mixed biofilm samples analyzed by negative-mode LAESI.

### Discussion

*P. aeruginosa* and *S. aureus* cause many chronic-, diabetic-, and combat-wound polymicrobial infections often involving antibiotic-resistant strains of these organisms (Petersen et al. 2007; Murray 2008; Yates et al. 2009). Their prevalence in coinfections has led to the concept of polymicrobial biofilm infections (Dowd et al. 2008) and the creation of mono- and mixed-biofilm models containing these two organisms for *in vitro* and *in vivo* study (Zhao et al. 2010; Dalton et al. 2011; Yang et al. 2011; Pastar et al. 2013).

This study is the first attempt to use LAESI-MS to detect the co-localization of bacteria within, and the effect of treatment on, a mixed biofilm. LAESI-MS was applied directly to analyze the localization of *P. aeruginosa* and *S. aureus* in a polymicrobial biofilm formed on polycarbonate filters. Biofilms on polycarbonate filters are relevant for *in vivo* studies, and can be used as a vehicle for the application of biofilm in wound models studies (Zhao et al. 2010).

As an example anti-biofilm treatment for this study, AMP LL-37 was used. LL-37 has been shown to disrupt preformed biofilms of *S. aureus* and *P. aeruginosa* biofilms in monoculture (Overhage et al. 2008; Dean et al. 2011a, 2011b), and to cure a mixed *S. aureus–P. aeruginosa* infection of wax-moth larvae (data not shown). The mechanism by which LL-37 brings about these effects is unknown for the case of *S. aureus*, and is suggested as the dysregulation of biofilm regulatory systems and quorum sensing in *P. aeruginosa* (Overhage et al. 2008).

| Table 1. Unique ions of *S. aureus* and *P. aeruginosa* identified with LAESI-MS. |
| m/z | *S. aureus* | *P. aeruginosa* |
|-----|-------------|----------------|
| 170.2 | | |
| 173.2 | | |
| 241.4 | | |
| 255.3 | | |
| 269.3 | | |
| 281.4 | | |
| 295.3 | | |
| 297.3 | | |
| 716.6 | | |
| 927.6 | | |
| 941.6 | | |

Notes: The unique ions identified in the scans of the *S. aureus* or the *P. aeruginosa* monocultured biofilms by LAESI-MS are indicated. The cumulative pattern of unique ions identified for each species represents an ion ‘fingerprint’ of that species, and can be used to identify each species within the mixed-culture biofilms.
In order to obtain an accurate picture of the effects of LL-37 peptide on an established *S. aureus* and *P. aeruginosa* mixed biofilm, selective media methods and plating were used to ensure that the ratio of *P. aeruginosa* and *S. aureus* was ~ 1:1 while testing the effect of LL-37 treatment. This aspect of the study was essential in light of reports that *P. aeruginosa* can negatively affect the replication of *S. aureus* (Pastar et al. 2013).

On the filter, a decrease in the total number of cells of either *S. aureus* or *P. aeruginosa* per filter following treatment with 2 μM LL-37 compared to untreated samples was not observed (data not shown), suggesting that LL-37 was not antimicrobial at a low concentration, in agreement with previous findings (Dean et al. 2011a, 2011b). Next, again using traditional microbiological techniques, LL-37 treatment was shown to disperse preformed polymicrobial biofilm under both static and flow conditions, similar to the effects seen in monoculture. In some cases, not all of the biofilm was eliminated in this experiment, but the possibility of resistance to LL-37 was not investigated further in this study. Polymicrobial biofilm has been shown to promote antibiotic resistance (Vega & Gore 2014), but it is not known whether this phenomenon applies to AMPs. Using confocal microscopy of fixed and processed biofilms, *S. aureus* and *P. aeruginosa* within the mixed biofilm showed significant co-localization of the cells, rather than self-segregation.
Figure 4. LAESI-MS analysis of mixed biofilms and LL-37 treatment. LL-37 treatment alters the relative amount of the different ions (left column). Representative contour maps of species-specific ions from mixed biofilms of *S. aureus* and *P. aeruginosa* using LAESI-MS (right column). Contour maps of mixed biofilm treated with 2 μM LL-37. The color bar indicates ion abundance. The color bar scaling is normalized separately for each row so that intensities can be compared.
Table 2. Correlation between S. aureus and P. aeruginosa specific ions in a mixed biofilm.

|         | - LL-37 | + LL-37 |
|---------|---------|---------|
| Sa 241.4 Pa 255.3 | 0.75 ± 0.16 | 0.54 ± 0.42 |
| Sa 241.4 Pa 281.4 | 0.72 ± 0.06 | 0.37 ± 0.46 |
| Sa 241.4 Pa 716.6 | 0.05 ± 0.09 | 0.06 ± 0.18 |
| Sa 941.6 Pa 255.3 | 0.07 ± 0.18 | 0.08 ± 0.27 |
| Sa 941.6 Pa 281.4 | 0.03 ± 0.13 | 0.05 ± 0.25 |
| Sa 941.6 Pa 716.6 | 0.34 ± 0.15 | 0.18 ± 0.12 |

Notes: Two-dimensional Pearson correlation coefficients were calculated from the indicated m/z ion data, e.g., the comparison between S. aureus m/z 241.4 and P. aeruginosa m/z 255.3 without LL37 treatment has a mean correlation coefficient of 0.75, SD 0.16 (n = 3).

Based on these data, LAESI-MS was carried out to determine whether it would perform consistently with traditional microbiological techniques while offering additional understanding of the distribution of bacteria within the polymicrobial biofilms and the effect of anti-biofilm treatment. The first test was to determine whether LAESI could be used in a practical setting to rapidly assess the presence and relative co-localization of both species of bacteria in a filter-formed biofilm without extensive sample processing, for example, to check biofilms immediately prior to their use in a wound study, or prior to a secondary analysis. The second test was to determine whether LAESI could be quickly performed on a wet or frozen biofilm to rapidly assess the presence of both bacteria using the spectral fingerprints provided above. Furthermore, it was hypothesized that by generating a topographical map of each m/z ion, its positional information and intensity information would be easy to comprehend, and that from these data, the relative location of the bacteria within the biofilm could be determined.

With the use of mass spectral databases and identifications in imaging mass spectrometry, the probable identities of the ions used in this study can be provided. Since most of the ions observed are deprotonated fatty acids and other lipids when ESI-MS techniques are used in negative ion mode (Eberlin et al. 2010), it seemed likely that most of the major ions observed in this study would be deprotonated bacterial lipids. In previous reports m/z 281.4 has been assigned to the carboxylate ion of oleic acid (C18:1 cis-9) present in the negative ion scans of biological samples (Eberlin et al. 2011; Hsu et al. 2013). The MassBank database gives m/z 281 as the predominant peak from ESI-MS/MS for oleic acid (Horai et al. 2010). The negative ion m/z 255.3 has been assigned previously to deprotonated palmitic acid (C16:0) (Fauland et al. 2013). The negative ion m/z 716.6 may be phosphatidylethanolamine, identified previously in samples from Salmonella membrane (Dalebroux et al. 2014). Negative ion m/z 241.4 from S. aureus in other studies has been attributed to C15:0 fatty acid (Vaisman & Oren 2009; Baronio et al. 2010) in MS experiments on halophile bacteria. C-15 fatty acids are known to play a role in membrane fluidity in S. aureus and other pathogens (Singh et al. 2008). There are a few publications attributing m/z 941.6 to a common ion found in biological samples, but none regarding bacteria. To help confirm the identities of these ions, LAESI-MS/MS was performed on ions m/z 255.3 and 281.4 of P. aeruginosa and m/z 241.4 of S. aureus on monoculture filter biofilms. However, it was found that tandem MS did not improve the resolution of the mass spectra (data not shown).

If the main unique negative ions identified are indeed fatty acids, there is a precedent in the literature for using fatty acid profiles for identification and clustering of bacterial species (Haack et al. 1994). This suggests that LAESI could allow for fatty acid profile differentiation within a biofilm on a two-dimensional coordinate system. Using these ions as indicators of the location of Pseudomonas or Staphylococcus, it was shown that these two pathogens were significantly co-localized in a mixed biofilm. Co-localization observed in the LAESI study was consistent with the co-localization observed using CLSM.

Interestingly, the level of co-localization was decreased with LL-37 treatment. The small ions used to track S. aureus and P. aeruginosa in the mixed biofilm increased in intensity with LL-37 treatment. Considering their probable identities as lipids, these ions may be viewed as dynamic metabolites of S. aureus and P. aeruginosa within the mixed biofilm. The increase in intensity could be attributed to the fact that sessile bacteria within biofilms have an inherently low metabolic rate compared to motile bacteria. Since LL-37 is known to induce motility of Pseudomonas cells released from a biofilm, and increase stress responses, it is expected that an
increase in metabolism would be observed with LL-37 treatment (Dean et al. 2011a, 2011b; Strempel et al. 2013). In addition, AMPs are known to significantly dysregulate gene expression of multiple metabolic systems, including lipid biosynthetic pathways (Overhage et al. 2008). Thus, the observed increase may be indicative of the resulting altered bacterial metabolism by LL-37 treatment compared to that of the sessile Pseudomonas cells, and not a direct measure of biofilm thickness for example. Of note is that larger ions used as species-specific ions for S. aureus and P. aeruginosa did not display the same increased intensity with LL-37 treatment as the small lipid ions. These larger ions (m/z 941.6 for S. aureus and m/z 716.6 for P. aeruginosa) may then be components of systems not affected by metabolic fluxes. The identities of these ions will be the subject of further study.

Fast, direct, non-invasive LAESI-MS analysis of biofilm could significantly hasten biofilm studies and provide previously unavailable information on both the structure of biofilms and the chemical effect of anti-biofilm treatment. Using LAESI on biofilms enables quick and efficient analysis of biofilm composition. The use of a fingerprint from the spectral profiles of each biofilm grown individually allows the composition of the mixed biofilm to be assessed. This method has many advantages over CLSM-based imaging for analysis of the relative composition and spatial organization, since there is no need to label organisms and sample preparation is greatly simplified (Schillinger et al. 2012). For example, clinical samples from a patient’s infected wound-bed could be rapidly analyzed using this IMS technique. Since LAESI does not require extensive sample processing or fixation prior to analysis, as do MALDI-based techniques (Watrous et al. 2013) or immunofluorescence, confidence that the biofilm is studied in an unperturbed state increases. This study showed that LAESI, in addition to giving compositional information, was able to supplement the traditional microbiological and imaging techniques used to study polymicrobial interactions.

Conclusion
This study demonstrates that LAESI can be used as a rapid, non-destructive, selective technique for direct analysis of biofilms of single or multiple bacteria and the effects of anti-biofilm treatment. These attributes may enable LAESI to facilitate the study of biofilms and the drugs used to inhibit or disperse them, because it does not require complicated culturing or sample preparation.

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