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Biomolecular signatures of diabetic wound healing by structural mass spectrometry

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Abstract

Wound fluid is a complex biological sample containing byproducts associated with the wound repair process. Contemporary techniques, such as immunoblotting and enzyme immunoassays, require extensive sample manipulation and do not permit the simultaneous analysis of multiple classes of biomolecular species. Structural mass spectrometry, implemented as ion mobility-mass spectrometry (IM-MS), comprises two sequential, gas-phase dispersion techniques well suited for the study of complex biological samples due to its ability to separate and simultaneously analyze multiple classes of biomolecules. As a model of diabetic wound healing, polyvinyl alcohol (PVA) sponges were inserted subcutaneously into non-diabetic (control) and streptozotocin-induced diabetic rats to elicit a granulation tissue response and to collect acute wound fluid. Sponges were harvested at days 2 or 5 to capture different stages of the early wound healing process. Utilizing IM-MS, statistical analysis, and targeted ultra-performance liquid chromatography (UPLC) analysis, biomolecular signatures of diabetic wound healing have been identified. The protein S100-A8 was highly enriched in the wound fluids collected from day 2 diabetic rats. Lysophosphatidylcholine (20:4) and cholic acid also contributed significantly to the differences between diabetic and control groups. This report provides a generalized workflow for wound fluid analysis demonstrated with a diabetic rat model.

Introduction

Diabetes is a metabolic disease characterized by abnormally high blood glucose levels resulting from the body's inability to produce or use insulin. The Centers for Disease Control

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Supporting Information **Available:** Additional materials not included in the main text can be found in the supporting information document. This document includes several figures, reference in the main text as Figures S-1 through S-7, as well as the summarizing table (Table S-1). This material is available free of charge via the Internet at <http://pubs.acs.org>.

and Prevention (CDC) has reported that diabetes affects 8.3% of the U.S. population.¹ Patients with diabetes are at significantly greater risk of complications such as blindness, kidney failure, and heart disease, making it the seventh leading cause of death in the U.S.¹ Additional complications of diabetes occur in the extremities and include the loss or reduction of nerve sensation and decreased blood flow.^{2,3} The lack of nerve sensation is experienced by nearly 70% of diabetics and is particularly serious when it occurs in the lower extremities.¹ Due to decreased blood flow and microcirculation in the lower limbs, signaling defects in the cytokine response, and the potential for infection, injuries incurred on the feet or lower leg can suffer from a delayed healing process, resulting in the formation of a chronic ulcer at the wound site.^{2,3} These chronic diabetic ulcers contribute significantly to the high number of lower-limb amputations performed per year on diabetics in the U.S. (65,700 in 2010).¹

The increased risk of chronic ulcer formation stems from disruption of the complex process of wound healing by the pathophysiological abnormalities associated with diabetes.^{3,4} Analysis of human diabetic ulcers has revealed the differential expression of growth factors, chemokines, cytokines and their receptors, which are crucial to several phases of the normal wound healing process.^{5,6} Matrix metalloproteinases (MMPs), a family of endoproteinases involved in tissue remodeling, are also differentially expressed in chronic wounds, causing the dysfunctional breakdown of the extracellular matrix.^{7,8} Macrophages isolated from the wounds of diabetic mice have exhibited decreased ability to remove dead cells, resulting in a prolonged inflammatory response.⁹ Although it is evident that diabetes and hyperglycemia cause widespread disruption of the wound healing process, studies of diabetic wound healing continue to provide only a narrow view of a large and complex process.

The protein detection methods of immunoblotting, microbead (Luminex), and enzyme immunoassays commonly utilized in wound healing studies rely on costly antibodies that are protein specific, requiring multiple antibodies for the analysis of multiple proteins. Proteomic analyses of wound healing have emerged recently with the broader focus of developing prognostic and diagnostic tools and potential therapies for chronic wounds.^{8,10,11} However, both proteomic and antibody-based analyses require rigorous and time-consuming sample preparation procedures to isolate the desired proteins that can alter the original state of the biological sample. To capture the full complexity of diabetic wound healing, it is desirable to utilize a more inclusive analysis requiring minimal sample manipulation.

Ion mobility-mass spectrometry (IM-MS) is a rapid method of analysis which requires minimal sample preparation and offers the flexibility to include pre-ionization separations, making it well suited for holistic studies of complex biological systems. IM-MS is a two-dimensional separation combining gas-phase ion mobility (IM) structural separations with the mass-to-charge (m/z) separations of mass spectrometry (MS). In gas-phase IM separations, ions travel under the influence of an electric field through a drift cell filled with neutral background gas. The number of collisions that occur between the ion and the neutral gas molecules results in a characteristic drift time, measured in micro- to milliseconds, that is dependent on the collision cross section (effective ion surface area). Within a class of biomolecules, such as lipids, proteins, or carbohydrates, a strong correlation between collision cross sections and m/z ratios is observed, resulting in the separation of each biomolecular class along unique mobility-mass correlation lines according to gas-phase packing efficiencies.^{12,13,14}

Integrating ion mobility with MS measurements provides four primary advantages over MS-only techniques in the analysis of complex biological samples for the fields of imaging,^{15,16} proteomics,^{17,18} glycomics,^{19,20} lipidomics,^{21,22} metabolomics,^{23,24} and systems biology.²⁵ Firstly, the IM-MS integration provides rapid separations, increased peak capacity, and

enhanced peak capacity production rate as a 2D separation over 1D separation dimensions. Secondly, IM-MS is inherently gas-phase and the timescales of separation are well suited for further integration with separations such as liquid chromatography (LC) to further increase peak capacity.^{17,18,26-28} Thirdly, different classes of biomolecules are readily distinguished from one another on the basis of their gas-phase structure. This provides higher signal-to-noise for low abundant species of one class (*e.g.* metabolites) that are separated from a species of a different class (*e.g.* peptides) having nearly, or the same, mass, which are unresolvable in the MS alone. Finally, ion activation following the IM separation, but prior to the MS separation provides the ability to obtain fragment ion spectra for all of the molecules nearly simultaneously.

This study demonstrates the use of IM-MS in identifying biomolecular signatures of diabetic wound healing. Wound exudate, a non-invasive sampling approach for human diabetic wounds, was collected from the wounds of diabetic and non-diabetic rats. The wound fluid was analyzed by IM-MS using electrospray ionization (ESI) after minimal sample preparation. Biomolecular signatures distinguishing diabetic and non-diabetic wound fluid and between wound fluids collected at two time points were revealed by direct comparison of IM-MS spectra and with the assistance of statistical analyses. Ultra-performance liquid chromatography (UPLC), tandem MS, and database searching were used in a targeted manner to identify and validate the biomolecular signatures of diabetic wound healing.

Experimental

Rat Model

Sixteen Sprague Dawley rats (300 g, male, Harlan Laboratories) were divided into treatment ($n=10$) and control ($n=6$) groups. The treatment group was dosed twice with streptozotocin (55 mg/kg), which induces diabetes by selective destruction of the insulin-producing pancreatic β -islet cells. A blood glucose concentration greater than 300 mg/dL confirmed the diabetic state in the treatment group, and all ten treated rats converted to the diabetic phenotype. Four sterilized 10 mm polyvinyl alcohol (PVA) sponges (Merocel, Medtronic, Mystic, CT) were inserted subcutaneously on the dorsal side of each animal. The PVA sponges were used to elicit a geometrically defined granulation tissue response as a model of wound healing and to retain wound fluid. Sponges were harvested two days post-implantation from five diabetic and three control rats. Sponges were removed from the remaining diabetic ($n=5$) and control ($n=3$) rats five days post-implantation. Approximately 40-50 μ L of wound fluid was extracted from each PVA sponge by centrifugation through spin columns (Pierce Spin Cups, cellulose acetate filter, Thermo Fisher Scientific, Waltham, MA). Protein concentrations in the wound fluids ranged from 36 – 114 μ g/mL. The remaining wound fluid was flash frozen and stored at -80°C .

Sample Preparation for ESI-IM-MS Direct Infusion Experiments

Scheme 1 illustrates a general workflow for the preparation and IM-MS analysis of wound fluid. The 64 different sponge wound fluid samples to be analyzed by ESI-IM-MS were prepared by diluting 20 μ L of wound fluid to 1 mL with 4% formic acid (Thermo Fisher Scientific, Waltham, MA) prepared in distilled deionized (DDI) water (18 M Ω cm; Millipore, Billerica, MA). The diluted wound fluid was desalted with 1 cc/30 mg Oasis HLB desalting cartridges (Waters, Milford, MA), using CHROMASOLV methanol (Sigma-Aldrich, St. Louis, MO) with 0.1% formic acid as the elution solvent. After desalting, samples were dried and reconstituted with 1 mL of 50% methanol containing 0.1% formic acid. Each of the 64 different samples were run in duplicate for technical replicates, resulting in 128 independent datasets.

Sample Preparation for UPLC-ESI-IM-MS Experiments

50 μL each from two of the previously prepared diabetic day 2 wound fluid samples were combined and diluted with 100 μL H_2O (CHROMASOLV-grade; Sigma-Aldrich, St. Louis, MO) for a total volume of 200 μL . The sample was centrifuged for 10 minutes at 14,500 rpm and transferred to an auto-sampler vial for UPLC separation.

Instrumentation

IM-MS was performed on a Synapt G2 (Waters, Milford, MA), which utilizes a traveling wave ion mobility cell and an orthogonal time-of-flight (TOF) mass spectrometer. The instrument was operated with electrospray ionization (ESI) and the TOFMS was operated in the single-stage reflectron, or “V,” configuration.^{29,30}

ESI-IM-MS—An external syringe pump (Harvard Apparatus, Holliston, MA) was used for direct infusion of the samples into the ESI source at a flow rate of 6 $\mu\text{L}/\text{min}$. The ESI source was operated in positive (+) mode. The electrospray voltage was 4.14 kV with sampling and extraction cone voltages of 20 V and 8 V, respectively. The desolvation temperature and gas flow were 150°C and 10 L min^{-1} , respectively. Ion mobility separation was performed through nitrogen gas with a traveling wave velocity of 550 m s^{-1} and an amplitude of 40 V. Mass calibration was performed with sodium iodide (2 $\mu\text{g}/\mu\text{L}$ in 50% 2-propanol) in the range of m/z 100 - 3000. Data were acquired over two minutes at a rate of 1 scan s^{-1} . The transfer region was used to perform post-mobility MS/MS experiments.

UPLC-ESI-IM-MS—An ACQUITY (Waters, Milford, MA) UPLC system with an ACQUITY HSS C18 column (1.8 μm , 1.0 $\times$ 100 mm; Waters, Milford, MA) was used for the chromatographic separations prior to IM-MS. The column was coupled to the Synapt G2 by the ESI source. The autosampler and column temperatures were maintained at 4°C and 45°C, respectively. Chromatographic separation was performed with a binary solvent system of 95% water/5% acetonitrile (CHROMOSOLV-grade; Sigma-Aldrich, St. Louis, MO) with 10 mM ammonium acetate (Solvent A) and 5% water/95% acetonitrile with 10 mM ammonium acetate (Solvent B). The mobile phase flow rate was set to 70 $\mu\text{L min}^{-1}$ and the injection volume was 5 μL . The initial gradient conditions were 99% A: 1% B for 1 min, followed by a linear gradient to 1% A: 99% B over the next 7 min. The solvent gradient was held at 1% A: 99% B for 2 min, and then linearly increased to 99% A: 1% B over the next 0.5 min. The positive mode (+) ESI conditions were as follows: capillary +3.5 kV; sampling cone: 35 V; extraction cone: 2 V; source temperature: 130 °C; desolvation temperature: 150 °C. The negative mode (-) ESI conditions were as follows: capillary -2.5 kV; sampling cone: -25 V; extraction cone: -2 V; source temperature: 100 °C; desolvation temperature: 150 °C. Desolvation and cone gas flow were 600 and 20 L/hr , respectively. A traveling wave with a velocity of 550 m s^{-1} and height of 40 V was used for IM separation. Leucine-enkephalin (m/z 556.2771) was used for lock mass correction. MS^E was performed in the transfer region with ramping collision energy from 10 – 30 eV.

Biostatistics

The MarkerLynx XS (Waters, Manchester, U.K.) software package was used to perform mass spectral peak detection, alignment and normalization. Mass spectral peak alignment was performed by the combined scan method, where peaks within a 0.1 Da mass window were combined, and all 120 scans in the 2 min ESI-IM-MS acquisitions were combined. The spectral peak detection threshold was set at 1000 counts. Normalization was performed by the constant sum method, where the intensities are normalized such that the sum of intensities of all peaks in a sample sums to 10,000. The aligned data were then exported to the Extended Statistics component of MarkerLynx XS for partial least-squares discriminant

analysis (PLS-DA) to show group differences and orthogonal partial least-squares discriminant analysis (OPLS-DA) S-plots to show the molecular species responsible for the group differences. Model parameters (R²_Y, Q²) for PLS-DA and OPLS-DA can be found in the supporting information documentation. Two sample t-tests (assuming unequal variance) were selectively performed to calculate significance *p*-values for the species revealed to contribute the most to the group differences in the OPLS-DA S-plot analysis of the ESI-IM-MS data. Peak areas from mobility-extracted mass spectra were used for the calculation of *p*-values, fold-changes and to prepare box-and-whisker plots.

Bioinformatics

Online database searching was performed by accurate mass through the MarkerLynx XS software package. The metabolite databases utilized included ChemSpider, the Human Metabolite Database (<http://www.hmdb.ca>), KEGG (<http://www.kegg.com>), LipidMAPS (<http://www.lipidmaps.org>), and METLIN (<http://metlin.scripps.edu/>). Searches were performed with a mass tolerance of 0.05 Da. Protein sequence similarity searches were performed with the UniProt BLAST program (<http://www.uniprot.org/?tab=blast>) and the UniProtKB protein database

Validation

The tentative identifications from the bioinformatics were validated by MS/MS fragmentation experiments. When available, chemical standards of the tentatively identified species were purchased from Sigma Aldrich (St. Louis, MO). To be considered a positive identification, all MS and MS/MS peaks of the standard were required to match those of the analyte.

Results and Discussion

Two-dimensional projections of IM-MS data representative of control day 2 and diabetic day 2 wound fluid are illustrated in Fig. 1(a),(b). The mass-to-charge (*m/z*) ratio is displayed along the x-axis, and drift time, measured in milliseconds (ms), is displayed along the y-axis. Intensity (counts), displayed on the z-axis in three-dimensional IM-MS spectra, is represented by false coloring in the two-dimensional projection. Integrating the drift time dimension of IM-MS data yields a mass spectrum, where intensity is displayed on the y-axis and *m/z* on the x-axis (Fig. 1(c)). Mass spectra may also be extracted from a user-defined region of mobility-mass space and are referred to as mobility-extracted mass spectra (Fig. 1(d)).

Several mobility-mass correlation lines were apparent in the IM-MS spectra of the control day 2 and diabetic day 2 wound fluid (Fig. 1(a),(b)). These lines were predicted to represent biomolecular species such as lipids, proteins, or carbohydrates. While most of these correlation lines were consistently present in all samples, a line of strong signals was observed between *m/z* 700-950 and 4.2-5.6 ms in the diabetic day 2 mobility-mass spectra (*e.g.*, white dashed box in Fig. 1(b)) that was not distinguishable in control day 2 samples or day 5 samples (day 2 control vs. diabetic: fold-change = 5.0; *p* = 9.56 × 10⁻⁵; see Fig. S-1(a)). Mass spectra extracted from the area outlined by the white rectangle in Fig. 1(b) revealed several highly charged signals (Fig. 1(d)). From the isotopic distributions, the signals were identified as the +11 to +14 charge states of an approximately 10.15 kDa species. The number of charges carried by this species and its corresponding molecular weight suggested it was a small protein.

While these signals were strongly apparent in the ion mobility-mass spectra, identifying these unique features of the diabetic day 2 wound fluid from conventional mass spectra would be more challenging. The region between *m/z* 600-1200 in the mobility-mass

spectrum of diabetic day 2 wound fluid (Fig. 1(b)) contained another correlation line of highly charged signals which was separated in drift time from the series of nearly isobaric signals corresponding to the 10.15 kDa protein. Without separation in the drift time dimension, these signals were collapsed onto one another in the corresponding mass spectrum (Fig. 1(c)). A mass spectrum extracted from the region of the mobility-mass spectrum containing the 10.15 kDa species (outlined in Fig. 1(b)) showed a significant reduction in the chemical noise baseline (Fig. 1(d)). For the +13 peak, m/z 781.7, a 2.7-fold improvement in the signal-to-noise ratio (S/N) was observed between the extracted mass spectrum and the full mass spectrum. Tandem MS (MS/MS) experiments are another approach widely used in attempts to identify unknown species, but the low S/N due to the overlapping isotopic distributions of several species would provide fragmentation spectra from which it would be challenging to make confident identifications. Two-dimensional IM-MS analysis allows ready discrimination of the chemical noise in a typical MS measurement from isobaric chemical species and overlapping isotopic distributions that are not resolved in one-dimensional separations (for examples, see Fig. S-2 and Fig. S-3).^{12,24,30}

The challenges presented to MS/MS experiments by isobaric signals and overlapping isotopic distributions can be overcome with the addition of ion mobility.³¹ The present instrumentation enables MS/MS experiments to be performed before or after IM separation, termed pre-mobility or post-mobility MS/MS, respectively, or both in series. While pre-mobility MS/MS (also known as ion activation) provides the unique drift times of precursor and fragment ions, post-mobility MS/MS yields fragment ions with the same drift time as the precursor ion. Fragmentation spectra are then mobility-organized, separating the precursor of interest and its corresponding fragment ions from isobaric signals in drift time. In contrast to conventional MS/MS, the current IM-MS/MS instrumentation does not require preselecting particular ion species for fragmentation analysis, since the current IM-MS/MS instrument supports multiplexed MS/MS measurements during the initial sample run, with a wealth of fragmentation data readily available for subsequent analysis without rerunning the sample for targeted MS/MS based upon an initial analysis.

Post-mobility MS/MS experiments were performed to identify the differentially expressed protein observed in the diabetic day 2 wound fluid. The MS/MS spectra taken for several charge states of this species yielded the amino acid sequence NFEEFLVLV from a series of triply charged y -ions (Fig. S-4). A UniProt BLAST sequence search of "NFEEFLVLV" yielded a match with 100% identity, a score of 70, and an E-value of 0.29 to residues 67-75 of protein S100-A8 (89 residues), also known as Calgranulin-A. The experimental monoisotopic molecular weight was calculated from the raw data to be $10,144.35 \pm 0.04$ Da, which corresponds to the calculated monoisotopic molecular weight of S100-A8 with 26 ppm mass accuracy. Protein S100-A8 is a member of the S100 family of proteins, characterized by the presence of two calcium binding domains which regulate cell functions such as cell stress, signal transduction, chemotaxis and inflammation.^{32,33} The heterodimer S100-A8/9 has been found at increased levels in the serum of type I diabetics.³⁴ S100-A8 and S100-A9 were also found to be differentially expressed in wounds caused by scalpel incisions, and the role of S100-A8 in wound healing is suspected to be the differentiation of fibroblasts and accumulation of monocytes in areas of inflammation.^{35,36}

To identify additional signatures of diabetic wound healing, statistical analysis was performed using MarkerLynx XS software. Data were aligned by mass-to-charge only, as MarkerLynx XS did not have the capability of aligning by drift time. Partial least-squares discriminant analysis (PLS-DA) and orthogonal partial least-squares discriminant analysis (OPLS-DA) were used to identify signals which contributed most significantly to the differences between control and diabetic samples. The results of these statistical tests were visualized in score plots and S-Plots (Fig. 2). PLS-DA scores depict the separation of control

and diabetic wound fluid by group (Fig. 2(a),(c),(e)), while OPLS-DA S-Plots (Fig. 2(b),(d), (f)) depict which molecular signals are contributing to the group separation. As shown by the arrows in Fig. 2(b), movement of a variable away from the origin in the x-direction correlates to its contribution to, or abundance in, a specific condition. Movement away from the origin in the y-direction is correlated with increased confidence in that variable's uniqueness to a specific condition, *i.e.*, the variable displays a substantial change between the diabetic and control groups. Features in the lower left quadrant of the S-Plots (Fig. 2(b), (d),(f)) relate to the respective control samples, while features in the upper right quadrant relate to the respective diabetic samples.

Three signals strongly contributed to the differences between the diabetic and control groups: m/z 544.4, 373.3, and 355.3. The maximum group separation was observed in the PLS-DA score plot for control day 2 and diabetic day 2 wound fluids (Fig. 2(a)). In the corresponding S-Plot (Fig. 2(b)), this separation was attributed to the signals m/z 544.4, 355.3 and 373.3, which were at their maximal separations from the other molecular signals. The signal m/z 544.4 contributed to the separation of control day 5 and diabetic day 5 (Fig. 2(c),(d)) as well as control day 2 and diabetic day 5 (Fig. 2(e),(f)) wound fluid; however, overall group separation decreased due to the lower contribution of m/z 355.3 and 373.3 to the diabetic groups. This result inferred that the diabetic day 5 wounds became more similar to the control wounds. The absence of S100-A8 in the control day 2, control day 5, and diabetic day 5 wound fluids (Fig. 1(a),(b)) supported this inference.

A four-fold increase in the intensity ($p = 3.66 \times 10^{-11}$, Fig. S-1(b)) of m/z 544.4 occurred in the control day 2 group relative to the diabetic day 2 group, and a 2.7-fold increase was observed in the control day 5 group relative to the diabetic day 5 group ($p = 8.37 \times 10^{-6}$, Fig. S-1(b)). In the IM-MS spectra of control day 2 wound fluid, this species was located above the region of the mobility-mass spectra containing the protein S100-A8, with a drift time of 5.03 ms (red circle in Fig. 1(a)). The area of mobility-mass spectra above the protein region (*i.e.*, with longer drift times) has been demonstrated previously as the region which lipids occupy, and localization of m/z 544.4 in this general region indicated it was a lipid species.^{13,14}

Extracted post-mobility MS/MS spectra of m/z 544.4 yielded fragments m/z 104 and m/z 184 (Fig. S-5). This fragmentation pattern is diagnostic of a glycerophosphocholine (GPCCho) lipid species, as the fragment m/z 184 is indicative of the loss of the lipid's phosphocholine head group. A mass-based database search in MarkerLynx XS suggested that m/z 544.4 was lysophosphatidylcholine (20:4) (LysoPC 20:4, LPC 20:4). Interrogation of the mobility-mass spectra had previously revealed this species to be a lipid due to its location above the protein mobility-mass correlation region, and the fragmentation and database results provided additional confirmation. Lipidomic analyses of human plasma have revealed decreased levels of lysophosphatidylcholines (LPCs), in general, for individuals with pre-diabetic conditions such as insulin resistance and glucose intolerance.³⁷⁻⁴⁰ LPCs have also been studied in regards to their involvement in inflammation,⁴¹ and recent work has indicated potential anti-inflammatory properties of polyunsaturated LPCs such as LPC (20:4).^{42,43}

Nine- ($p = 2.3 \times 10^{-10}$; Fig. S-1(d)) and 2.6-fold ($p = 2.5 \times 10^{-8}$; Fig. S-1(c)) increases in intensity were observed for m/z 373.3 and 355.3, respectively, in the diabetic day 2 group relative to the control day 2 group. Furthermore, m/z 355.3 and m/z 373.3 presented similar trends in intensity across the four sample groups, in which intensity was most abundant in the diabetic day 2 group, but also had increased intensity in the diabetic day 5 group relative to its control (Fig. S-1(c),(d)). The species m/z 355.3 and 373.3 were located within the same mobility-mass correlation line in the IM-MS spectra of diabetic day 2 wound fluids,

with respective drift times of 3.28 and 3.50 *ms* (Fig. S-6), and generally located between the lipid and protein regions of the mobility-mass spectra. The proximity of these two signals in drift time and in *m/z*, as well as the similarity in expression across the sample groups, indicated that these signals shared a common structure and perhaps a similar function. The structural similarities of these species, as indicated by their adjacent drift times, strongly guided further analysis to identify the nature of these signals.

The mass difference of 18 Da between *m/z* 355.3 and 373.3 was suspected to correspond to a neutral water loss and to occur via in-source fragmentation. A pre-ionization separation by UPLC was paired with IM-MS in a targeted analysis to determine a common precursor from which these species were generated in the ionization process. In the positive-mode targeted UPLC-IM-MS analysis of diabetic day 2 wound fluid, *m/z* 355.26 and 373.27 were observed to co-elute from the column with a signal at *m/z* 426.32 at 5.31 min (Fig. S-7(a),(b)), confirming the two signals were chemically, as well as structurally, similar. Mobility-extracted fragmentation spectra from post-mobility MS/MS of *m/z* 373.27 were dominated by a fragment at *m/z* 355.26. The loss of 18 Da from *m/z* 373.26 to form the fragment at *m/z* 355.26 was consistent with a neutral water loss. Both the chromatographic and fragmentation data supported the hypothesis generated from the mobility data that *m/z* 355.26 and 373.27 are homologous species.

The targeted UPLC-IM-MS analysis was repeated in negative ionization mode and a peak at 5.31 min was observed in the chromatogram (Fig. S-7(c)), similar to that observed in the positive mode UPLC chromatogram (Fig. S-7(a)). The mass spectra at 5.31 min contained a signal at *m/z* 407.2809 (Fig. S-7(d)), on which post-mobility fragmentation was performed (Fig. 3(a)). An accurate mass-based search of the METLIN metabolomics database (<http://www.metlin.scripps.edu>) for *m/z* 407.2809 suggested that this species was the [M-H]⁻ signal of cholic acid, with an experimental mass accuracy of 1.47 ppm.

Tandem MS data provided by METLIN and Lipid Maps (<http://www.lipidmaps.org>) matched that obtained from the analysis of diabetic day 2 wound fluids (Fig. 3(a)), strongly suggesting that *m/z* 407.2809 was cholic acid. Fragmentation of a cholic acid standard (Fig. 3(b)) was performed to validate the assignment of *m/z* 407.2809 as cholic acid.

Database searches of the signals present in the ESI+ chromatographic peak at 5.31 min were also performed (Fig. S-7(a),(b)). A mass-based search for *m/z* 426.3216 strongly suggested that this species was the [M+NH₄]⁺ adduct of cholic acid, with an experimental mass accuracy of 0.47 ppm. The signals *m/z* 355.2627 and 373.2735 corresponded to three and two neutral water losses from cholic acid, indicating that these species may be the result of in-source fragmentation of cholic acid in positive ionization mode. An ESI+ analysis of the cholic acid standard confirmed that fragments corresponding to two and three neutral water losses are the primary species observed from cholic acid in positive ionization mode (Fig. 3(c)), while the [M-H]⁻ of cholic acid is the primary species generated by ESI-. Bile acids, such as cholic acid and its derivatives, and their receptors are emerging as important factors in the regulation of glucose homeostasis and diabetes.⁴⁴⁻⁴⁶

Conclusions

We have illustrated the utility of IM-MS in the analysis of complex biological samples for the study of diabetic wound healing. After minimal sample preparation, IM-MS spectra were rapidly collected for 64 samples of diabetic and non-diabetic wound fluid and four biomolecules distinguishing diabetic and control wound fluid were identified. IM-MS spectra revealed a 10.15 kDa protein, in the form of a unique mobility-mass correlation line, was highly enriched in diabetic day 2 wound fluid. Post-mobility MS/MS aided in the

identification of this protein as S100-A8. The separation of S100-A8 in ion mobility drift time from another highly charged, high mass protein provided a 2.7-fold increase in S/N over the MS-only spectrum of the +13 charge state of S100-A8. An OPLS-DA statistical analysis revealed three additional species distinguishing diabetic from control wound fluid, and subsequent UPLC separations and post-mobility MS/MS were performed in a targeted manner to identify and validate these species. The locations of these species in mobility-mass space greatly aided in their identification, as the drift times indicated that m/z 544.4 was a lipid and the proximity of m/z 355.3 and 373.3 in drift time revealed they were structurally homologous species. A combination of fragmentation experiments and accurate mass database searching led to the identifications of lysophosphatidylcholine (20:4) and cholic acid, which were found at increased intensities in control and diabetic wound fluid, respectively.

The methodology demonstrated in this study can be applied to the analysis of a variety of complex biological systems to rapidly identify biomolecular signatures of diseases and biological processes with IM-MS. In a disease such as the diabetic model examined herein, there can be both increased and decreased (or absent) abundance of chemical species relative to the control. After an intervention, such as the wounding of the tissue, the kinetics of the intervention-induced changes may also differ between the disease model and control. In this study, we focused on the three most abundant and most significantly different species in the first and third quadrants of the S-plot, but with a sufficient investment in effort, an IM-MS/MS workflow as outlined in this paper could be applied to every species in Fig. 2(b),(d),(f). We expect that such an examination of just the subsets of species that lie within the three we have studied provides, in an efficient manner, an even deeper physiological investigation of the processes that differ between normal and diabetic wound healing. We believe that such an analysis lies midway between narrowly targeted and totally untargeted searches for important contributors to the wound healing process in health and disease.

The growing reliance on structural mass spectrometry stems in part from the flexibility of IM-MS systems, which span smoothly the range between fully targeted and totally untargeted analyses. The workflow that we present demonstrates that a single instrument can both identify and validate chemical species whose concentrations differ statistically between two experimental conditions. One of the contemporary challenges in metabolomic measurements on complex samples is to transition from untargeted to targeted analysis, not because of instrumentation limitations, but because of the need for both standard samples for target validation and new bioinformatic and biostatistical tools optimized for identification of the salient features in differential and time-series experiments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Centers for Disease Control and Prevention. National diabetes fact sheet: national estimates and general information on diabetes and prediabetes in the United States, 2011. Atlanta, GA: U.S. Department of Health and Human Services, Centers for Disease Control and Prevention; 2011.
2. Jeffcoate WJ, Harding KG. *Lancet*. 2003; 361:1545–1551. [PubMed: 12737879]
3. Falanga V. *Lancet*. 2005; 366:1736–1743. [PubMed: 16291068]
4. Sibbald RG, Woo KY. *Diabetes/Metab Res and Rev*. 2008; 24:S25–S30.
5. Werner S, Grose R. *Physiol Rev*. 2003; 83:835–870. [PubMed: 12843410]
6. Galkowska H, Wojewodzka U, Olszewski WL. *Wound Rep Reg*. 2006; 14:558–565.
7. Lobmann R, Ambrosch A, Schultz G, Waldmann K, Schiweck S, Lehnert H. *Diabetologia*. 2002; 45:1011–1016. [PubMed: 12136400]
8. Eming SA, Koch M, Krieger A, Brachvogel B, Kreft S, Bruckner-Tuderman L, Krieg T, Shannon JD, Fox JW. *J Prot Res*. 2010; 9:4758–4766.
9. Khanna S, Biswas S, Shang Y, Collard E, Azad A, Kauh C, Bhasker V, Gordillo GM, Sen CK, Roy S. *PLoS One*. 2010; 5:e9539. [PubMed: 20209061]
10. Fernandez ML, Broadbent JA, Shooter GK, Malda J, Upton Z. *Br J Dermatol*. 2008; 158:281–290. [PubMed: 18070206]
11. Broadbent J, Walsh T, Upton Z. *Proteomics: Clin Appl*. 2010; 4:204–214. [PubMed: 21137044]
12. Fenn LS, McLean JA. *Anal Bioanal Chem*. 2008; 391:905–909. [PubMed: 18320175]
13. McLean JA. *J Am Soc Mass Spectrom*. 2009; 20:1775–1781. [PubMed: 19646898]
14. Fenn LS, Kliman M, Mahsut A, Zhao SR, McLean JA. *Anal Bioanal Chem*. 2009; 394:235–244. [PubMed: 19247641]
15. Jackson SN, Ugarov M, Egan T, Post JD, Langlais D, Schultz JA, Woods AS. *J Mass Spectrom*. 2007; 42:1093–1098. [PubMed: 17621389]
16. McLean JA, Ridenour WB, Caprioli RM. *J Mass Spectrom*. 2007; 42:1099–1105. [PubMed: 17621390]
17. McLean JA, Ruotolo BT, Gillig KJ, Russell DH. *Int J Mass Spectrom*. 2005; 240:301–315.
18. Valentine SJ, Plasencia MD, Liu X, Krishnan M, Naylor S, Udseth HR, Smith RD, Clemmer DE. *J Prot Res*. 2006; 5:2977–2984.
19. Plasencia MD, Isailovic D, Merenbloom SI, Mechref Y, Clemmer DE. *J Am Soc Mass Spectrom*. 2008; 19:1706–1715. [PubMed: 18760624]
20. Fenn LS, McLean JA. *Phys Chem Chem Phys*. 2011; 13:2196–2205. [PubMed: 21113554]
21. Kim HI, Kim H, Pang ES, Ryu EK, Beegle LW, Loo JA, Goddard WA, Kanik I. *Anal Chem*. 2009; 81:8289–8297. [PubMed: 19764704]
22. Kliman M, Vijayakrishnan N, Wang L, Tapp JT, Broadie K, McLean JA. *Mol BioSyst*. 2010; 6:958–966. [PubMed: 20379606]
23. Dwivedi P, Wu P, Klopsch SJ, Puzon GJ, Xun L, Hill HH. *Metabolomics*. 2008; 4:63–80.
24. Dwivedi P, Schultz AJ, Hill HH. *Int J Mass Spectrom*. 2010; 298:78–90. [PubMed: 21113320]
25. Enders JR, Marasco CC, Kole A, Nguyen B, Sevugarajan S, Seale KT, Wikswo JP, McLean JA. *IET Syst Biol*. 2010; 4:416–427. [PubMed: 21073240]
26. Ruotolo BT, Gillig KJ, Stone EG, Russell DH. *J Chromatogr, B: Anal Technol Biomed Life Sci*. 2002; 782:385–392.
27. Ruotolo BT, McLean JA, Gillig KJ, Russell DH. *J Mass Spectrom*. 2004; 39:361–367. [PubMed: 15103649]
28. Liu X, Plasencia M, Ragg S, Valentine SJ, Clemmer DE. *Briefings Funct Genomics Proteomics*. 2004; 3:177–186.
29. Giles K, Pringle SD, Worthington KR, Little D, Wildgoose JL, Bateman RH. *Rapid Commun Mass Spectrom*. 2004; 18:2401–2414. [PubMed: 15386629]
30. Pringle SD, Giles K, Wildgoose JL, Williams JP, Slade SE, Thalassinos K, Bateman RH, Bowers MT, Scrivens JH. *Int J Mass Spectrom*. 2007; 261:1–12.

31. Hoaglund-Hyzer CS, Li JW, Clemmer DE. *Anal Chem.* 2000; 72:2737–2740. [PubMed: 10905301]
32. Raftery MJ, Geczy CL. *Anal Biochem.* 1998; 258:285–292. [PubMed: 9570842]
33. Eckert RL, Broome AM, Ruse M, Robinson N, Ryan D, Lee K. *J Invest Dermatol.* 2004; 123:23–33. [PubMed: 15191538]
34. Bouma G, Lam-Tse WK, Wierenga-Wolf AF, Drexhage HA, Versnel MA. *Diabetes.* 2004; 53:1979–1986. [PubMed: 15277376]
35. Hessian PA, Edgeworth J, Hogg N. *J Leukocyte Biol.* 1993; 53:197–204. [PubMed: 8445331]
36. Wu NJ, Davidson JM. *Wound Rep Regen.* 2004; 12:327–336.
37. Graessler J, Schwudke D, Schwarz PEH, Herzog R, Shevchenko A, Bornstein SR. *PLoS One.* 2009; 4:e6261. [PubMed: 19603071]
38. Zhao X, Fritsche J, Wang J, Chen J, Rittig K, Schmitt-Kopplin P, Fritsche A, Haering HU, Schleicher ED, Xu G, Lehmann R. *Metabolomics.* 2010; 6:362–374. [PubMed: 20676218]
39. Rhee EP, Cheng S, Larson MG, Walford GA, Lewis GD, McCabe E, Yang E, Farrell L, Fox CS, O'Donnell CJ, Carr SA, Vasan RS, Florez JC, Clish CB, Wang TJ, Gerszten RE. *J Clin Invest.* 2011; 121:1402–1411. [PubMed: 21403394]
40. Barber MN, Risis S, Yang C, Meikle PJ, Staples M, Febbraio MA, Bruce CR. *PLoS One.* 2012; 7:e41456. [PubMed: 22848500]
41. Sevastou I, Kaffé E, Mouratis MA, Aidinis V. *Biochim Biophys Acta Mol Cell Biol Lipids.* 2013; 1831:42–60.
42. Hung ND, Kim MR, Sok DE. *Prostaglandins Other Lipid Mediators.* 2009; 90:105–111. [PubMed: 19819343]
43. Hung ND, Sok DE, Kim MR. *Inflammation Res.* 2012; 61:473–483.
44. Duran-Sandoval D, Mautino G, Martin GV, Percevault F, Barbier O, Fruchart JC, Kuipers F, Staels B. *Diabetes.* 2004; 53:890–898. [PubMed: 15047603]
45. Lefebvre P, Cariou B, Lien F, Kuipers F, Staels B. *Physiol Rev.* 2009; 89:147–191. [PubMed: 19126757]
46. Cai S, Huo TG, Xu JH, Lu XM, Zheng SN, Li FM. *J Chromatogr, B: Anal Technol Biomed Life Sci.* 2009; 877:3619–3624.

Figure 1.

Three-dimensional ion mobility-mass spectra (a and b), two-dimensional mass spectra (c and d) representative of the ESI-IM-MS analysis of the 64 wound fluid samples. (a) IM-MS spectrum representative of control day 2 wound fluid. The red circle corresponds to m/z 544.4 as discussed in the text. (b) IM-MS spectrum representative of diabetic day 2 wound fluid. The region outlined by the white dotted rectangle contains the +11 to +14 charge states of protein S100-A8. (c) MS-only spectrum of the region m/z 700-950 (as indicated by the grey dotted lines) of diabetic day 2 wound fluid (b), obtained by collapsing the IM dimension. (d) Mobility-selected mass spectrum of the region outlined by white-dashed lines in (b). A 2.7-fold increase in the chemical signal-to-noise ratio (S/N) of the +13 charge state signal of S100-A8, m/z 781, was observed between the two-dimensional mass spectrum (c) and mobility-extracted mass spectrum (d) of the same m/z region.

Figure 2.

Partial least-squares discriminant analysis (PLS-DA) scores (a,c,e) and orthogonal partial least-squares discriminant analysis (OPLS-DA) S-Plots (b,d,f) for ESI-IM-MS data of control day 2 and diabetic day 2 wound fluids (a,b), control day 5 and diabetic day 5 wound fluids (c,d), and control day 2 and diabetic day 5 wound fluids (e,f). PLS-DA scores (a,c,e) display the separation of control and diabetic wound fluid groups. S-Plots (b,d,f) display the molecular signals contributing the group differences depicted by the PLS-DA scores. The interpretation of S-Plots is depicted by the arrows in (b) and is further discussed in the text. The signal m/z 544.4 in the lower left quadrants of the S-Plots contributes most significantly to the respective control group, while the signals m/z 355.3 and 373.3 in the upper right quadrant contribute most significantly to the respective diabetic group. Model parameters (R^2Y , Q^2) can be found in the supporting information documentation.

Figure 3.

(a) Post-mobility MS/MS of m/z 407 (ESI-) in diabetic day 2 wound fluid revealed fragments characteristic of cholic acid. (b) Fragmentation of a standard reference of cholic acid ($1\text{ }\mu\text{g/mL}$) was performed to validate the assignment of m/z 407 as cholic acid. (c) The primary species observed from the positive ionization (ESI+) MS analysis of the cholic acid standard were m/z 355 and 373. These signals corresponded to three and two neutral waters losses from cholic acid, respectively.

Scheme 1.

Workflow for the preparation and IM-MS analysis of wound fluid for biomolecular signatures of diabetic wound healing. Briefly, wound fluid collected from PVA sponges was desalted and analyzed by ESI-IM-MS. An orthogonal partial least-squares discriminant analysis (OPLS-DA) statistical analysis of the ESI-IM-MS data revealed biomolecular signatures of diabetic wound healing. UPLC-IM-MS/MS was performed in a targeted fashion to obtain accurate mass and fragmentation data for select biomolecular signatures. These data were then used for database searching, from which tentative identifications of the biomolecular signatures were determined.