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Letter

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Acoustic Mist Ionization Platform for Direct and Contactless Ultrahigh-Throughput Mass Spectrometry Analysis of Liquid Samples

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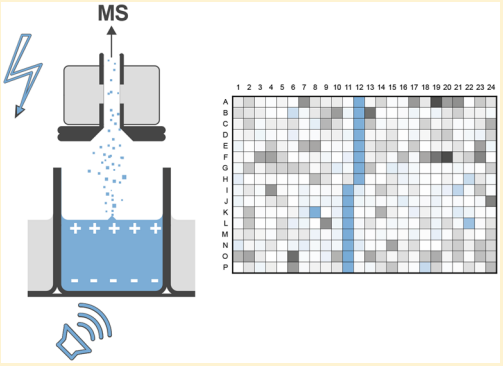
Supporting Information

ABSTRACT: Mass spectrometry (MS) has many advantages as a quantitative detection technology for applications within drug discovery. However, current methods of liquid sample introduction to a detector are slow and limit the use of mass spectrometry for kinetic and high-throughput applications. We present the development of an acoustic mist ionization (AMI) interface capable of contactless nanoliter-scale "infusion" of up to three individual samples per second into the mass detector. Installing simple plate handling automation allowed us to reach a throughput of 100 000 samples per day on a single mass spectrometer. We applied AMI-MS to identify inhibitors of a human histone deacetylase from AstraZeneca's collection of 2 million small molecules and measured their half-maximal inhibitory concentration. The speed, sensitivity, simplicity, robustness, and consumption of nanoliter volumes of sample suggest that this technology will have a major impact across many areas of basic and applied research.



Modern medicine discovery increasingly relies on detailed and accurate chemical analyses of complex samples from *in vitro* and *in vivo* studies. Mass spectrometry (MS) has been widely adopted over the past few decades, mainly thanks to the invention of electrospray ionization (ESI).[1] The development of countless ESI-related methods[2−6] and sample preparation protocols,[7] together with more sensitive and user-friendly mass detectors, has enabled routine profiling, quantification, and even imaging most of the molecular makeup of a cell ranging from small molecular weight metabolites to large proteins.

One of the important remaining bottlenecks in ESI-MS analysis is the rate at which individual liquid samples can be introduced into the mass detector, preventing wide utilization of ESI-MS in kinetic and high-throughput applications that are key to early drug discovery. Researchers still mostly rely on expensive labelled detection technologies (e.g., fluorescence, chemiluminescence) or coupled enzyme systems[8] to monitor biological reactions, substantially increasing the cost of identifying new drug candidates as well as the risk of finding false positives.[9]

The fastest commercially available ESI-based MS platform (RapidFire, Agilent, CA) is able to introduce a sample every 5−10 s and therefore can be used to run medium-sized (e.g., 100 000 molecules) biochemical screening assays.[10−12] Direct infusion ESI-MS is also a promising technique for untargeted profiling of hundreds of metabolites and lipids from crude cell lysates with the fastest reported front-end able to inject up to 1500 samples per day.[13,14] Other MS technologies, not based on ESI, with the potential to process high numbers of samples

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are laser diode thermal desorption (LDTD) and matrix-assisted laser desorption ionization (MALDI), achieving rates of just above 2 s per sample.[15−18] Yet, these techniques require additional preparation time for sample deposition and drying onto a specialized surface or plate prior to analysis. This increases the cost of screening and prevents using these techniques in kinetic analyses.

In 2016, we reported the design of a prototype acoustic mist ionization mass spectrometry (AMI-MS) platform as a next-generation high-throughput electrospray-type MS technology.[19] This platform uses contactless acoustic energy to load ionized liquid samples from a microtiter plate directly to a mass spectrometer, achieving rates of 3 samples per second. Here, we describe further improvements of the AMI-MS platform and present its first uses in real life drug discovery projects for biochemical assay development and high-throughput screening to identify active compounds that inhibit enzymes of relevance for biomedical research.

The AMI-MS platform consists of an externalized transducer assembly from an Echo 555 liquid handler (Labcyte), a moving *XY*-stage (Labcyte custom-made), an aperture connected to a high-voltage power supply (RIGOL) and a heated transfer tube (Waters custom-made) leading to a Xevo G2-XS quadrupole time-of-flight mass spectrometer (Waters) (Supplemental Figure 1 and Supplemental Video). Ionization of the sample is achieved by applying high voltage (0.5−4 kV) above the test well, which results in charge separation in the sample prior to droplet generation (e.g., in positive ion mode, negative voltage is applied above the well and results in concentration of positive charge at the meniscus) (Figure 1a). Applying pulses of acoustic energy to the liquid surface then "lifts up" a population of charged droplets. These charged droplets get entrained through the charging cone into the heated transfer interface where most of the desolvation occurs. High-voltage and electrically insulated wells are required for AMI to show any MS signal, which demonstrates that droplet charging does not happen on transit and distinguishes AMI from solvent-assisted inlet ionization (SAII)[5] and surface acoustic wave nebulization (SAWN)[6] methods.

The ultrasonic pulse of the transducer in AMI-MS ejects a mist of droplets with an average diameter of 5.5 μm (approximately 87 fL volume) and with an average initial vertical velocity of 2 m/s (Figure 1b,c and Supplemental Figure 2). We raised the acoustic misting repetition rate from 500 to 1400 Hz, significantly increasing the flow rate of misted sample from the well (from 2.3 to 12.6 μL/min) and sensitivity (i.e., data quality) per unit of time (Supplemental Figure 3). We also optimized the acoustic mist generation for pure organic solvents (acetonitrile, methanol, DMSO) and for any mixture of these with water.

Since the original glass capillary inlet design led to issues with condensation and ion trapping,[19] we introduced an inverted charging cone and electrically insulated it from the transfer optics (Figure 1a). This allowed heating to be applied throughout the stem of the transfer tube and resulted in improved desolvation, ionization efficiency, and transfer of sample to the ion source of the mass spectrometer. Entire 384-well plates could now be sampled robustly at rates of 0.3 s per sample (Figure 1d). Typical coefficient of variation (CV) for raw signal between individual sampling events (inter- or intrawell) is 25% and is reduced to 3.5% when using isotope ratios (Supplemental Figure 4).

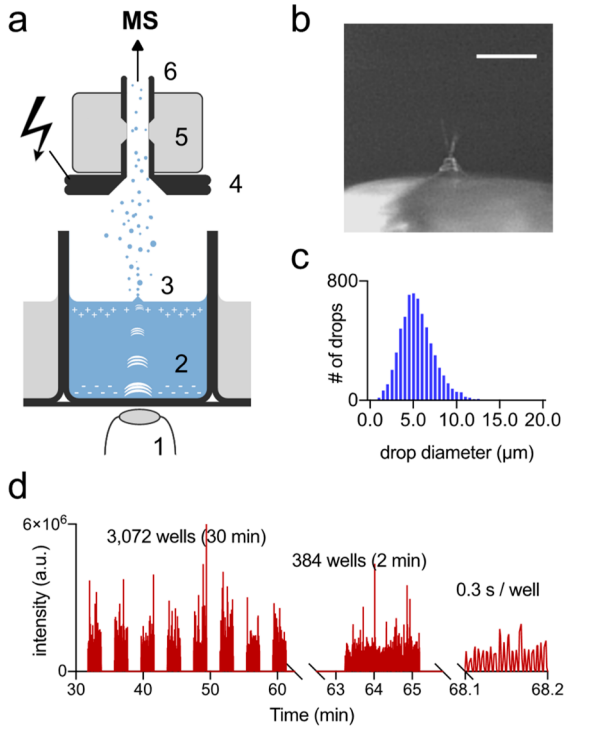

Figure 1. Key features of the AMI-MS platform. (a) Schematic representation of the mechanism of ambient ionization. An externalized acoustic transducer (1) from a Labcyte Echo liquid handler emits sound waves into a liquid sample in a 384-well microtiter plate (2) located on a moving *XY*-stage (not shown). High voltage is applied to a charging cone (4) suspended directly above the sample plate, inducing charge separation in the sample. A mound (3) forms on the meniscus and micrometer-sized charged droplets spray through an electrical insulator piece (5) into a heated transfer tube (6) leading to the source of a mass spectrometer. Not to scale. (b) A movie frame of an acoustic misting event taken in a cut-down 384-well plate at 50 000 frames per second. Scale bar 500 μm. (c) Histogram of drop diameter distribution (bin size 0.5 μm, n = 6385). (d) Typical raw data (total ion current) generated from an automated batch of 384-well plates read at a rate of 0.3 s per sample or >6000 samples per hour, including plate handling.

A plate handling robot with a capacity of 150 384-well plates (Access station, Labcyte) was integrated with the system (Supplemental Figure 5). To maximize the throughput, plates are loaded into simple plate stacks, each plate acts as the lid for the plate below, removing the need for separate lids or seals. Plates are picked from the top of each stack, this ensures that there are no mechanical components in the stackers that could fail and no requirement for electronic handshake between the plate storage and the robot. This simple robot handler allows an unattended batch analysis of 57 600 samples to be run in under 10 h and therefore provides in excess of 100 000 data points per day.

To process the large amount of data generated by the system, we acquire an entire 150-plate batch into an individual raw data file using MassLynx and perform a real-time recording of plate barcode and well location for every MS scan via a custom application called DataSync (Waters). A customized postprocessing macro then combines all scans originating from the same well and saves a single mass spectrum per sample as a text file (i.e., 57 600 files for a 150-plate batch). Finally, we designed a Web-based application to facilitate searching these mass spectra for the ions of interest (e.g., a substrate and a

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product of a biochemical assay or an internal standard if present). The application simply sums the intensities within a given mass-to-charge (m/z) window and generates a result file for each 384-well plate in a format suitable for importing directly into a commercial data visualization and management tool Genedata Screener (Genedata, Basel, CH) (Supplemental Figure 6). The data processing step can be carried out offline while the next batch of plates is being acquired and currently takes around 3 h to complete using a standard laboratory computer.

We first tested whether the redesigned AMI-MS platform is suitable for measuring a wide range of biologically relevant analytes frequently used in biochemical assays such as peptides, nucleotides, *S*-adenosylmethionine, or a small protein. Using isotopically labelled internal standards, we obtained a linear concentration range of more than 4 orders of magnitude for most analytes with limits of detection in water as low as 10 nM acquired at 0.4 s per data point and consuming only 15 nL of sample (Supplemental Figure 7). Carryover was only observed when a blank sample was infused following a very high concentration of an analyte (e.g., 10 mM), giving a signal corresponding to less than 200 nM (i.e., 0.002% carryover). Like standard ESI, the vast majority of ions generated by AMI-MS are either $[M + H]^+$ or $[M - H]^-$, with some contribution from Na^+ and K^+ salt adducts in the positive ion mode. We found that unlike ESI, the current prototype is unable to efficiently ionize large molecules above 25 kDa.

Next, we tested several common buffers and detergents to understand their ionization potential and ion suppression effects in both positive and negative ion modes. While phosphate-based buffers such as 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) caused severe signal suppression, a combination of tris(hydroxymethyl)-aminomethane (Tris) and Triton X-100 as the buffer-detergent system produced clean mass spectra of substrates/products at concentrations ranging from 1 to 10 μM. This simple buffer was able to maintain the activity of selected example enzymes, including various protein kinases, a histone deacetylase, a methyltransferase, and a disulfide reductase (Supplemental Figure 8). Unlike label-based assay readouts (e.g., fluorescence or chemiluminescence), we found that the contents of label-free assays stopped with a weakly acidic or basic buffer can be stable for many weeks and can be reread without loss of data quality.

To determine the performance and robustness of the platform in high-throughput biochemical screening, we developed an assay to identify inhibitors of a human histone deacetylase (HDAC) from an internal library of 2 million compounds. The assay was performed directly in acoustic 384-well plates using a combination of Echo liquid handlers and Multidrop dispensers. Compounds were preincubated with HDAC enzyme prior to the addition of acetylated HDAC substrate, allowing the production of 3 μM deacetylated peptide (15% conversion) in vehicle controls over 90 min (Figure 2a). The reaction was stopped by the addition of aqueous acetic acid to pH 4, which further diluted the product to 1.2 μM. This relatively low analyte concentration still generated a high AMI-MS signal in the positive ion mode and allowed a sufficient signal window to robustly distinguish between active and inactive compounds (Figure 2b,c and Supplemental Figure 9).

During early validation experiments, we observed occasional wells showing significantly lower total ion current compared to

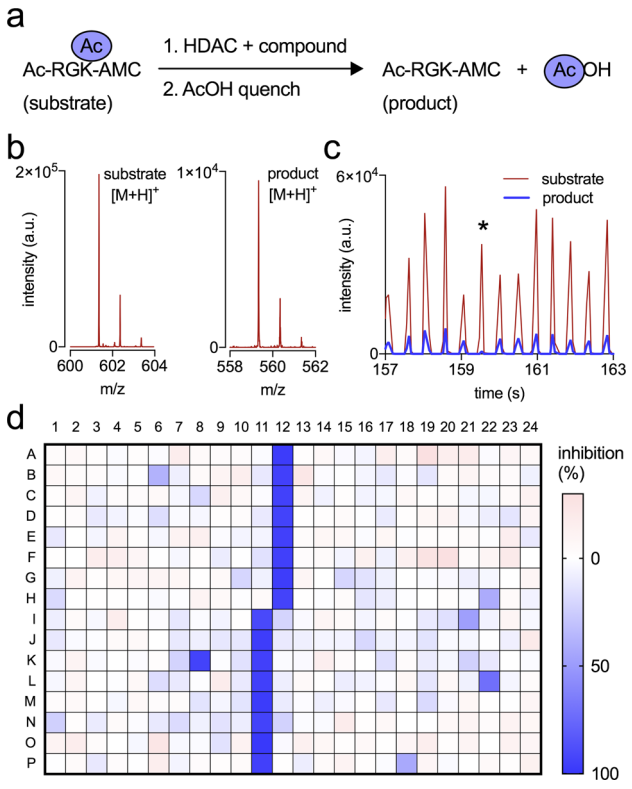

Figure 2. HDAC inhibition assay development. (a) Overview of HDAC inhibition assay based on a commercial peptide substrate. (b) Example of raw MS data obtained directly from a single stopped assay well in 0.45 s. (c) Example overlaid extracted ion chromatograms of substrate and product acquired at 0.45 s per sample or 0.75 s per sample including automated plate handling. An identified inhibitor is highlighted with an asterisk. (d) Example of processed data for a 384-well plate read in under 3 min. Columns 11 and 12 contain 32 control wells (DMSO and inhibitor). Full inhibition was observed from a compound in well K8.

the rest of the wells on the microtiter plate. We believe these low-signal wells are related to an uneven meniscus that prevents efficient formation of charged acoustic mist. We found that the meniscus can be levelled off acoustically by first ejecting 15 nL of sample at the wrong polarity (e.g., forming negatively charged mist in positive ion mode that is invisible to the mass spectrometer), followed by 15 nL of sample at the correct polarity (e.g., positively charged mist in positive ion mode, producing mass spectra). This approach extended the run time by approximately 0.15 s per sample (now 0.45 s per sample, 2.88 min per plate) but ensured a robust signal and higher data quality for every well on a plate (Supplemental Figure 9).

We next processed a validation set of 19 plates (6580 compounds at 10 μM and 608 controls) through the entire assay, in duplicate on two separate occasions. We identified hits as compounds that inhibited substrate conversion by more than 5 robust standard deviations from the median conversion across the plate (Z score of −5, corresponding to around 50% inhibition). We obtained a reasonable hit rate of 0.12% and a very good agreement between the two data sets (Figure 3a).

The main screen took only 7 weeks during which we analyzed a total of 2.75 M wells (7 164 plates) on a single AMI-MS platform. We found that it was possible to process more than 100 000 samples per day or 500 000 per week, with very high assay quality throughout (average robust Z'-factor

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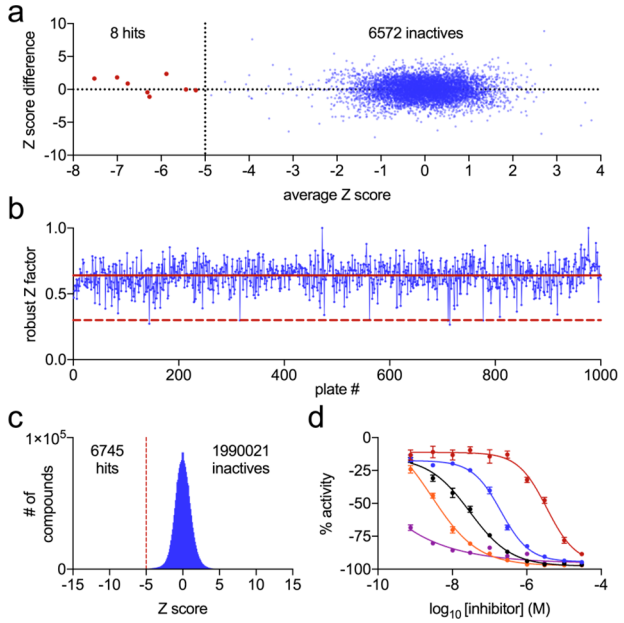

Figure 3. AMI enables label-free MS-based HTS at scale. (a) Concordance between two independent replicates of a validation run comprising 6580 compounds. All hits (average Z score below −5, highlighted in red) agreed between the two runs. (b) Consecutive series of 1000 plates (384 000 samples) shows an average robust Z' of 0.64 (solid line) with only 5 plates below the quality acceptance limit of 0.3 (dashed line). (c) Histogram of Z score for all screened compounds (~2 000 000), bin size = 0.1. 6745 compounds were below the arbitrary Z score limit of −5 (0.3% hit rate). (d) 10-point concentration−response curves for selected inhibitors generated using AMI-MS at 3.1 s per well. Shown are mean ± SD of three repeat wells.

well above 0.6) (Figure 3b). Given the stability of the stopped assay, plates with insufficient data quality (Z'-factor below 0.3) could be reanalyzed with the next batch of plates without the need to repeat the assay. We experienced minimal instrument downtime and only needed to wipe clean the charging cone once every 2−3 days to maintain high sensitivity.

We identified a total of 6 745 inhibitors (0.3% hit rate) (Figure 3c), 10% of which contained a hydroxamic acid motif known to inhibit histone deacetylases.[20] The remaining hits included novel chemical entities previously unreported as interacting with histone deacetylases. We generated a 10-point concentration−response (IC_{50}) curve for 4872 selected compounds, discarding 1141 as inactive and leaving 3731 compounds with pIC_{50} between 4.5 and 9.1 (77% confirmation rate). A number of examples of IC_{50} curves are shown in Figure 3d.

Overall, we have created a powerful ultrahigh-throughput MS-based analytical platform. This was achieved by developing a new mass spectrometer interface with robust custom-built hardware and software to acquire and process data. Acoustic mist ionization is much like electrospray in analyte coverage and the ability to produce multiply charged species in both ion modes. The throughput is achieved by using contactless charge separation in the liquid sample and contactless acoustic ejection of charged mist.

As with most direct sampling MS methods, AMI suffers from ion suppression which can be limiting for some challenging analytes and assays. We have shown that traditional biochemical assay buffer components can be adapted to be compatible with AMI-MS, enabling label-free biochemical screening directly from an assay well with no sample preparation and with substrate concentrations down to sub-μM. Currently, the well-to-well variability is higher than other MS screening platforms which leads to lower Z' values (0.6 vs 0.75 for RapidFire or MALDI),[10,21] however, further improvements in sensitivity and reproducibility are expected as this prototype platform develops. This could be achieved by reducing the average droplet size[22] in the acoustic mist, which we expect would also improve desolvation and give access to molecular species larger than 25 kDa.

We believe that AMI-MS will find applications in a variety of fields where electrospray ionization is currently being used, such as drug discovery, fine chemical analyses, quality control, environmental applications, forensics, or microbiome research.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications Web site. and Supplemental Figures 1−9 (PDF) Supplemental Video (MOV) The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.9b00142.

Experimental procedures; photo of AMI hardware and automation package; measurements of drop velocity; comparison between acoustic misting at 500 Hz and 1500 Hz; raw MS intensity of ^{12}C and ^{13}C isotopes of a short peptide in a TRIS-based biochemical assay buffer; CAD drawings of the automated system used to feed plates into the AMI-MS system; schematic of the data acquisition and processing workflow; calibration curves; and mass spectra (PDF)

Video of a 384-well plate read by AMI-MS at 0.5 s per sample (AVI)

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Author Contributions

#I.S. and M.B. have contributed equally. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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