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Re-Imagining Drug Discovery using Mass Spectrometry

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This contribution is dedicated to Prof. Helmut Schwarz, in recognition of his keen insights across chemistry and his major role in extending the depth of research in mass spectrometry over the past five decades.

Abstract: It is argued that each of the three key steps in drug discovery, (i) reaction screening to find successful routes to desired drug candidates, (ii) scale up of the synthesis to produce amounts adequate for testing, and (iii) bioactivity assessment of the candidate compounds, can all be performed using mass spectrometry (MS) in a sequential fashion. The particular ionization method of choice, desorption electrospray ionization (DESI), is both an analytical technique and a procedure for small-scale synthesis. It is also highly compatible with automation, providing for high throughput in both synthesis and analysis. Moreover, because accelerated reactions take place in the secondary DESI microdroplets generated from individual reaction mixtures, this allows either online analysis by MS or collection of the synthetic products by droplet deposition. DESI also has the unique advantage, amongst spray-based MS ionization methods, that complex buffered biological solutions can be analyzed directly, without concern for capillary blockage. Here, all these capabilities are illustrated, the unique chemistry at droplet interfaces is presented, and the possible future implementation of DESI-MS based drug discovery is discussed.

Keywords: Desorption electrospray ionization · automation · bioanalytical · tandem mass spectrometry · late-stage functionalization · high-throughput screening

Introduction

Mass spectrometry was at a turning point in the mid-1970's. The aim to achieve ionization of biological molecules had been set in place by the work of Beckey on field desorption[1] and Macfarlane on plasma desorption.[2] This focus would last another 20 years before Fenn,[3] and Hillenkamp and Karas,[4] unlocked the genie with electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI) with subsequent explosive results for biology. The mid-seventies also saw the first direct analysis of complex mixtures, using collision-induced dissociation (CID), as introduced by Jennings[5] and McLafferty[6] for the characterization of individual ions, but transformed into a mixture analysis method by Cooks[7] using CID in combination with soft ionization. It was at this time that Helmut Schwarz began his independent career, with investigations into the reactions of metal ions with organic compounds,[8] a subject which he shared with Freiser[9] and Armentrout,[10] and which he later perfected by developing and fully elucidating gas-phase catalytic reactions.[11] The existence of a true catalytic cycle in a gas-phase reaction was greeted with surprise, even skepticism, but Schwarz's remarkable command of the twin fields of mass spectrometry and computational chemistry, coupled to a broad knowledge of all of Chemistry, allowed him to validate these claims. His studies on room-temperature, selective C–H bond activation in methane used an enormous variety of catalysts including metal oxides, metal clusters and organometallics[12] in work that laid the foundation for the rational design of C–H activation. Simultaneously, he produced extraordinarily detailed data on the chemistry of another unreactive molecule, CO_2, providing a wealth of kinetic and thermochemical information as well as electronic structural effects for metal-catalyzed CO_2 reduction to form CO, the HCO_2 anion, and C–C coupled products.[13,14] The work of Prof. Schwarz involves highly intricate catalytic chemistry but it is also of the greatest practical importance. With the example of Schwarz's work in mind, this paper describes progress towards another important practical goal, that of drug discovery, starting in the same unlikely branch of physical science, mass spectrometry.

Mass spectrometry has long played a significant role in metabolite characterization,[15] especially in the form of the MS/MS experiment long used to screen for inborn errors of metabolism in newborns using heel-prick blood samples[16] and in ADME (absorption, distribution, metabolism, and excretion) pharmacokinetics,[17] a key aspect of drug development. The role of mass spectrometry as the foundational technology in the field of proteomics, marked by the pioneering efforts of Hunt[18] in particular, is noteworthy. Similarly, the field of molecular imaging has been transformed by MS, especially by MALDI imaging in the hands of Caprioli.[19] This paper deals with desorption electrospray ionization (DESI),[20] an ioniza-

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tion method that allows the examination of ordinary objects in their native environment, the defining capability of the suite of techniques known as ambient ionization.[21]

Drug discovery, as now practiced, involves three stages which can be described as pre-, core- and post-discovery activities. Pre-discovery actions include literature studies, computer-aided searches[22] and structural studies of biological targets[23] which identify molecules as potential drug candidates. Post-discovery activities, which take the longest time, include safety and efficacy tests in vitro and in vivo.[24] Core activities, on which this paper keys, involve (i) establishing synthetic routes to particular drug candidates, (ii) preparing small amounts of these candidate molecules, and (iii) performing bioassays that examine candidate drug/biological target interactions (such as chemical binding or enzyme kinetics) at the molecular and sometimes the cell culture level. This report includes experimental data and arguments for the view that the core activities in drug discovery (chemical analysis, small-scale synthesis and bioassays) can be performed using MS. This expectation is based in a larger view of the future of MS which includes an underlying conviction that, in the next few years "… MS will transition from being a predominantly analytical technique to a synthetic technique. (…) It's only natural that MS should be an important component of bond-forming chemistry."[25]

Results and Discussion

Overall Aim

High-throughput (HT) DESI-MS has the potential to accelerate drug discovery by combining chemical analysis with in situ accelerated chemical synthesis. An automated HT DESI-MS system that combines HT screening, HT synthesis, and HT bioanalysis is illustrated schematically in Figure 1. This platform utilizes small sample volumes (50 nL, equivalent to nanogram amounts of material) of reaction or bioassay mixtures, in the form of high-density arrays (up to 6,144 samples per array). Sampling is automated at rates better than 1 Hz with automatic real-time data analysis.[26,27]

The approach espoused here privileges speed, and hence wider coverage, over accuracy of individual measurements in searching for synthetic routes and identifying hits. It will miss some routes and some drug candidates (i.e. give false negatives) which is acceptable given the enormity of chemical space (ca. 10^{60} compounds).[28–30] It might also result in false positives, which are also acceptable to a degree in an initial large screen as they will be removed in subsequent rigorous (and slower) screens covering much smaller sets of molecules.

Reaction Screening Using Accelerated Reactions in Microdroplets

DESI is a micro-extraction method in which charged micro-droplets (low micron size range) are pneumatically accelerated, then impacted upon a surface, so splashing and releasing secondary droplets (ca. 1 micrometer diameter). The latter

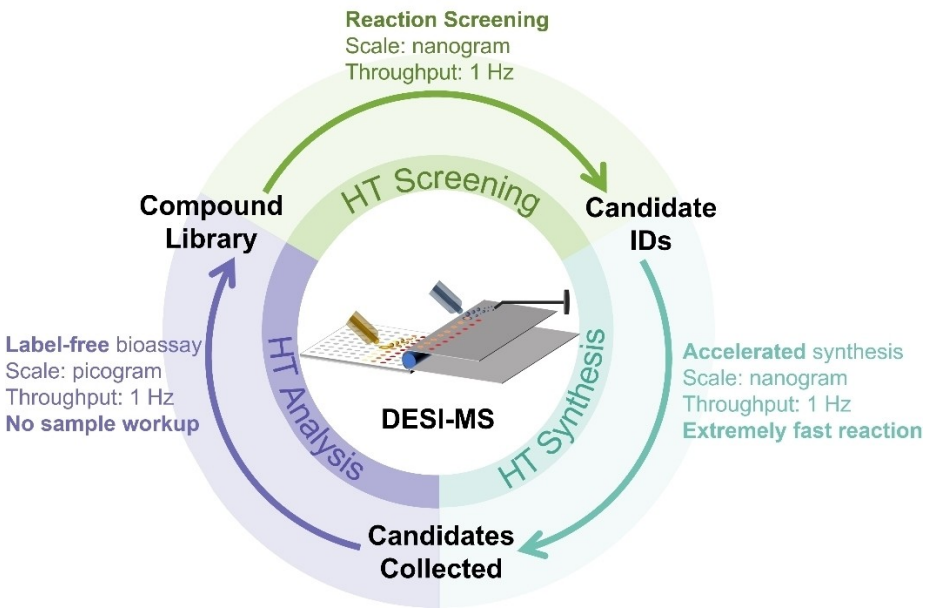


Figure 1. Schematic representation of an automated platform for HT drug discovery based on DESI-MS.

evaporate and undergo coulombic fission as they move towards and within a mass spectrometer (flight times around a few tens of ms), until they yield dry ions that are then mass analyzed.[20–22,29] The contactless nature of DESI allows direct analysis of complex and buffered reaction/biological mixtures without any sample workup,[26,31–34] while the phenomena of reaction acceleration[35–37] in microdroplets provides access to extremely fast reactions. Reaction acceleration, often expressed as the ratio of the reaction rate constant in microdroplets vs. that in the corresponding bulk reaction, can be very high indeed, up to 10^6.[35,36,38] There is wide agreement[35–37,39,40] that this is due to interfacial reactions, although there is also a contribution from droplet evaporation for some solvents. The role of partial solvation in increasing reaction rates at the droplet interface,[35,41] together with the effect of a strong interfacial electric field,[42,43] the superacid/superbase droplet surface,[44,45] and the presence of other highly reactive species,[46–49] has been proposed to underlie the observation of reaction acceleration.

Organic solvents are typically used to study microdroplet reactions although the presence of some water has been shown necessary to drive some of these processes.[45] Far from being simply a solvent, unexpected chemistry has been shown to occur in water droplets. For example, spontaneous redox reactions have been observed in aqueous microdroplets,[46,47,50,51] where the proposed oxidant is the water radical cation, likely in the form of its dimer, which either exists as a hydroxyl radical/hydronium complex (Structure 1, Figure 2A) or a O–O single-electron bound dimer (Structure 2, Scheme 1A)[52]. The reactivity of the water radical cation towards X=Y heteroatom double bonds yields a covalent adduct (presumably the geminal diol radical cation, Structure 3 in Scheme 1B). The tendency to generate this ion as a major product of spray ionization has been demonstrated through a large-scale study (ca. 21,000 non-proprietary compounds) using HT DESI-MS.[48,53] The cleavage of the heterobond in this adduct leads to the observed oxidation product, e.g. sulfones to sulfonic acids or ketones to carboxylic acids (Scheme 1B and Figure 2B). Condensation (i.e. water loss) reactions, such as the formation of peptide bonds between amino acids, has also been shown to occur in aqueous microdroplets due to the particular environment at the droplet surface.[54] This result is a strong indicator of the potential relevance of micron-size water droplets (e.g. aerosols, sea spray) in the origin of life in the prebiotic world.

While it is certainly true that not every reaction mixture that forms products in microdroplets will give the same products in bulk solution, this is overwhelmingly the case in the systems so far studied, as shown by the listing of some successful microdroplet reactions in Table 1 (for a complete

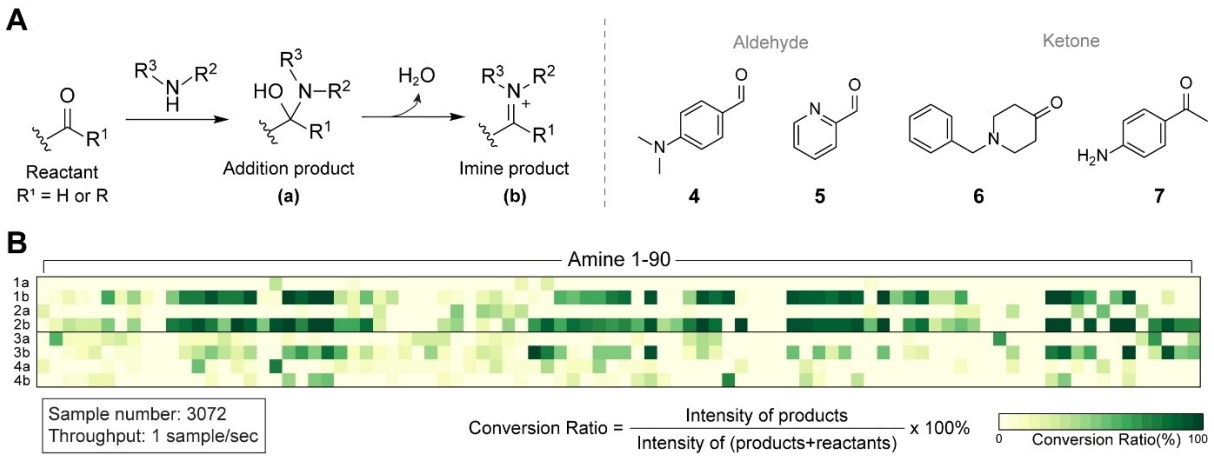
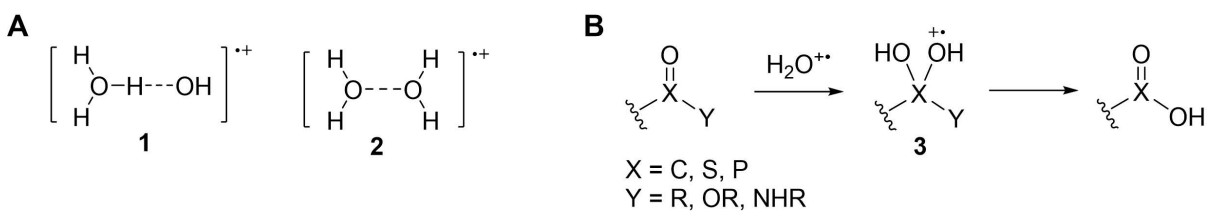


Figure 2. Imine formation screening using HT DESI-MS. (A) Schematic of the reaction screened including the carbonyl substrates (**4**–**7**) explored. (B) Heatmap showing conversion ratios for the addition product (intermediate) and for the final imine product. Conversion ratios, calculated as denoted in the figure, indicate the extent of reaction observed through DESI-MS screening without pre-incubation.



Scheme 1. Reactivity of water radical cations. (A) Possible structures (from computation)[55] of the water dimer radical cation. (B) Water radical adduction with X=Y heteroatom double bond and subsequent bond cleavage yielding the oxidation products.

Table 1. Chemical reactions observed in microdroplets. Detailed chemical transformation, corresponding references, and comments are listed in the Supporting Information.

Reaction type	Demonstrated accelerated reactions
Addition	Michael addition, aza-Michael addition, carbamic acid formation, epoxide ring opening, hydration of alkene, [3 + 2] cyclo addition
Elimination	Dehydration, pinacol rearrangement, Hoffman elimination
Addition-Elimination	Schiff base formation, Katritzky reaction, ketoxime formation, benzimidazole formation, hydrazide formation, amide bond formation, esterification, Claisen-Schmidt condensation, ester hydrolysis, substitution (S_N2), amine acylation
Redox	Amine oxidation, sulfone oxidation, oxidative cleavage of C=C, epoxidation, stevens oxidation, aerobic oxidation, carboxylic acid formation, Baeyer-Villiger reaction, phosphonylation, sulfide oxidation, electro-oxidative C–N coupling, spontaneous reduction, nanoparticle synthesis, Dakin reaction, CO_2 reduction, phosphonate redox reaction, carbonyl reduction, dye photo-oxidation
Multicomponent	Mannich reaction, Betti reaction, Hantzsch reaction, Biginelli reaction, spiro-pyrolidine formation, N-alkylation of indole
Cross-coupling	Suzuki coupling, decarboxylative amination, $C(sp^3)$–N coupling, $C(sp^2)$–N coupling

list including references and observations, *see* Supporting Information). It is worth highlighting that almost all of these bimolecular reactions are accelerated relative to bulk. Note too that only reactions reported in microdroplets with a gas/solution interface (as opposed to water in oil systems) are included as the former give much greater acceleration factors.

Yields of products from microdroplet reactions vary, depending on the surface area of the droplets. This dependence means that very small droplets are most effective in terms of fast reactions, provided enough material is generated for subsequent operations. Note that accelerated reactions in microdroplets can sometimes be scaled up by using higher concentrations or larger volumes or, most effectively, by recycling and respraying the collected liquid. Yields of grams/hour can be reached in this latter way although at significant cost in terms of time.[56]

Accelerated reactions in microdroplets enable the use of DESI-MS as a HT reaction screening tool where the reaction products can be formed during the DESI process and immediately detected by MS without the need for incubation steps. An example is reaction screening for imine formation as shown in Figure 2. The use of DESI-MS allows for the identification of both reaction products and intermediates (Figure 2A), as well as reactivity trends across different reactants (Figure 2B). For instance, aldehydes show higher reactivity than ketones, as do aliphatic amines compared to aromatics. The high throughput of the automated platform and the high density of the arrays used facilitates the rapid exploration of a large number of reaction conditions or reactant combinations. In this particular experiment, reactant pairs were prepared using fluid handling robotics (< 5 min) and 3,072 spots including independent and instrumental replicates were screened (ca. 45 min), providing average conversion ratios (yields expressed in ion abundance ratios) through a user-friendly heatmap interface in under an hour.

The discussion of accelerated reactions so far has dealt with simple compounds and classical chemical reactions. However, drug discovery is often concerned with modifications to complex molecular skeletons that show an encouraging degree of biological activity. This approach, referred to as late-stage functionalization (LSF),[57] changes targeted functional groups without protecting others in multifunctional drug candidates. The first applications of HT DESI-MS for selective functional group modification using LSF were recently demonstrated.[58]

LSF has been implemented in the case of the novel μ-opioid receptor agonist PZM21.[59] This compound has phenol, tertiary amine and thiophene functional groups available for modification. Multiple reaction types (fluorosufurylation, sulfur-fluoride exchange or SuFEx click reaction, ene-type reaction, and N-alkylation) and hundreds of reaction conditions were screened by HT DESI-MS to reveal potential new drug candidates formed by accelerated reactions in the DESI microdroplets, as shown in Figure 3A for the SuFEx LSF screening.[60] More than 50 functionalized drug candidate molecules were identified in this late-stage diversification of PZM21. By repeating the experiment on exactly the same sample (or a duplicate spot on the DESI slide) structural information on the drug candidates was provided by tandem mass spectrometry (Figure 3B).

High-Throughput Synthesis by Collecting Reacting Microdroplets

When using HT DESI-MS to screen for ions corresponding to products of interest, the automated system will take two actions (i) switch from single-stage MS to the MS/MS mode to fragment the ions corresponding to the putative drug candidates and perform structural characterization as discussed

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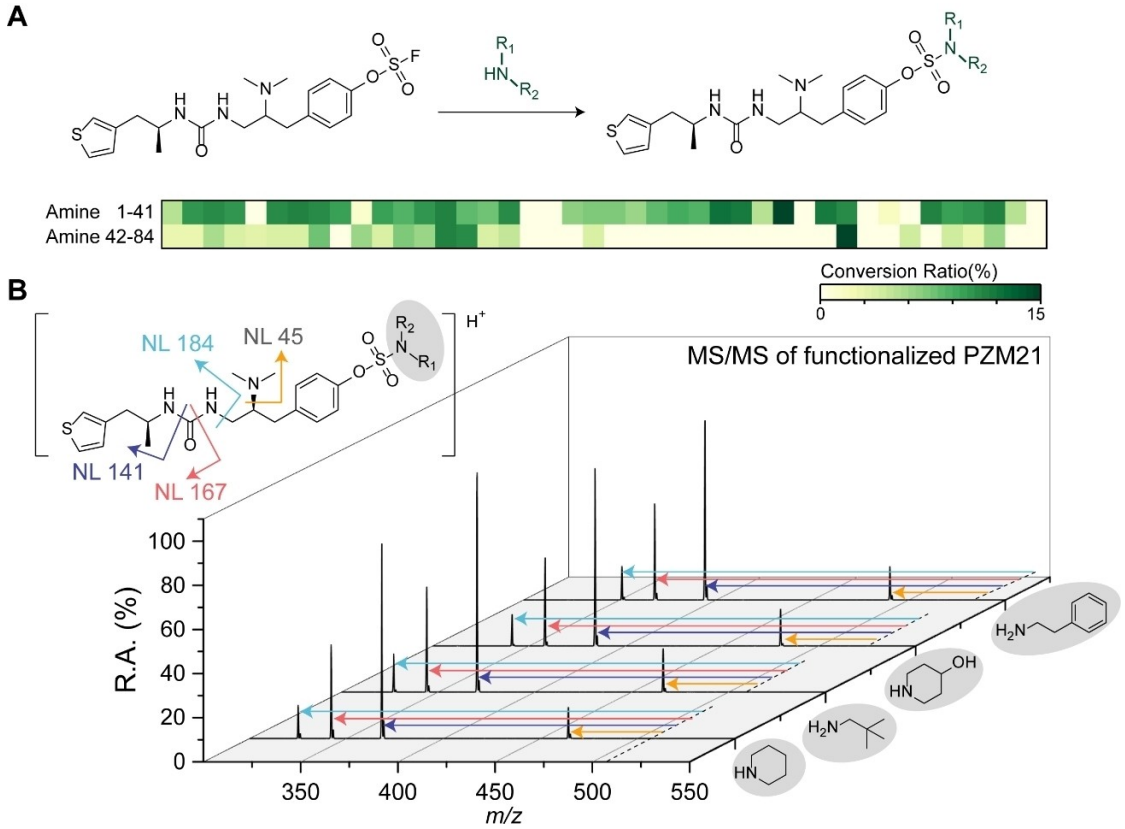

Figure 3. HT LSF of PZM21 using SuFEx click reactions in microdroplets. (A) Heatmap of ion conversion ratios for the final SuFEx products after screening 84 different amines. (B) Tandem mass spectrometry characterization of four PZM21 analogs obtained through DESI-MS LSF. Characteristic neutral losses (NL) present in the unmodified substrate are preserved in the functionalized versions, as expected, indicating the success of the reactions and the selectivity towards the phenol moiety.

above, and then (ii) switch the secondary droplet stream from the mass spectrometer to a collecting surface. This capability, which is currently being automated, will ensure that the particular species produced through the microdroplet accelerated reactions and seen in the analytical mass spectrum, are collected. A schematic illustration and a photograph of the first generation of the instrumentation used for collection of the synthetic products is shown in Figure 4. Note that while

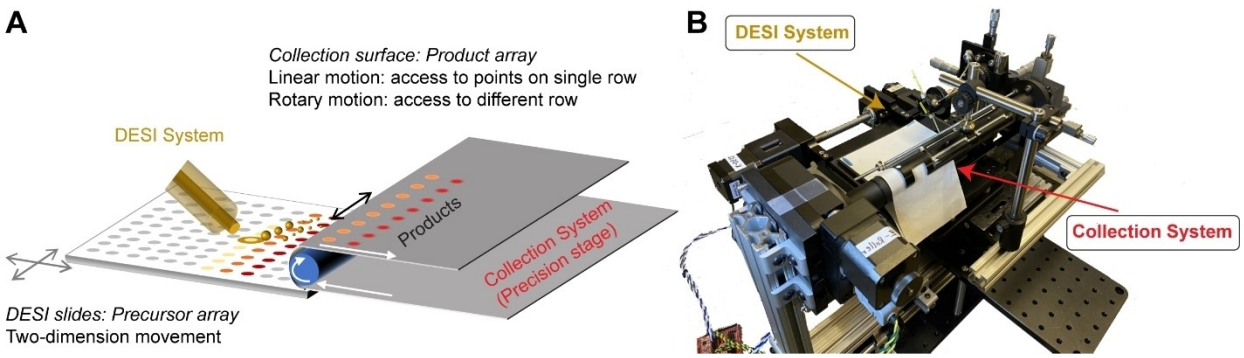

Figure 4. DESI-based system for collection of microdroplet reaction products. (A) Schematic illustration of the instrumentation being used for collection of drug candidates in the reacting droplet plume generated by DESI from reaction mixtures on particular spots in an array. Note the array-to-array transfer with maintenance of relative position in each array, achieved by controlled motion of the DESI spray along a row and then by the row-to-row motion by a mechanism that is based on principles used in 20th century typewriters. (B) Photograph of the actual device.

provision could be made for some form of product purification, the underlying philosophy of our approach is to maximize speed and avoid sample work up.

The collection system contains two arrays: (i) a precursor array, containing the reaction mixtures as spotted onto a DESI slide, and (ii) a product array, where the microdroplet reaction products are deposited (Figure 4A). The two-dimensional movement of the DESI slide makes accessible spots of interest in the precursor array (those which yielded expected products in the previous screening stage before chemical transformation achieved by in the DESI microdroplets). The two degrees of motion of the collection system then allow the generation of a product array: products are deposited on a single row at different positions using linear movement while the rotary movement provides access to different rows. A video of a proof-of-concept experiment involving spatially resolved collection of a single row of products onto a strip of filter paper is included as Supplementary Material. The reaction mixtures on the DESI slide were impacted by DESI droplets, and the reagents in the splashed secondary droplets underwent accelerated reactions yielding products that were deposited on the collecting surface. Re-analysis of the surface by DESI confirms the presence of spatially resolved reaction products proving the concept of collecting the reacting DESI microdroplets from different reaction mixtures at different positions.

High-Throughput Label-Free Bioassays

The nanogram amounts of microdroplet reaction products collected suffice for initial bioactivity assessments; these are carried out using the same automated DESI-MS platform instead of traditional spectrophotometric or radiometric systems. The use of DESI-MS for biological assays brings several advantages compared to conventional bioassay approaches as MS is an inherently label-free technique with high sensitivity and chemical specificity. This not only maximizes the versatility of the platform, reducing the cost and time required to develop new methods for each different substrate or biological target, but it also eliminates safety concerns related to radioactive materials as well as interferences due to the use of non-native substrates or coupled secondary reactions that are often required to obtain outputs suitable for spectrophotometric determinations.[26,32,61,62] Additionally, the use of DESI in particular as the ionization method allows the direct analysis of complex bioassay mixtures as it has a high tolerance for high concentrations of non-volatile buffers, salts and detergents,[31,32,62] all typical components of biological assay matrices and commonly not compatible with direct MS analysis.

Multiple examples of biological assays carried out using HT DESI-MS with identical throughput to that of the DESI organic reaction screening (i.e. 1 Hz) have been described.[26,32,61,63] Characterization of enzymatic systems through kinetic studies,[32,61,63] substrate scope assessment,[26] as well as screening and characterization of inhibitors and reactivators,[26,32,61,63] have all been demonstrated using this automated platform. It is worth highlighting that, in contrast to organic reaction screening, precise quantitative analysis is required for the application of DESI-MS to bioassays. The use of (i) internal standards or (ii) substrate-to-product ratios together with external calibration curves, gives excellent quantitative performance (coefficients of variance commonly better than 10%) as consistently shown with HT DESI-MS.[31,32,61] Additionally, due to the analysis speed and high density of the sample arrays, robust results can be obtained by efficiently analyzing a large number of replicates (both independent, aided by automatic preparation using fluid handling robotics, as well as instrumental, generated by pinning replicates of the samples on the DESI slide).

Applications of HT DESI-MS to measure the activity of enzymes related to neurodegenerative diseases,[32] cancer,[61,63,64] and metabolic regulation[26] have been explored. Here we present a new example taken from the study of β-lactamases, which are important enzymes in the context of bacterial antibiotic resistance.[65,66] As observed in Figure 5, automated HT DESI-MS can be used to monitor the hydrolytic activity (Figure 5D) of the *Bacillus cereus* penicillinase towards amoxicillin (Figure 5A) and ampicillin (Figure 5B and 5E), typical first-line antibiotic treatments.[67,68] The hydrolysis of these two compounds can be monitored over time as a function of the ratio between the hydrolysis product (detected as an $[M+H]^+$ ion) and the antibiotic substrate (principally detected as an $[M+Na]^+$ species). Note that ion ratios can be easily calibrated to provide absolute concentrations using an external calibration created with standards of both reactants and products.[32] As expected, no enzymatic activity is observed against meropenem, a third generation β-lactam antibiotic for which *Bacillus cereus* does not commonly show natural resistance[69,70] (Figure 5C and 5F). This demonstrates the versatility of the label-free DESI-MS approach for rapidly assessing enzymatic activity against multiple substrates without the need for any additional method development. Furthermore, the DESI-MS system can be used to identify and characterize enzymatic inhibition, as illustrated here for the standard pharmaceutical combinations ampicillin/sulbactam (Figure 5G) and amoxicillin/clavulanate (Figure 5H and 5I), where sulbactam (IC_{50} determined as 10.8 ± 0.9 μM) and clavulanate (IC_{50} found as 6.1 ± 0.3 μM) are β-lactamase inhibitors that prevent the antibiotic hydrolysis.[71] These results showcase the potential of DESI-MS for the screening of new β-lactamase resistant antibiotics as well as the identification of novel antibiotic-inhibitor combinations, both relevant goals to face the current antimicrobial resistance crisis.[72–74]

Conclusions

The work described in this paper illustrates a significant current development in mass spectrometry, namely the addition of a role in chemical synthesis to its common use in

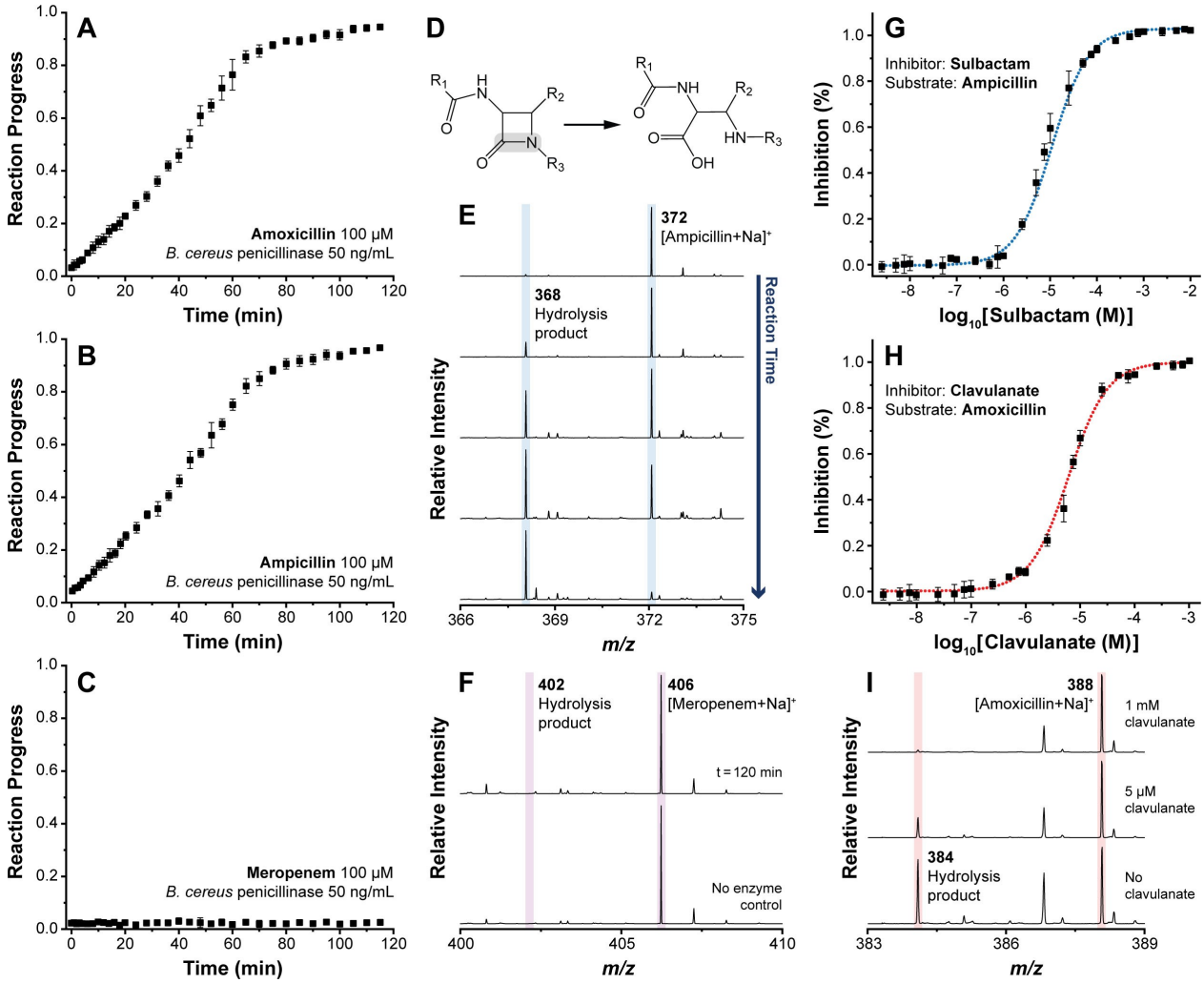

Figure 5. Results of the HT DESI-MS label-free β-lactamase assay. (A) Reaction progress curve obtained using amoxicillin as substrate. The reaction progress is monitored as the ion intensity ratio between the hydrolysis product (detected as an $[M+H]^+$ ion) and the substrate (detected primarily as an $[M+Na]^+$ ion). (B) Reaction progress curve obtained using ampicillin as substrate. (**C**) Reaction progress curve obtained using meropenem as substrate. No hydrolytic activity was observed for this antibiotic. For all curves, 16 independent replicates were prepared for each time point and 4 instrumental replicates were spotted and analyzed for each sample. For each substrate, 30 time points were collected, for a total of 1,920 spots analyzed per curve (ca. 30 min of analysis). (D) Hydrolysis reaction catalyzed by β-lactamases. The target C–N bond is highlighted. (E) Representative HT DESI-MS spectra corresponding to the ampicillin reaction over time. Note the increase in product intensity and the simultaneous decrease in substrate intensity as the reaction time increases (denoted by the arrow on the right). (F) Representative HT DESI-MS spectra corresponding to the meropenem reaction. No differences were observed after 2 hours of reaction compared to control samples without enzyme. (G) Dose-response curve for the inhibition of the β-lactamase by sulbactam using ampicillin as substrate. The corresponding sigmoidal fit (adjusted R^2 0.998) is included as a dotted line. (H) Dose-response curve for the inhibition of the β-lactamase by clavulanate using amoxicillin as substrate. The corresponding sigmoidal fit (adjusted R^2 0.999) is included as a dotted line. For both curves, 16 independent replicates were prepared for each concentration and 4 instrumental replicates were spotted and analyzed for each sample. For each inhibitor, 24 concentrations were tested, for a total of 1,536 spots analyzed per curve (ca. 25 min of analysis). (I) Representative HT DESI-MS spectra corresponding to some of the dose-response experiments using clavulanate as inhibitor and amoxicillin as substrate. Note the decrease in product intensity as clavulanate concentration increases. In all cases error bars represent standard deviations.

chemical analysis. It is worth noting that the use of mass spectrometry in synthesis is not confined to droplet chemistry. Ions are being used as chemical reagents to modify surfaces, prepare catalysts,[75–77] and create devices involving a variety of types of fundamental processes that include reactive scattering and ion soft landing.[78,79] Earlier examples of ion beam chemical synthesis were associated with ion/surface chemistry.[80] The ion synthesis role illustrated here represents an extension of the earlier topic of ion/molecule reactions, one of the bedrocks of mass spectrometry in the second half of the

20th century, often discussed under the simple title *ion chemistry*. The droplet chemistry discussed in the present paper is also ion chemistry – but at the gas/solution interface – and as such it has close similarities to conventional solution phase chemistry while retaining the speed advantages of ion/molecule reactions in vacuum.

As of now, there are no complete end-to-end examples of drug discovery based on high throughput accelerated droplet reactions. However, detailed studies have been completed on each of the three component steps, (i) reaction screening to find successful routes to desired drug candidates, (ii) collection of microdroplets to produce amounts of products adequate for testing, and (iii) bioassays of the candidate compounds. Further instrumental developments are underway, including building of an entirely new DESI-based drug discovery system (NCATS ASPIRE UG3 TR004139). This development should increase the speed of drug discovery.

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Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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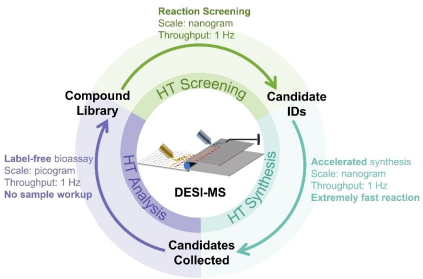

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Re-Imagining Drug Discovery using Mass Spectrometry