A Mass Spectrometry Plate Reader: Monitoring Enzyme Activity and Inhibition with a Desorption/Ionization on Silicon (DIOS) Platform

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A surface-based laser desorption/ionization mass spectrometry assay that makes use of Desorption/Ionization on Silicon Mass Spectrometry (DIOS-MS) has been developed to monitor enzyme activity and enzyme inhibition. DIOS-MS has been used to characterize inhibitors from a library and then to monitor their activity against selected enzyme targets, including proteases, glycotransferase, and acetylcholinesterase. An automated DIOS-MS system was also used as a high-throughput screen for the activity of novel enzymes and enzyme inhibitors. On two different commercially available instruments, a sampling rate of up to 38 inhibitors per minute was accomplished, with thousands of inhibitors being monitored. The ease of applying mass spectrometry toward developing enzyme assays and the speed of surface-based assays such as DIOS for monitoring inhibitor effectiveness and enzyme activity makes it attractive for a broad range of screening applications.

Introduction

Recent advances in enzyme engineering and discovery have created new demands for automated, rapid, and reliable primary screening methods for enzyme inhibition and other small molecule–protein interactions.[1–3] Fluorescence plate readers have become the dominant tool in these efforts because they are simple and easy to operate, in many cases replacing radiometric assays because they provide similar sensitivity to radiolabels and can be employed in high-throughput format.[4–8] Typical enzyme-based drug screening with fluorescence plate readers can achieve a throughput rate that meets the demand of most pharmaceutical companies (10000 inhibitors per day). However, fluorescence-based assays suffer from three significant problems: 1) substantial assay development time, 2) the incorporation of a fluorescent moiety can affect enzyme reactivity, and 3) false positives or false negatives from inhibitors that fluoresce or that quench fluorescence.

Mass spectrometry, which accomplishes the detection of a wide range of molecules with high sensitivity, can effectively address the limitations of fluorescence plate readers.[9,10] Mass spectrometry is capable of detecting most natural substrates and/or products of enzymatic reactions, and therefore assay development is rapid (typically less than a day), and false positives and false negatives are rare (*m/z* overlap with a contaminant). Only in the area of speed and multiplexed analytical capabilities has mass spectrometry been deficient with respect to optical techniques; however, considerable progress in instrument hardware, robotics, and sample handling has significantly increased sample throughput.[11,12]

Electrospray ionization mass spectrometry (ESI-MS) has proven to be a viable technique for the identification and screening of effective ligand-binding receptors, new catalysts, and enzyme inhibitors.[9,13,14] ESI-MS has been the primary approach for enzyme-activity experiments because of its potential to be used for a wide array of molecules, its analytical specificity, and its accuracy. However, ESI and new chip-based ESI technology are limited in their high-throughput capability. We describe here the application of Desorption/Ionization On Silicon mass spectrometry (DIOS-MS), a surface-based approach,

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that allows for high-throughput analyses as samples are analyzed by scanning the silicon surface.

DIOS-MS is similar to matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS), yet DIOS-MS does not require a matrix and is performed by placing the sample directly on the silicon surface followed by laser desorption/ionization. It offers high sensitivity, no matrix interference, and has a mass range amenable to drug candidate analysis. Recent experiments illustrate the broad applicability of DIOS-MS, with analyses encompassing small-molecule characterization, proteomics, forensics, peptide and small molecule sequencing, and peptide detection from cultured cells on the porous silicon (pSi) surface.[15–19] Moreover, with the incorporation of electrospray sample deposition (ESD), the accuracy and reproducibility of ESD DIOS-MS make it well suited for quantitative analysis.[20] In this study, we extend the utility of DIOS-MS as a high-throughput drug-screening tool, taking advantage of its sensitivity and accuracy to analyze inhibitor libraries and enzyme activity. As with all mass spectrometry-based methods, no chromophore or radiolabels are required.

Results and Discussion

Analysis of small molecules

The general applicability of DIOS-MS to small molecules has been demonstrated previously.[15–17] Here the relatively high-throughput screening of an array of 100 different compounds consisting of amino acids, a small drug-discovery library, peptides, and protein digests was demonstrated. Typically, 0.5 μL samples of 100 μM concentration of these solutions were deposited on the etched spots of a photopatterned pSi plate (Figure 1) and were allowed to dry in air. The DIOS plate was then loaded into the mass spectrometer and analyzed by using automated scanning. The analyses of the samples were accomplished in less than in 8 min when utilizing a relatively

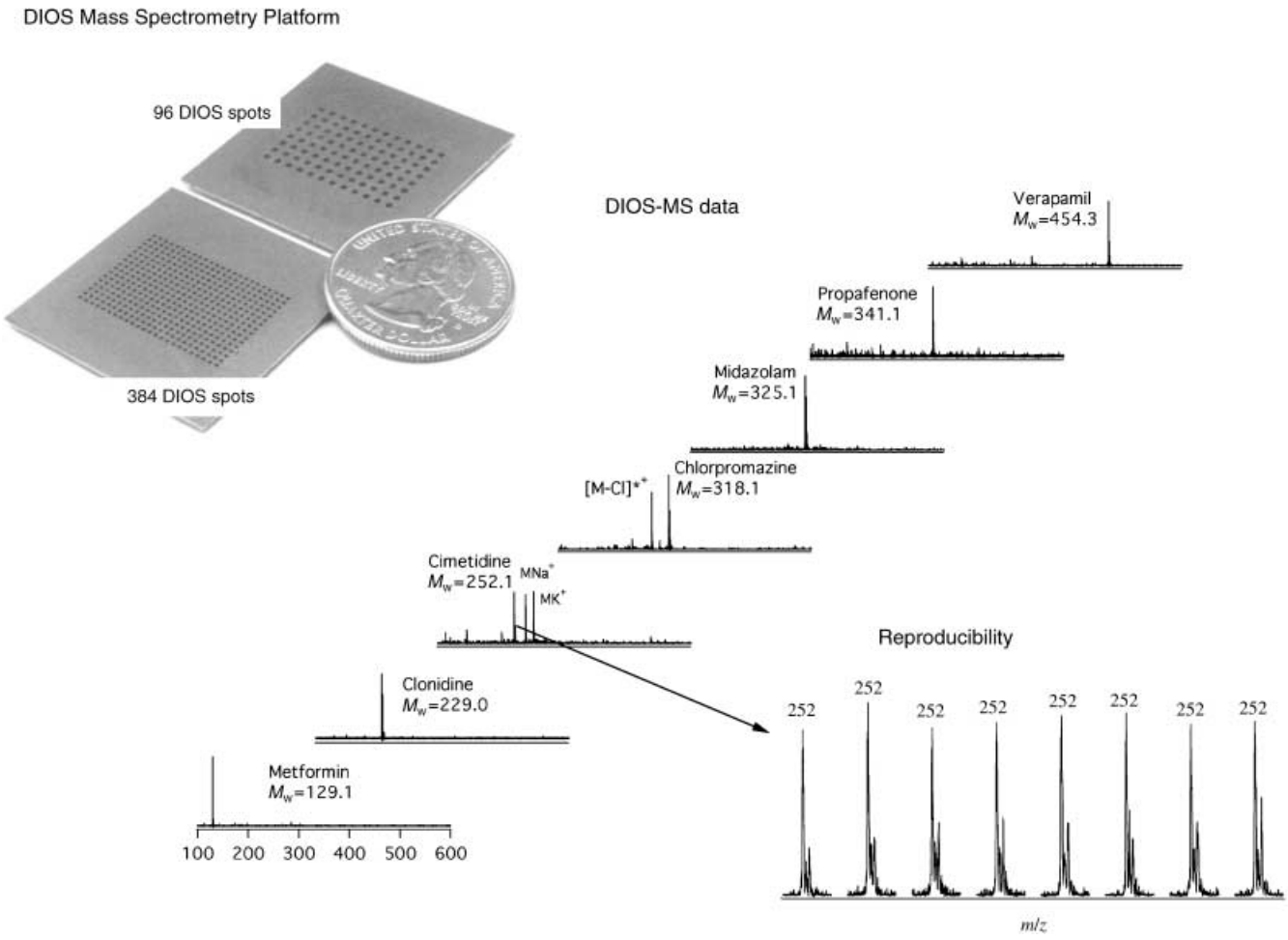

Figure 1. *Top left: Photopatterned (96 and 384) DIOS chips with arrays of dark spots corresponding to porous Si photopatterned onto the Si wafer by using photo-enhanced electrochemical etching. (The chips are shown adjacent to a quarter-dollar coin to give an idea of their size.) Center: DIOS mass spectra of small-drug molecules from each porous silicon spot containing 50 pmol of sample. Each spectrum shown is an average of over 16 laser shots (laser repetition rate 5 Hz) and was obtained in, on average, 5 s: 1 s to move from one sample spot to the next and 4 s to initiate laser firing and data collection. This gives a rate of 720 samples per hour. Bottom right: DIOS-MS spectra reproducibility of eight distinct sample spots containing cimetidine. The standard deviation for the signal-intensity variation was below 20%.*

low laser repetition rate of 5 Hz. Figure 1 shows the DIOS chips and representative sample spectra of the high-throughput screening of the 100 compounds with good resolution and high signal-to-noise ratio.

Enzyme discovery

The primary focus of the enzyme-discovery effort was to apply DIOS-MS as a high-throughput screen for the discovery of new enzymes with properties similar to phenylalanine hydroxylase (PAH). In the initial set of experiments, we explored the high-throughput capability of the DIOS-MS plate reader assay to monitor the activity of PAH and, further, to identify new enzymes that would have the same activity as PAH (Figure 2). PAH deficiency is directly associated with the disease phenylketonuria (PKU), an inherited, metabolic disorder[21] that can result in severe mental retardation if untreated at birth. PKU is caused by a deficiency of the enzyme PAH, which is necessary to convert phenylalanine to tyrosine. The goal is to develop the DIOS-MS plate-reader assay to screen mutant forms of endogenous and exogenous PAH enzymes that could potentially be used as a drug therapy.[22–24]

A current standard approach for screening for PAH enzymes is based on electrospray ionization tandem mass spectrometry with a throughput of one sample every 120 seconds.[25,26] PAH activity assays based on colorimetric or radioactive detection are not as reliable, sensitive, or accurate as mass spectrometry techniques. Due to the specificity of mass spectrometry, assay screening with DIOS-MS allows quick identification of Tyr and Phe signals in the incubation mixture. Although pure Tyr and Phe standards yield strong signals in DIOS under both positive and negative ion modes, we found that in the PAH assay, the use of negative ion mode produces less low mass interference from the incubation mixture. As shown in Figure 2, a spectrum of the PAH reaction product has a peak at $m/z = 164$ (corresponding to unreacted L-phenylalanine) and a peak at $m/z = 180$ (corresponding to enzymatically produced L-tyrosine). An internal standard for each analyte of interest (deuterium-labeled L-Phe and L-Tyr, $[D_5]$-ring-Phe, $[D_4]$-ring-Tyr, respectively) makes it possible to quantify the transformation of L-Phe to L-Tyr, and thereby calculate the specific activity of the enzyme by varying substrate concentration and measuring product formation for a fixed reaction time.

Enzyme reaction monitoring and inhibition

We have implemented DIOS-MS as an assay to monitor the reaction time course of α-(2,6)-sialyltransferase in the sialylation of *N*-acetyllactosamine (LacNAc). Sialyltransferase is an important class of glycotransferase that catalyzes the transfer of the sialic acid of cytidine monophosphate–sialic acid (CMP-NeuAc) to the nonreducing end of the carbohydrate moiety of glycoconjugates.[27] This enzyme exhibits high acceptor–substrate specificity and plays an important role in cell adhesion and molecular recognition events.[28] Although the enzyme can accept several acceptor–substrate combinations due to some degree of flexibility around their glycosidic linkages, the sialylation site of the α-(2,6)-sialyltransferase used in this study is in the C-6′ of the galactose group of LacNAc. The production of α-(2,6)-sialylated trisaccharide was monitored for a single-step reaction catalyzed by α-(2,6)-sialyltransferase from a corresponding lactoside (Figure 3). Under optimal conditions

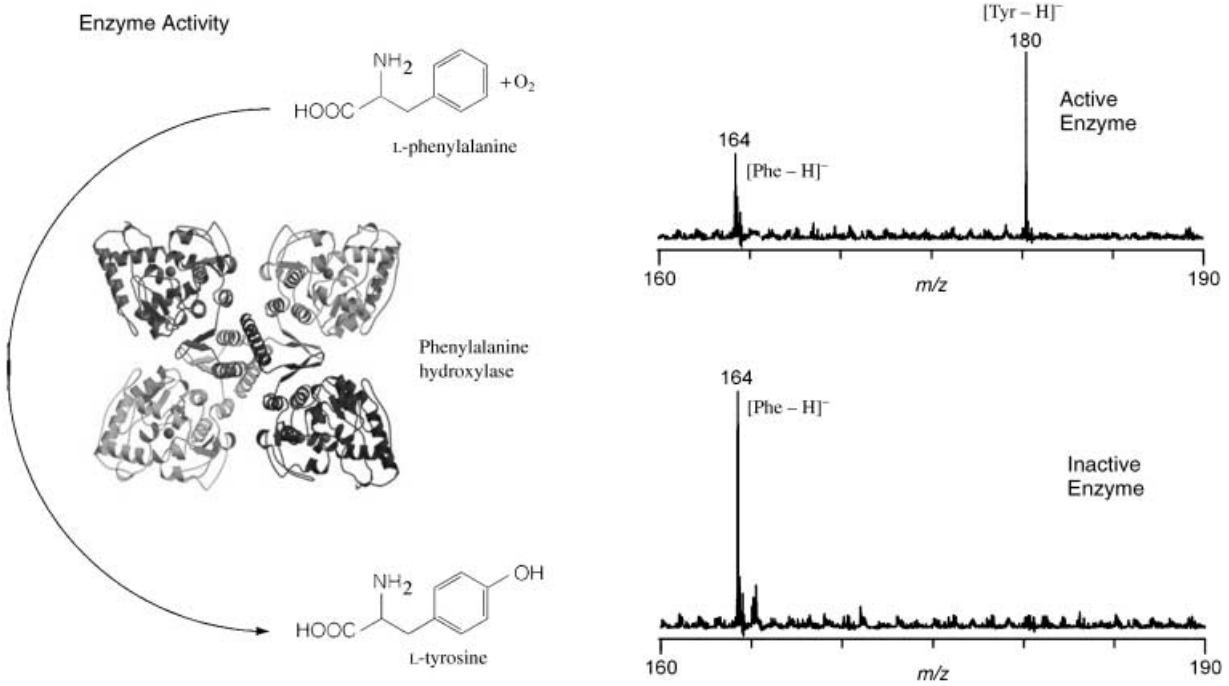

Figure 2. *Monitoring the activity of phenylalanine hydroxylase (PAH), which converts phenylalanine to tyrosine. DIOS-MS is used as an assay to screen recombinant PAH as potential drugs. An active enzyme is denoted by the formation of tyrosine in the DIOS-MS analysis.*

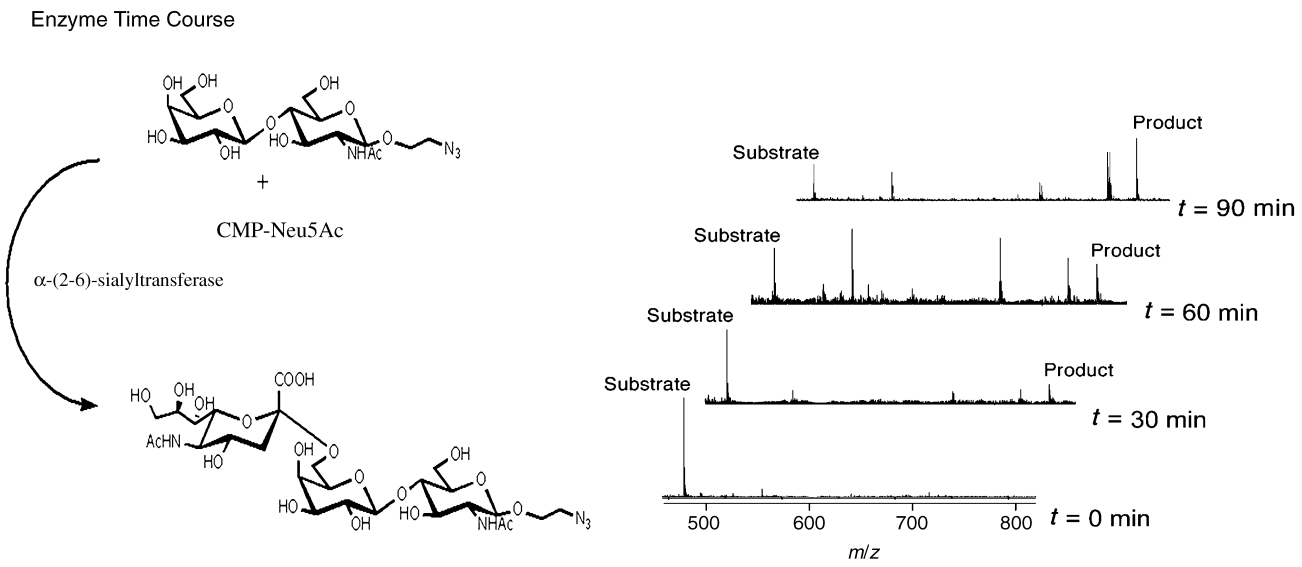

Figure 3. *Monitoring the α-(2,6)-sialyltransferase enzymatic time course through the DIOS-MS of α-(2,6)-sialylated trisaccharide (m/z = 788) generated from the corresponding lactoside (m/z = 475).*

(pH 6.5, 37 °C), the formation of the sialylated trisaccharide was detected after 15 minutes. Figure 3 shows the DIOS-MS sialylation reaction with both the sodiated glycosyl substrate at $m/z = 475$ and the product peak at $m/z = 788$. Once the sialylation reaction had been observed, we monitored the enzyme activity by measuring the formation of products at different reaction times using the same substrate concentration.

The ability of DIOS to rapidly monitor the inhibition of other enzyme-catalyzed reactions was investigated with four different proteases. The DIOS assays for all of the enzymes were each developed within a couple of hours, and the subsequent monitoring of inhibition was readily achieved. DIOS-MS was successfully applied to monitoring the inhibition of four different proteases including trypsin, endoproteinases Arg-C, Lys-C, and Glu-C with bovine serum albumin (BSA) serving as the model protein. DIOS mass-spectral data of BSA digested with trypsin and the endoproteinase enzymes generated the expected proteolytic fragments. In contrast, when the enzyme and inhibitor mixture were incubated for at least 30 minutes at 4 °C prior to the addition of BSA (100 nM and 2 μM) and ammonium citrate buffer solution, the known inhibitors completely inhibited digestion of BSA as observed with DIOS-MS and further confirmed with MALDI-MS. In this inhibition experiment, an enzyme/enzyme inhibitor molar ratio of 1:6 was used to achieve complete inhibition.

Having demonstrated the applicability of the DIOS-MS plate reader assay in monitoring enzyme activity, we also illustrated the effectiveness of DIOS for monitoring multiple inhibitors against a single enzyme. Acetylcholinesterase (AChE) is an important enzyme of the nervous system that terminates the action of acetylcholine released into the cholinergic synapses.[29] AChE catalyzes the hydrolysis of the neurotransmitter acetylcholine to choline, (Figure 4) and has been the focus of inhibitor studies in Alzheimer's disease therapy to boost the endogenous levels of acetylcholine in the brain and thereby to enhance cholinergic neurotransmission. Because of this, as well as its biological relevance, it was an early focus as a target enzyme for screening enzyme inhibition. In the experiment we incorporated the ESD method of sample deposition to monitor the pseudo first-order AChE reaction performed at optimal conditions (pH 8, 37 °C) with $[D_9]$choline as internal standard. The production of choline was monitored as a function of time with a starting substrate concentration of 200 μM. The kinetics plot generated for the AChE reaction was consistent with an earlier study with smaller error bars due to a marked improvement in the sample homogeneity across the pSi surface (Figure 5).

The screening for potential AChE inhibitors was examined by monitoring the AChE activity in the presence of potential inhibitors from a small-molecule library by using the DIOS plate reader. The inhibition of the deacylation reaction of acetylcholine was performed by incubation of candidate small molecules with the enzyme and substrate for 30 minutes (pH 8, 37 °C), followed by measurement of the acetylcholine/choline ratio by DIOS-MS (Figure 4). Known AChE inhibitors (tacrine and huperzine) could be identified through the observation of high acetylcholine/choline ratios; no false positives or false negatives were observed among the potential inhibitors. The throughput of such enzyme-inhibitor screening was approximately 1 sample every 3.5 seconds, or 5 minutes for a set of 100 compounds with a standard commercial instrument with a 10 Hz laser. Four thousand samples were analyzed in the course of a five-hour period. In a separate set of experiments with a 200 Hz laser, a sampling rate 1.6 seconds per sample was achieved, with a plate of 100 samples being analyzed in 160 seconds. Interestingly, the rate-limiting step in these experiments was not the data acquisition but the time required to move from spot to spot. Because the method is inherently sensitive, simple, and fast, the screening method should greatly facilitate inhibitor discovery.

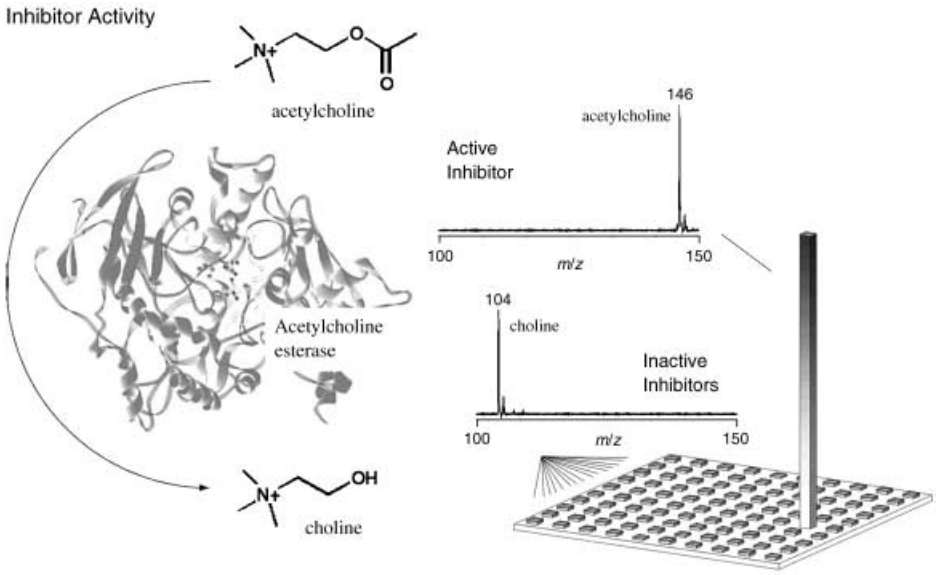

Figure 4. *The inhibition of acetylcholinesterase was determined by monitoring the substrate-to-product ratio with a high-throughput DIOS-MS in the presence of potential inhibitors at a rate of one sample every 1.6 s. The 100-compound inhibitor library was screened in less than 3 min.*

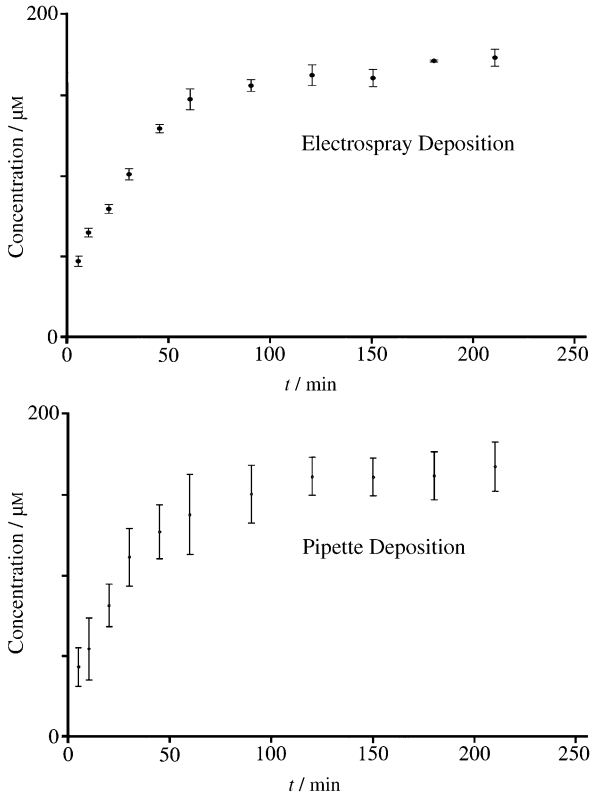

Figure 5. *Quantitative analysis of enzyme activity with DIOS-MS was improved by using an electrospray deposition (ESD) approach as compared to the traditional pipette deposition. ESD allows for more uniform deposition and provides a more homogeneous signal.*

Conclusion

We have demonstrated the capability of DIOS-MS for enzymatic assay development and for screening potential inhibitors. With the increasing importance in screening large numbers of compounds with disparate activities, this plate-based approach offers the advantages of high sensitivity and specificity, robustness, ease of use, and the ability to measure low-molecular-weight compounds. In addition, the DIOS-MS assay does not require the use of chromophore- or radio-labeled material, therefore real substrates and products are monitored in enzymatic reactions, and the analysis is more representative of the reaction than traditional approaches requiring fluorescent tags. Because of its inherent high-throughput capability with a scanning MS plate-based approach,[30] well over 10000 enzyme inhibitors can be screened on commercially available instrumentation. Moreover, the addition a 1000 Hz repetition rate laser, improvement in the plate movement, and software development for rapid acquisition of data will even further enhance sample throughput.[19] Interestingly, sample deposition is proving to be a key factor for high accuracy,[20] and our laboratory is currently exploring new high-throughput deposition approaches for high-throughput quantitative analyses.

Experimental Section

The laser desorption/ionization measurements were performed with a Waters-Micromass (Milford, MA) M@LDI-R time-of-flight mass spectrometer, and an Applied Biosystems (Framingham, MA) Voyager STR time-of-flight reflectron, as well as 4700 TOF/TOF mass spectrometers. The DIOS chips were attached to the MALDI target plates by using conductive carbon tape, and samples were irradiated with a nitrogen laser operated at 337 nm at 5–200 Hz (3 ns pulse duration) and attenuated with a neutral density filter. Ions produced by laser desorption were energetically stabilized during a delayed extraction period of, typically, 150 ns and then accelerated through the linear time-of-flight reflectron mass analyzer with a 20 kV pulse. The instruments were equipped with automated, multisampling capabilities to facilitate data collection and analysis. Between five and twenty five scans were typically averaged to obtain an adequate signal-to-noise ratio for each sample (Figure 1). The total time spent for each sample spot analysis was between 1.6 and 5 s, depending upon the application. This included acquisition time for averaging scans, a delay for firing the laser, and repositioning of the sample plate. Photopatterned DIOS chips were manufactured to allow the accurate allocation of 96 to 384 sample spots (Figure 1) and up to 1536 sample spots could be generated per wafer. It should be noted that the DIOS sample reservoirs are

not "wells" but etched regions of porous Si approximately 2 mm in diameter that contain pores approximately 70–120 nm in diameter with a pore depth of up to 200 nm.

DIOS chips were prepared by electrochemical etching of n-type ⟨100⟩ silicon wafers (0.005–0.02 Ω cm resistivity) in 25% HF/EtOH under white light illumination for 2 min at a constant current density of 5 mA cm^{-2}. The resulting porous silicon surfaces were oxidized by O_3 and then treated with 5% HF in H_2O, which has been found to provide more regular pore morphology and improved DIOS performance.[16] Photopatterning of the DIOS chips is necessary in order to utilize the autosampler mode during sample deposition and data acquisition. Irradiation through a simple mask during etching has allowed effective photopatterning of DIOS-active spots, since the etching rate on n-type silicon is strongly dependent on light intensity. In order to create patterns on the DIOS surface, the light from a fiber-optic light source was passed through a printed mask and two achromatic lenses, then focused on the silicon surface. This simple procedure reproducibly produces sharply defined porous silicon spots.

All analyses were performed in the automated mode. The automation of the DIOS analyses was designed to search for an analyte signal from each sample spot by monitoring signal strength as a function of laser intensity and laser position. The starting point for the relevant parameters (laser intensity, step size in each "well", and *m/z* range) was established manually for each plate. The laser intensity was initially set at the minimum level necessary for desorption and then allowed to increase by approximately 10 μJ per pulse per step until an acceptable data signal (signal-to-noise ratio >100 within the specified mass range) was acquired, up to a maximum of 50 μJ per pulse. If no signal was observed, the laser beam was repositioned on the well, and DIOS-MS analysis was resumed at the lower laser power. To adjust the laser position on the sample plate, a preprogrammed spiral search pattern was used that began in the center of each circular well and spiraled outward in 0.2 mm increments. In practice, the autoadjustments of laser position were never triggered since a strong and reproducible signal was obtained on the first attempt. Standard deviation of the signal intensities of the same cimetidine sample (50 pmol) deposited on different spots was less than 20% (Figure 1).

Analytes from the libraries were typically dissolved in water or water/methanol/DMSO (54.5:45:0.5%). Freshly prepared DIOS surfaces are hydrophobic; this allows aqueous solutions to bead and dry in a relatively uniform manner. Nonpolar solvents spread across the hydrophobic porous silicon wafer and are therefore to be avoided if possible. Aliquots (0.1–0.5 μL; containing 1–500 pmol of analyte) were deposited directly onto the porous Si surfaces and allowed to dry in air.

The acetylcholinesterase (AChE) assay involved monitoring inhibition of more than 900 compounds including known inhibitors. Typically, the reaction mixture of AChE (1 μM), potential inhibitor (10 μM), and acetylcholine (100 μM, Sigma) in ammonium bicarbonate buffer (10 mM, pH 8) was incubated for 30 min at 37 °C prior to analysis. The samples were then deposited on DIOS chips and analyzed for choline and acetylcholine.

For the proteolytic assay, BSA (Sigma) was incubated overnight at 37 °C in ammonium citrate (5 mM, pH 7.5 for tryptic digest and pH 8.5 for the endoproteinases). The reactions reached completion within 18 h. Samples were deposited on optimized double-etched DIOS chips (etching conditions: 50 mW, 2 min, and 5 mA). Known inhibitors, obtained from Sigma, including aprotinin, sulfanilamide, 4-acetomidaphenol, nystatin, chloropram, phenoxymethyl penicillinic acid, cimetidine, and furosemide were tested.

PAH assays for DIOS-MS analysis involved mixing wild-type with mutant forms of recombinant human-PAH purified protein (0.06 mg mL^{-1}) or mutant PAH over-expressed *E. coli* lysate (1% of the final volume) with ammonium bicarbonate buffer (25 mM, pH 7.4), bovine catalase (0.1 mg mL^{-1}), and L-phenylalanine (1 mM). This mixture was incubated at 25 °C for 5 min followed by addition of Fe^{2+} (1 μM) to the reaction for 1 min at 25 °C. The hydroxylation reactions were started with the addition of dithiothreitol (DTT, 6 mM) and cofactor (6R-BH_4, 75 μM). Mixtures were allowed to react for 30 min at 37 °C and were then stopped with the addition of acetic acid. Samples (0.5 μL aliquots) were deposited onto a DIOS chip, allowed to dry, and analyzed by DIOS-MS.

The sialyltransferase assays for DIOS-MS were conducted in a 50 μL final volume, containing 20 mM each of the acceptor 2-azidoethyl(β-D-Galactopyranosyl)-(1→4)-*O*-2-acetamido-2-deoxy-β-D-glucopyronoside and the donor CMP-*N*-acetylcetylneuraminic acid in cacodylate buffer (50 mM, pH 6.5), and NaCl (20 mM). The reaction was initiated by the addition of α-(2,6)-sialyltransferase (7.5 μL, ≈1 mU = 100 mg). The reaction mixture was incubated at 37 °C for 90 min, and 5 μL aliquots were taken every 15 min. For each aliquot, the reaction was stopped by the addition of acetic acid. Samples (0.5 μL) were then spotted on the DIOS plate, allowed to dry in air, and analyzed.

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